Review Article

Hemolytic causes of neonatal jaundice: diagnosis and treatment

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Contributions: (I) Conception and design: All authors; (II) Administrative support: None; (III) Provision of study materials or patients: None; (IV) Collection and assembly of data: All authors; (V) Data analysis and interpretation: All authors; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

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Abstract: Jaundice is very common in newborns. Hemolysis is a major component in the pathophysiology of hyperbilirubinemia, and newborns with severe hemolysis appear to be at greater risk of developing bilirubin neurotoxicity. In this review, we highlight cardinal concepts in the pathophysiology of hyperbilirubinemia, define hemolysis, discuss current evidence for hemolysis as a bilirubin neurotoxicity risk factor, and review the most common specific causes of hemolysis in newborns. In addition, we discuss modern methods of detecting and quantifying hemolysis. End-tidal carbon monoxide, corrected for ambient carbon monoxide (ETCOc), appears to one of the most specific methods to detect and quantify hemolysis because CO and bilirubin are produced in equimolar quantities as red blood cell breakdown occurs. The incidence of alloimmune hemolytic disease of the fetus and newborn has decreased since the development and routine use of Rh immune globulin in Rh-negative mothers but is still rampant in developing countries. Nonetheless, alloimmune hemolysis due to various antibody/antigen pairs continues be a major cause of hemolysis. In cases where non-immune hemolysis is diagnosed and causes hyperbilirubinemia that is prolonged or difficult to control, next-generation sequencing technology and hemolysis-specific gene panels have been shown to be high-yield in uncovering the etiology of the hemolysis. We review literature on the use of these technologies for diagnostic purposes.

Keywords: Jaundice; hyperbilirubinemia; hemolysis

Received: 02 February 2021; Accepted: 26 August 2021; Published: 28 November 2021.
doi: 10.21037/pm-21-14
View this article at: https://dx.doi.org/10.21037/pm-21-14

Introduction

Some degree of visible jaundice appears in 80% of otherwise healthy, term newborns (1,2). Usually the icterus, the result of immature activity of the bilirubin conjugating enzyme UDP-glucuronosyltransferase (UGT)1A1 in combination with increased red blood cell (RBC) turnover, is self-limited and mild. In some instances, due to an imbalance between bilirubin production and elimination processes (3), total serum bilirubin (TSB) concentrations may exceed the 95th percentile on the hour-specific bilirubin nomogram (4). On rare occasions, further imbalance may cause the indirect TSB component to progress to even higher levels with the danger of the unbound bilirubin (UB) component entering vulnerable brain cells, especially in the basal ganglia and auditory system. Acute bilirubin encephalopathy (ABE), the first manifestation of bilirubin neurotoxicity, frequently progresses to what is known as the kernicterus spectrum disorder (KSD) (5), with the most threatening aspects include severe and permanent choreoathetotic cerebral palsy, and auditory dyssynchrony including deafness. Long after the hyperbilirubinemia has passed, bilirubin

neurotoxicity in the neonatal period continues to have detrimental effects on neurodevelopment, contributing to childhood developmental disorders persisting into childhood and beyond. It is the avoidance of bilirubin-associated neurologic damage which is the motivating force behind our efforts to understand, predict, prevent, diagnose, and treat neonatal hyperbilirubinemia. As will be discussed, hemolysis is a major component in the pathophysiology of hyperbilirubinemia and newborns with hemolysis appear to be at greater risk of developing bilirubin neurotoxicity.

In this review, it is not our intention to comprehensively review all aspects of neonatal hemolysis. Rather, we aim to highlight and bring attention to contemporary issues and aspects which are currently under discussion in the medical literature, and which have potential to change our approach to the treatment of neonatal hyperbilirubinemia.

Imbalance between bilirubin production and elimination: a cardinal concept in the pathophysiology of hyperbilirubinemia

During the first postnatal days, there is a moderate, transient, physiological imbalance between bilirubin production and elimination. The activity of the bilirubin conjugating enzyme, UGT1A1, is diminished in term newborns to about 1% of that of healthy adults. Preterm newborns have even lower UGT1A1 activity (6) and are therefore at higher risk for developing hyperbilirubinemia than term infants. With regard to term and late preterm newborns, the risk increases with each decreasing week of gestation from 42 to 35 weeks (7). However, as both production and elimination processes contribute to the TSB level at any point in time, the concept of equilibrium between the hemolytic and conjugative forces is all important (3). Some newborns may have a high rate of bilirubin production, but in the face of a relatively mature UGT1A1 activity, may not develop hyperbilirubinemia. On the other hand, moderate hemolysis and relatively immature bilirubin conjugation may be instrumental in the development of hyperbilirubinemia. The importance of equilibrium between bilirubin production and conjugation, rather than the individual effect of either of these processes, has been shown mathematically (3). An index of the combined forces of bilirubin production and conjugation, constructed from the blood carboxyhemoglobin corrected for ambient carbon monoxide (COHbc) (an indicator of bilirubin production), divided by serum total conjugated bilirubin (an indicator of bilirubin conjugation), correlated more strongly with TSB than with either of the components individually. This confirmed the importance of the combined processes, rather than either bilirubin production or conjugation individually, in the pathophysiology of neonatal hyperbilirubinemia. However, because diminished bilirubin conjugation, a major contributor to bilirubin elimination, is universal in newborns, neonatal hyperbilirubinemia is frequently modulated by increased bilirubin production or hemolysis.

