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Chapter

Neonatal Anemia

Laura M. Dionisio and Thamires A. Dzirba

Abstract

Neonatal anemia and iron deficiency are frequent founds in neonatal intensive care units (NICUs). The three major causes of anemia in neonates are blood loss, reduced red blood cell production, and increased degradation of the erythrocytes. Premature infants in ICUs have high levels of iron deficiency, and ascertaining the cause of anemia in this group of patients can be a challenge in clinical practice. This chapter provides an updated review of neonatal anemia. It will concern the pathophysiology of neonatal anemia in term and preterm infants and a detailed discussion of the traditional and innovative laboratory tests for diagnosis and assessment of this condition in the ICUs.

Keywords: anemia of prematurity, hemolysis, neonatal anemia, neonatal intensive care, physiologic anemia

1. Introduction

Neonatal anemia is a frequent found in neonatal intensive care units (NICUs). The diagnostic approach for neonatal anemia must establish its underlying cause for determining the proper treatment. However, due to the complexity of patients under NICUs (prematurity, infections, other diseases, ventilatory support) and the variety of laboratory tests available, determine the cause of neonatal anemia could be a challenge for clinicians.

After birth, all infants experience a gradual decrease in hemoglobin that results in varying degrees of anemia. During the first weeks of life, the hemoglobin concentration decreases significantly as a consequence of the reduced production of erythrocytes due to reduced production of erythropoietin (EPO) and also by the decreased erythrocyte lifespan during this period. Although all neonates develop the physiologic anemia, preterm infants experience a greater degree of anemia, the anemia of prematurity, which results from a combination of physiologic and pathological factors.

The iatrogenic blood loss due to phlebotomy for laboratory testing in NCIU is an important contributor for the anemia of prematurity. Because low birth-weight and premature infants usually need close monitoring, they suffer the loss of great amounts of blood.

The three major non-physiologic causes of anemia in neonates are blood loss, reduced red blood cell production, and increased degradation of the erythrocytes.

The diagnostic approach usually includes the patient history (familial, maternal, patient), physical examination and laboratory tests.

This chapter is an updated review of neonatal anemia. It concerns the pathophysiology of neonatal anemia, anemia of prematurity and non-physiological causes of anemia in NICUs patients.

We also provide a detailed discussion of the traditional and innovative laboratory tests for diagnosis and assessment of this condition in the ICUs.

2. Developmental erythropoiesis

To properly understand the pathophysiology aspects of neonatal anemia, is essential to comprehend how the erythroid blood cells are formed during pregnancy and the extrauterine life.

The erythropoiesis in the fetus starts in the liver, with progenitors that migrate from the yolk sac and infiltrate the liver circulation. Approximately at the sixth week of gestation, the liver becomes the main hematopoietic site, remaining its role during the second trimester of pregnancy. After the sixth month of gestation, the bone marrow is the major site of erythropoiesis, gradually replacing the liver in this function until birth [1, 2]. Fetal erythropoiesis is an extremely active process, mainly during the last 2 months of gestation, where the red blood cell (RBC) production is 3 to 5 times greater than the healthy adult ones [3].

The erythropoiesis process is regulated by cytokines and growth factors, where the erythropoietin (EPO) exerts a major role, working synergically with the other factors. EPO acts as the main regulator of erythropoiesis, leading to the differentiation, proliferation and survival of red blood cells. In the fetuses, the liver is a primary site of EPO production, which is replaced by the kidney in the extrauterine life. The synthesis of EPO and its receptor (EPO-R) is not directly regulated by hemoglobin levels, but by the systemic availability of oxygen, in a strictly regulated feedback loop [4].

During the first week of life, the hemoglobin levels of the term newborn infants start to decrease progressively for the next 6 to 8 weeks. This process occurs in both term and preterm neonates. The postnatal plasma levels of EPO exhibit a predictable pattern of change, which is inversely associated with hemoglobin levels. Also, there is a directly proportional association between the EPO levels and the reticulocyte counts [5].

3. Physiologic anemia of the newborn

After birth, newborn infants undergo dramatic changes in tissue oxygenation. When the lungs replace the placenta as the source of oxygen, the arterial oxygen saturation increases about two-fold, which is followed by a decrease in EPO levels, resulting in the characteristic transient decay in hemoglobin levels, referred to as the "physiologic anemia of the newborn". Furthermore, the reduction in hemoglobin levels is also accompanied by a gradual decrease in the RBC count and the mean corpuscular volume (MCV). In term neonates, the physiologic anemia occurs at 4 to 6 weeks of life, persisting until the hemoglobin levels falls to approximately 10 to 11 g/dL, thus stimulating the EPO production and release by the kidneys, as a compensatory process [6].

Full-term neonates have from 50 to 80% of hemoglobin F and 15 to 50% of hemoglobin A, yet the new erythrocytes produced after birth will contain mostly hemoglobin A, allowing better tissue oxygenation, as the last has higher oxygen affinity the hemoglobin F. Another adaptative mechanism that improves oxygenation at this phase is the increase of 2,3-diphosphoglycerate concentration inside the erythrocytes, thus improving the oxygen release from hemoglobin [5, 7].

The gradual decrease in EPO production is not described as the unique cause of the physiologic anemia of the newborn. Reduced red cell lifespan and hemodilution are also involved in this process.

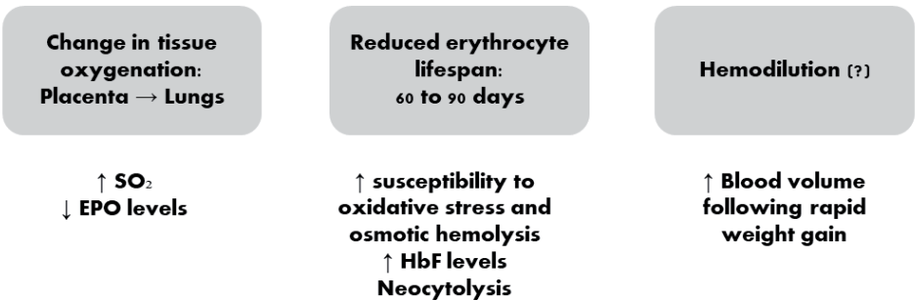


Figure 1.
Causes of physiologic anemia of the newborn.

The main causes of the physiologic anemia of the newborn are presented in **Figure 1**.

Differently from normal adult erythrocytes that have a life span of 120 days, the neonate erythrocytes life span is only 60 to 90 days [8]. This shortened survival is attributed to greater sensitivity and osmotic hemolysis and oxidative injury than the adult red blood cells. The neonatal erythrocyte has lower levels of antioxidant enzymes such as glutathione peroxidase, increasing thus their susceptibility to oxidative stress, and subsequential formation of Heinz bodies and methemoglobin, which contributes to reducing their lifespan [3, 9]. Additionally, a process named neocytolysis is also responsible for the reduced life span of the erythrocytes. It consists of the active removal of young erythrocytes generated in a relatively hypoxic condition when the individual is exposed to normoxic or hyperoxic conditions, as occurs in the change from placental to lung