Does hemolysis increase the risk of developing bilirubin neurotoxicity?

The risk of developing bilirubin neurotoxicity appears to be increased in neonates with a predominantly hemolytic etiology for their hyperbilirubinemia. Whether hemolysis results in a bilirubin threshold-lowering effect for neurotoxicity, or whether the increased bilirubin production results in extreme, rapidly reached peak TSB concentrations, sometimes of prolonged duration, with greater penetration of the blood-brain barrier (BBB) is unclear.

In the past, it was believed that the risk of developing kernicterus in newborns without evidence of hemolysis was less than in cases where hemolysis was present (8-10). However, in a review of cases of kernicterus referred to them for litigation, Maisels and Newman reported affected, but previously healthy, newborns in whom no evidence of hemolysis could be found. No cause, other than breastfeeding, could be found for their hyperbilirubinemia (9). Confirming that hemolysis is not a prerequisite for kernicterus are cases of Crigler-Najjar syndrome, a genetic condition associated with kernicterus, in which there is no bilirubin conjugative activity, but also no hemolysis (10).

Increased hemolysis is associated not only with classic choreoathetotic cerebral palsy, but also with neurologic abnormalities and lower IQ. Of 102 children, who had been treated in infancy for indirect hyperbilirubinemia, a positive direct Coombs' test, or direct antiglobulin test (DAT), presumed to be a marker of hemolysis, was associated with lower IQ scores and a higher incidence of neurologic abnormalities (11). Similarly, of 55 Norwegian males born in the early 1960s who developed neonatal hyperbilirubinemia, those who had a positive DAT, and hyperbilirubinemia for greater than five days, had significantly lower IQ scores than average (12).

The 5-year outcomes of 140 Californian children who had TSB levels >25 mg/dL (428 µmol/L) in the neonatal

period were similar to controls, and there were no cases of kernicterus. However, a subgroup of 9 children, who had a positive DAT and were therefore presumed hemolytic, had significantly lower IQ values than those hyperbilirubinemic counterparts who were DAT negative (13). In a re-analysis of data from the Collaborative Perinatal Project, Kuzniewicz and Newman found no relationship between maximum TSB and IQ scores, but the presence of a positive DAT in those with a TSB ≥25 mg/dL (428 µmol/L) was associated with a 6.7 point decrease in IQ scores (14).

Of 249 newborns admitted to a children's hospital in Cairo, Egypt, with TSB values ≥25 mg/dL (428 µmol/L), there was a poor correlation between the TSB at admission and the development of ABE (15). However, those with risk factors for hemolysis, such as Rh incompatibility or sepsis, were at greater risk for developing ABE than those without these factors. The effect of hemolysis in exacerbating bilirubin neurotoxicity has recently been reviewed (16).

Although there is to date no evidence demonstrating higher levels of UB in hemolyzing neonates, it is generally accepted that hemolysis is a potential factor increasing the risk for bilirubin-related brain damage. While a TSB concentration of 20–24 mg/dL (342–410 µmol/L) may be associated with kernicterus in a neonate with Rh isoimmunization, a healthy, term non-hemolyzing infant will rarely be endangered by TSB concentrations in this range. Hemolysis due to DAT positive Rh, ABO immunization, or other isoimmunizations as well as glucose-6-phosphate dehydrogenase (G6PD) deficiency may therefore pose an increased threat to an otherwise healthy newborn.

Historically, the first correlation between increasing TSB concentrations and bilirubin encephalopathy was reported by Hsia *et al.* in babies with severely hemolytic Rh isoimmunization (17). The more the TSB rose above 20 mg/dL, the greater chance of developing kernicterus. Although virtually eliminated nowadays in industrialized Western countries, kernicterus due to Rh disease is still rampant in developing countries (18,19). Kernicterus was first reported in Greece in the 1960s in association with another hemolytic condition, G6PD deficiency (20), and continues to be encountered in recent times both in developing countries with a high indigenous G6PD deficiency frequency as well as in countries where G6PD deficiency has been introduced as a result of immigration and population migration (21).

Hemolytic conditions continue to top the list of identified etiologies of hyperbilirubinemia and ABE in modern times (22). For example, in the US-based Pilot Kernicterus Registry of 125 newborns, 57 (45.6%) had an identified hemolytic condition. 28 (22.4%) had ABO blood group heterospecificity, with or without a positive DAT, 26 (20.8%) were G6PD-deficient, and 3 (2.4%) had hereditary spherocytosis (23). An additional 23 (18.4%) had non-specific hematologic evidence of hemolysis. Hemolytic conditions, especially ABO incompatibility and G6PD deficiency, are predominant in registries of KSD cases from the US (24,25), Canada (26), the United Kingdom, and Ireland (27,28). The trend continues in very recent reports. In Utah, an ABE registry from 2009 to 2018 showed that, though the incidence of ABE was low, hemolytic disease was a unifying feature of all seven cases. Two had DAT-positive hemolysis. The remaining five had mutations in genes that resulted in hereditary hemolytic disease (e.g., G6PD deficiency or hereditary spherocytosis) (29). In Sweden, of 67 newborns ≥35 weeks of gestation with TSB >30 mg/dL (513 µmol/L), born between 2008–2016, hemolytic disease was found in 33 (49%), of which ABO blood group incompatibility and G6PD deficiency were the most common (30). In a study of 408 newborns born in Denmark between 2000 and 2015 who developed a TSB ≥26 mg/dL (425 µmol/L), ABO blood group incompatibility, with or without positive DAT, was the most common etiology among those in whom an etiology was identified. Other hemolytic conditions included G6PD deficiency and hereditary spherocytosis (31). Of 123 term newborns in Wuhan, China, who underwent exchange transfusion for hyperbilirubinemia [peak TSB 25.7±5.4 mg/dL (440±92 µmol/L)], 41 (33%) were ABO incompatible (of whom 7 developed ABE), 22 (17.8%) had Rh disease (4 ABE), and 8 (6.5%) (4 ABE) were G6PD-deficient (32). Of note is the high incidence of ABE among each of these hemolytic causes of hyperbilirubinemia. G6PD deficiency is a common cause of kernicterus in Central and West Africa, especially in Nigeria (33,34). In that country, Vidavalur *et al.* recently estimated a high prevalence of hyperbilirubinemia with resultant kernicterus and mortality due to G6PD deficiency (35).

The Subcommittee on Hyperbilirubinemia of the American Academy of Pediatrics (AAP), in its 2004 guideline, emphasizes the high risk of developing hyperbilirubinemia associated with hemolysis (36). The guideline warns that rapid onset of jaundice developing within the first 24 hours (a clinical sign of hemolysis), blood group incompatibility with a positive DAT, and known hemolytic disease including G6PD deficiency, are all associated with increased hemolysis, and, as a result,

Table 1 Some important, or commonly occurring, causes of hemolysis in the newborn

1. Immune hemolysis
ABO blood group incompatibility (mother group O, newborn group A or B, DAT either positive or negative)
Rh (D) isoimmunization
Some rarer immune setups
Anti-c
Anti-C
Anti-e
Anti-E
Anti-Kell
Anti-Duffy
2. Nonimmune hemolysis
RBC enzyme deficiencies
G6PD deficiency
PK deficiency
RBC membrane defects
HS
Elliptocytosis
Ovalocytosis
Stomatocytosis
Pyknocytosis
Unstable hemoglobinopathies
Sepsis
Extravascular extravasated blood collections
Cephalhematoma
Adrenal hemorrhage

high risk for developing hyperbilirubinemia. In addition to increased risk for subsequent hyperbilirubinemia, risk factors for neurotoxicity are emphasized in the guideline and in a subsequent "update with clarifications" (7). Neurotoxicity risk factors may increase the risk of brain damage in newborns with hyperbilirubinemia. Topping this list of neurotoxicity risk factors are hemolytic conditions including isoimmune hemolytic disease and G6PD deficiency (7). The practical aspects of both increased risk for hyperbilirubinemia and neurotoxicity in association with hemolysis are manifest in the recommendations of the 2004 AAP guideline, in which a more aggressive approach is adopted in babies with hemolysis than in those without an obvious hemolytic condition. The AAP recommends initiating phototherapy or performing exchange transfusion at lower levels of TSB in neonates with hemolytic conditions than in non-hemolyzing counterparts.

A (non-comprehensive) list of some more important and/or commonly occurring causes of hemolysis are listed in *Table 1*.

Some specific causes of hemolysis in neonates

It is not our intention in this section to comprehensively describe all or many hemolytic conditions in the newborn, but rather to focus on those which continue to cause hemolysis with the potential of bilirubin neurotoxicity and the KSD, and highlight ongoing communications in the medical literature.

G6PD deficiency

G6PD deficiency is probably one of the most important hemolytic conditions contributing to the persistence of hyperbilirubinemia and KSD. While common in developing countries, especially those in which G6PD deficiency is endemic (Central and West Africa, the Mediterranean Basin, the Middle and Far East), because of migration patterns, modern day ease of travel, and Trans-Atlantic slave trade, population movements, the condition is also encountered nowadays in almost any region of the world. The Americas are not exempt: G6PD deficiency is overrepresented in the US-based Pilot Kernicterus Registry relative to the background G6PD frequency (23). G6PD deficiency is not infrequent in Central and South Americas (37), and in Canada (26). Similar overrepresentation of G6PD deficiency was found in a study from the United Kingdom and Ireland (27). Parents of hyperbilirubinemic newborns should always be questioned with regard to their family's origins. The neonatal aspects of G6PD deficiency and the role of G6PD screening in combination with parental education in the prevention of bilirubin neuropathology has been comprehensively reviewed (38,39).

The role of hemolysis in the generation of hyperbilirubinemia has been challenged (40). This misconception may be due to the finding that in newborns with the common and more moderate form of hyperbilirubinemia, a major role for diminished bilirubin conjugation, the result of a polymorphism in the TATAA box of the gene promoter encoding the bilirubin conjugating

enzyme UGT1A1 (*UTA1A1*28*) has been reported (41,42). Furthermore, COHbc or end tidal CO (ETCO) levels, accurate indices of heme catabolism, therefore reflecting hemolysis, is universally increased in neonates who have G6PD deficiency, and not necessarily only in those who develop the moderate form of hyperbilirubinemia (43,44). Blood count indices are notoriously non-reflective of hemolysis in obviously hemolyzing G6PD-deficient neonates (45,46). However, in the extreme form of G6PD-deficiency-associated hyperbilirubinemia, the situation is different. Only extreme hemolysis can explain the sudden and exponential increase in TSB to neurotoxic levels (47). Cases of hyperbilirubinemia have been reported in which either COHbc (48) or ETCOc (49) values have been increased, confirming the role of hemolysis in G6PD-deficient neonates with hyperbilirubinemia.

Notwithstanding to the usual response to phototherapy in the more moderate form of G6PD deficiency-associated hyperbilirubinemia, these newborns are still at risk and should be vigilantly observed for rebound hyperbilirubinemia following lowering of their TSB levels by phototherapy (21,50). Diminished bilirubin conjugation in combination with some degree of increased hemolysis, as described above, place these neonates at special risk. Any further exposure to a hemolytic agent, or further diminution of bilirubin conjugation, such as in late prematurity, may further upset the equilibrium between bilirubin production and elimination, and precipitate hyperbilirubinemia.

Screening for G6PD deficiency, in combination with parental education for preventing hemolysis and detecting hyperbilirubinemia, has been introduced and has been successful in some countries, as reviewed (39). Screening cannot be expected to prevent hemolytic episodes, but can be expected to increase parental and medical caretaker awareness of the increased risk inherent in a G6PD-deficient newborn, therefore facilitating earlier referral for treatment, and lowering of the TSB levels prior to reaching neurotoxic levels.

Because it is an X-linked condition, female heterozygotes are common, but their diagnosis may be difficult unless molecular genetic methods of diagnosis are used. Qualitative screening tests are designed to identify only those with very low G6PD activity, while quantitative tests may give either a deficient, intermediate, or normal result, depending on the degree of X-chromosome inactivation (51). Heterozygotes have been demonstrated to have higher levels of COHbc than G6PD-normal counterparts and correspondingly higher rates of hyperbilirubinemia requiring phototherapy (52). A case of neonatal hyperbilirubinemia resulting in death occurred in a heterozygote for the $G6PD^{Mediterranean}$ mutation, in combination with heterozygosity for *UGT1A1*28* (53). Female newborns from high-risk population groups, whose quantitative testing results in intermediate or even normal values, should nevertheless be closely followed up for the development of hyperbilirubinemia.

ABO isoimmunization

While DAT-positive ABO-incompatible neonates have been shown by COHbc studies to have increased hemolysis (54), as with G6PD deficiency, only a fraction of affected neonates will develop hyperbilirubinemia and potential bilirubin encephalopathy. Nevertheless, as mentioned above, ABO heterospecificity is high on the etiology list in a recent publication of cases of hyperbilirubinemia and bilirubin encephalopathy. The hyperbilirubinemia frequently occurs within the first 24 hours and many meet AAP indications for phototherapy (54). In our Jerusalem, Israel, experience (M.K.), the use of intravenous immune globulin (IVIG) has been effective in limiting the rise in TSB in many cases in which phototherapy has been unable to stay the bilirubin increase and which approaches exchange transfusion indications.

Rh isoimmunization

The development of Rh immune globulin (RhIG) with successful preventive programs has made Rh immune hemolysis a rarity in industrialized countries. In Jerusalem, we occasionally encounter cases in immigrants from countries in which Rh prophylaxis has not been introduced. The situation in developing countries is very different. In systematic reviews and meta-analyses of Rh disease and neonatal hyperbilirubinemia, Bhutani *et al.* estimated a high mortality and morbidity associated with these conditions. Three-quarters of the mortality occurred in sub-Saharan Africa and South Asia. They concluded that failure to prevent Rh sensitization and manage neonatal hyperbilirubinemia resulted in 114,100 avoidable neonatal deaths in 2010 and that many affected children grow up with disabilities. Proven solutions remain underused, especially in low-income countries (19).

Hereditary spherocytosis

Hereditary spherocytosis (HS) is a disorder characterized

by defects in RBC membrane proteins resulting in a loss of RBC membrane surface area and thus sphere-shaped, hyperdense, poorly deformable RBCs. The cells have a shortened lifespan which causes a hemolytic anemia found worldwide in individuals from all races and ethnicities. HS is the most common inherited hemolytic anemia in people with Northern European ancestry with an estimated incidence of 1 per 1,000 to 1 per 3,000 births (55,56). Seventy-five percent of cases are inherited in an autosomal dominant manner and the remaining are inherited in an autosomal recessive manner. The most commonly mutated gene associated with HS in the Northern European population is *ANK1* (Ankyrin-1, 40–65%). Other genes known to cause hereditary spherocytosis include *SPTB* (Spectrin beta chain), *SPTA1* (Spectrin alpha chain-1), *SPTA2* (Spectrin alpha chain-2), *SLC4A1* (Band 3 anion transport protein), and *EPB42* (Protein 4.2).

The astute practitioner should consider HS when a neonate has Coombs-negative hyperbilirubinemia, anemia, and/or hemolysis, and has microcytosis. Christensen and Henry in 2010 also showed that a mean corpuscular hemoglobin concentration (MCHC) >36 g/dL in newborns with a TSB >20 mg/dL (342 µmol/L) is both sensitive and specific for HS (57). In a subsequent publication by Christensen *et al.*, the authors estimate the ratio of MCHC/mean corpuscular volume (MCV) >0.36 to be 97% sensitive and >99% specific for HS (58).

The traditional diagnostic test for HS has been by the osmotic fragility test. It has been suggested that the diagnostic criteria for HS used in adults and older children are unreliable in newborn infants because both preterm and term infants' RBC membranes have an increased osmotic resistance. A relatively new diagnostic test for HS, eosin-5-maleimide (EMA)-flow cytometry, quantifies the erythrocyte membrane band 3 complex using an eosin-5-maleimide dye flow cytometric analysis (59,60). A reduction in band 3 complex is consistent with HS. Therefore, the test is commonly referred to as "band 3 reduction" test at reference laboratories that offer it. This test appears to perform very well in neonates even in the first days of life (61,62). If the patient has no family history of HS or the causal mutation is unknown, sequencing the genes that can cause HS is indicated. Different mutations result in a variety of disease severities. Identifying the causal mutation can also aid in identification of the inheritance pattern and reproductive risk for the proband.

Pyruvate kinase deficiency

Pyruvate kinase (PK) is responsible for the conversion of phosphoenolpyruvate to pyruvate with concomitant phosphorylation of ADP to ATP. PK deficiency is rare, with a prevalence estimated at <51 cases per million. It has a worldwide distribution, but it is more common among people of northern European ancestry. Despite being rare, though, PK deficiency is the most common enzymopathy in the glycolytic pathway and some have suggested that PK deficiency is the second most common hereditary variety of Coombs-negative, non-spherocytic hemolytic disease in the US (63).

The *PKLR* gene located on chromosome 1q22 encodes both the liver and RBC isozymes of the enzyme (55). PK deficiency follows an autosomal recessive inheritance pattern (often compound heterozygosity), and as such, heterozygous carriers of *PKLR* mutations are asymptomatic. Over 260 different *PKLR* mutations have been described and associated with hemolytic anemias, and most of which are single nucleotide missense mutations (64).

Newborns with PK deficiency present with hyperbilirubinemia that can be difficult to control with phototherapy (63). Though the severity of symptoms and chronic anemia vary greatly, the disorder is lifelong and may result in a need for chronic transfusions.

Defining hemolysis in neonates

The RBCs of a mid-trimester fetus or an extremely preterm infant are produced in the liver as well as in the bone marrow. At term gestation, the site of production is almost exclusively the marrow (65). RBC released from the production site into the blood should remain in the circulation intact and functional for a set number of days. This length of time is known as the "RBC lifespan" and can be measured using stable isotope labeling such as ^{15}N-glycine, or radioactive isotope labeling with ^{51}Cr, or with biotin labeling (66-68). Healthy adults have a RBC lifespan of 120 days (66,67). Upon reaching that age, RBC are actively removed from the circulation in a nonrandom manner and their elements recycled. In term infants the RBC lifespan is shorter than 120 days. Typical measurements indicate 80 days or about two-thirds of the adult RBC life span (69,70). In preterm infants, the RBC lifespan is shorter still, approximately proportionate to

gestational age (69-71). The definition of *hemolysis* is a RBC life span significantly shorter than it should be (72,73).

The process of natural termination of RBCs is a physiological process known as *senescence* (73). *Neocytolysis*, is a well-described phenomenon of preferential destruction of RBCs following a change from low to high oxygen exposure. This mechanism accounts for the physiologic rapid over-correction of the relatively high hematocrit in neonates (74,75). In contrast to RBC senescence and physiologic neocytolysis in the newborn, *hemolysis* is pathological shortening of the RBC life span. Such shortening can be the consequence of a wide range of abnormalities, including genetically-based mutations in RBC structure or function, antibodies attached to erythrocytes leading to their premature removal, or issues extrinsic to RBCs such as physical disruption, injury by infectious agents, temperature or chemicals, or by disruption from tethering on fibrin strands in the circulation as occurs in disseminated intravascular coagulation (DIC) (76). Extravasation of blood into tissues is another cause of hemolysis that occurs with heavy bruising, cephalohematomas, subgaleal hemorrhages, or intraventricular hemorrhages (IVH), since RBC in tissues are metabolized much more rapidly than had they remained in the circulation (23).

One consequence of hemolysis is a "bilirubin load" considerably larger than normal. This is because heme is metabolized to bilirubin (76,77). The "bilirubin load" is the amount of bilirubin that must be taken-up by hepatocytes, conjugated, and excreted. When hemolysis produces a bilirubin load too large to be cleared efficiently by normally-functioning bilirubin metabolic mechanisms, the serum concentration of bilirubin rises, producing *hemolytic jaundice*. If the hemolytic rate exceeds the capacity of the bone marrow (and liver) to increase RBC production in compensation, the condition also proceeds to *hemolytic anemia* (16,76,77).

When neonatal jaundice is found to be the result of hemolysis, caregivers should be aware that: (I) the TSB level can rise rapidly; (II) the hyperbilirubinemia might be slow to fall even with intensive phototherapy; and (III) the hyperbilirubinemia is likely to rebound after phototherapy is discontinued. These consequences explain, in part, why neonates with *hemolytic* hyperbilirubinemia are at risk for developing ABE or kernicterus (16,23,29,77,78).

Methods of detecting hemolysis in neonates

Many laboratory assays have been considered and are used to detect hemolysis and quantify hemolysis in the context of hyperbilirubinemia. Because the hematocrit is a balance between RBC death and production, it may mask chronic mild to moderate hemolysis that has been compensated by reticulocytosis or upregulated RBC production. In addition, the hematocrit is significantly affected by fluid shifts characteristic of the first days of life, making it difficult to interpret as a marker of hemolysis. Similarly, multiple laboratory tests can suggest hemolysis, but each has limitations, particularly in neonates. *Table 2* summarizes ten laboratory tests that can be helpful in detecting hemolysis. For each, we describe test results that suggest hemolysis and problems that have been describe when each test is applied to neonates. A combination of these tests is more conclusive than any single test alone.

We hypothesize that the ETCOc measurement is one of the most sensitive markers of hemolysis. Therefore, the following section discusses this metric in greater depth.

Using ETCOc to differentiate hemolytic from non-hemolytic jaundice, and to quantify the hemolytic rate

When heme is metabolized, CO and bilirubin are produced in equimolar quantities (79,80). Thus, measuring ETCOc reveals the rate of RBC breakdown and the subsequent rate of bilirubin production. Our group and others have reported associations between ETCOc and hyperbilirubinemia risk (80-85). The clinical value of ETCOc measurements in newborns continues to be studied. A paper published by Christensen *et al.* found that ETCOc reference intervals were higher in healthy neonates during the first week after birth (5^{th} to 95^{th} percentile reference range, 1.4 to 1.7 ppm) than after seven days of age (all ≤1.0 ppm). In our clinical practice, we have successfully utilized the cutoff of 2.0 ppm to define hemolysis in neonates during the first week of life (83).

The measurement of ETCOc has multiple advantages over other methods of detecting hemolysis; it reflects RBC breakdown whether it is occurring at a physiologic or an accelerated rate. In addition, ETCOc should be elevated in all cases of hemolysis, regardless of etiology. As noted in the previous section, blood typing and DAT testing of all neonates born to type-O mothers have been used to screen for hemolysis. Alloimmunity, though, is only one cause of hemolysis in the newborn. A negative DAT will rule out alloimmune causes of hemolysis, but not other non-immune-mediated causes of hemolysis as shown by a 2002 study by Herschel *et al.* (86). Furthermore, not

Table 2 Laboratory determinations that can be performed on jaundiced neonates in order to evaluate whether *hemolysis* is contributing to the pathogenesis

Test	Test result suggesting hemolysis	Problems applying the test to neonates
Routine urine analysis (UA) to assess presence of heme	≥ Trace hemoglobin in urine	RBCs in urine are not uncommon in very low birthweight (VLBW) neonates. When present they render urinary hemoglobin an invalid test for detecting hemolysis. However, hemoglobinuria in the *absence* of any RBC on the UA suggests hemolysis
Low or absent serum haptoglobin	A value that is falling or is below the lower limit of detection	Haptoglobin should be detected in the serum of term infants. Jaundiced neonates who have a haptoglobin level below the lower limit of detection can be considered to have hemolytic hyperbilirubinemia. Preterm infants without hemolysis sometimes have very low serum haptoglobin concentrations but it should not be undetectable, unless they are hemolyzing
DAT (Coombs)	Positive direct Coombs	A positive DAT does not prove hemolysis. A negative DAT does not exclude the possibility of hemolysis
Elevated absolute reticulocyte count	A value above the upper reference interval for gestational and postnatal age	Reticulocytosis indicates increased RBC production. Thus in a jaundiced neonate an elevated reticulocyte count suggests the jaundice might be on the basis of hemolysis
Elevated immature reticulocyte fraction	Value above 7% at birth or above 3% after 24 h	Not well validated in VLBW neonates
Elevated nucleated RBC count	Upper limit 3000/μL at birth. Should be <500/μL after 3–4 days	Nucleated RBCs are typically higher in younger gestational age at birth
Abnormal RBC morphology on stained blood film	Microspherocytes, elliptocytes, bite and blister cells, echinocytes, schistocytes	Some degree of anisocytosis and poikilocytosis are frequently encountered in healthy neonates. In a neonate with jaundice, finding marked abnormalities of RBC size and shape suggest hemolytic jaundice. Abundant schistocytes suggests microangiopathic hemolytic disease
Heinz body prep	Heinz bodies support diagnosis of hemolysis due to precipitated (unstable) hemoglobin	Less well studied in preterm infants
Elevated ETCOc	First week the upper limit =1.7 ppm, thereafter upper limit = 1 ppm	Cannot perform test if neonate is intubated or on nasal cannula O_2. Difficult to get accurate reading if respiratory rate is >60 breaths/min
Carboxyhemoglobin by Co-oximetry	Upper limit =1.5% to 2.0%	Reference intervals for VLBW neonates are lacking, and the measurement is not sensitive

VLBW, very-low-birth-weight, <1,500 grams.

all neonates with a positive DAT will have significant hemolysis. A study by Elsaie *et al.* published in 2020 showed that only 27.2% of 191 DAT-positive infants were hemolyzing based on ETCOc ≥2.5 ppm, while 29.1% of DAT-negative infants were hemolyzing based on ETCOc ≥2.5 ppm (85). Only half of neonates with hemolysis had a positive DAT. Similarly, our group showed that, though the mean peak TSB is slightly greater in neonates who were blood group A or B and born to group O-positive mothers (and thus were at some risk for ABO hemolytic disease), these neonates were not more likely to develop hemolytic disease in the setting of universal predischarge screening for hyperbilirubinemia (87,88).

The 2004 AAP guideline for the management of hyperbilirubinemia in the newborn left open the question: "How will ETCOc affect management?" (36). In the beginning of 2020, the University of Utah Nursery implemented routine ETCOc measurements in newborns with hyperbilirubinemia at the time of phototherapy initiation to determine the degree of hemolysis contributing to hyperbilirubinemia. If a newborn has hemolytic hyperbilirubinemia, defined as an ETCOc ≥2.0 ppm, we initiate a phototherapy using a blanket in addition to an overhead device, instead of an overhead device alone. We

also recommend earlier outpatient follow-up following discharge. In addition, we only obtain a blood type and direct Coombs testing from stored cord blood samples of those babies who have ETCOc ≥2.0 ppm. We believe this is the first protocolized implementation of ETCOc measurements in a newborn nursery directed towards newborns who were about to receive phototherapy.

In an interim analysis of this project, we found that that measuring ETCOc on all phototherapy recipients was feasible and safe and that higher ETCOc values predicted earlier and longer phototherapy courses. Specifically, we found that for every 1 ppm increase in ETCOc, phototherapy was started 9.1 hours earlier (95% CI, 3.3–14.8, P=0.002). Likewise, ETCOc was associated with the total duration of phototherapy during the birth hospitalization. Specifically, for every 1 ppm increase in ETCOc an additional 9.3 hours (95% CI, 4.1–14.6, P<0.001) of phototherapy was provided. Three of the 145 patients included in the analysis required readmission following post-birth discharge. All three had elevated ETCOc at the time when phototherapy was initiated.

We conclude that infants with hyperbilirubinemia due to high levels of bilirubin production ("hemolytic hyperbilirubinemia'), including but not limited to those with alloimmune hemolytic disease, require phototherapy earlier and for longer durations. In addition, they may have an increased risk for developing KSD (89,90).

Modern genetic methods to identify underlying causes of neonatal hyperbilirubinemia

In a neonate with hemolytic jaundice, identifying the exact cause of the hemolysis is sometimes straightforward, but occasionally it is quite challenging. Finding the exact cause can be very helpful in devising anticipatory guidance for the neonate and can also inform expectant management of future pregnancies. Moreover, discovering the exact cause of neonatal hyperbilirubinemia will provide a clear explanation to parents who are otherwise left never knowing why their baby had this problem (91).

Even when the cause of the hemolysis seems apparent from initial laboratory testing, the pathogenesis might be more complex. For instance, the combination of one mutation that shortens RBC lifespan can be accompanied by a second mutation (or polymorphism) that reduces bilirubin conjugation (29). As an example, a neonate with hemolytic hyperbilirubinemia and abundant spherocytes on blood film and a positive Band 3 reduction test will be diagnosed appropriately as having HS (56). However, that patient might also have a *UGT1A1*28* variant significantly prolonging and worsening the hyperbilirubinemia (29). Combinations of mutations that affect both bilirubin production and clearance can be difficult to identify using standard laboratory testing but can be clarified using "next-generation sequencing" (NGS) panels.

Developed only a few years ago, these panels are currently being adopted by clinical reference laboratories as diagnostic tests for mutations or polymorphisms responsible for various genetically-based disorders. Because multiple candidate genes from one patient can be sequenced in parallel in a single run, the cost can be far less than sequencing candidate genes one at a time (55,92). Moreover, using a "barcoding" technique to identify which DNA fragments belong to which patients, the DNA of many patients can be sequenced in a single run, further reducing costs per patient. Another helpful aspect of NGS panels is that it detects not just which gene is involved, but it identifies the exact mutation in that gene. Also, when novel mutations or genetically complex conditions are present involving several mutations, NGS can identify the situation, while previous techniques were typically unable to do so (55,92).

The quality of genetic data generated by NGS, and the usefulness of the findings to clinical decision making, has already generated NGS availability for causes of otherwise idiopathic neonatal hyperbilirubinemia. Panels that identify the underlying genetic causes of neonatal hyperbilirubinemia are now available from several reference laboratories (55,92-94).

Although whole exome or whole genome sequencing techniques can provide more information than focused NGS panels, the overall complexity of data analysis, with frequent variants of unknown significance, is sometimes a drawback of exome or genome sequencing (95-97). Turnaround time, cost-effectiveness, and reimbursement fee schedules should also be kept in mind in deciding on which test methodologies to order.

Using our targeted NGS neonatal hyperbilirubinemia/anemia panel, we have discovered many novel mutations. These were initially described in the laboratory reports as "variant of uncertain significance", because no previous publications or listings of the putative mutation were found in databases. However, computational analysis, modeling, clinical pathological correlation, and finding previously described damaging mutations at or near a novel variant site, can all suggest that the variant is indeed a mutation responsible for the phenotype. Examples from our own experience include novel variants causing erythrocyte

membrane disorders (HS, hereditary elliptocytosis, and hereditary pyropoikilocytosis), G6PD deficiency, and PK deficiency (98-102).

Recently, we reported results of a study using our NGS panel among 268 patients with hemolytic anemia. The panel identified pathogenic mutations in 64/268 (24%) half of which were novel mutations (55). Overall, 29/268 (11%) of patients were homozygous for a promoter polymorphism in the *UGT1A1* gene A(TA)7TAA (*UGT1A1*28*), which leads to reduced expression of the *UGT1A1* gene and Gilbert syndrome (55,92,103).

When neonatal hemolytic hyperbilirubinemia can be diagnosed by family history, erythrocyte morphology (104) and erythrocyte enzymology, modern genetic methods of diagnosis are usually felt to be unnecessary. However, when the condition is idiopathic, these methods can add substantial value, clarifying the pathogenesis and informing physicians and families of best practices for this patient and perhaps future pregnancies in this family or kindred.

Summary

Hemolysis is defined as an abnormally short RBC lifespan. Common varieties of neonatal hyperbilirubinemia are not hemolytic, or have only a minor and transient hemolytic component. Hemolytic neonatal hyperbilirubinemia, although less common, imparts a greater risk of life-long adverse neurodevelopmental consequences.

Simple laboratory methods are available to assist in diagnosing hemolysis. Currently the "gold standard" we use for diagnosing hemolytic hyperbilirubinemia and quantifying the hemolytic rate is the ETCOc measurement. Once a case of neonatal hyperbilirubinemia has been identified to be hemolytic, the next step in assessment is to identify the underlying cause of the hemolysis. This is generally straightforward, but rare mutations or combinations of mutations render some cases perplexing. Cases where the cause of hemolysis is unclear can generally be diagnosed by NGS panels. Kernicterus due to G6PD deficiency remains a global problem. Greater focus is needed on rapid diagnosis, acknowledging the hemolytic presentation, and appreciating the pernicious combination of G6PD deficiency plus polymorphisms retarding bilirubin clearance.

Acknowledgments

Funding: The work was supported in part by grant U54DK110858 from the US Public Health Service.

Footnote

Provenance and Peer Review: This article was commissioned by the Guest Editors (David K. Stevenson and Ronald J Wong) for the series "Neonatal Jaundice" published in *Pediatric Medicine*. The article has undergone external peer review.

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://pm.amegroups.com/article/view/10.21037/pm-21-14/coif). The series "Neonatal Jaundice" was commissioned by the editorial office without any funding or sponsorship. The authors have no other conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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doi: 10.21037/pm-21-14
Cite this article as: Bahr TM, Christensen RD, Kaplan M. Hemolytic causes of neonatal jaundice: diagnosis and treatment. Pediatr Med 2021;4:32.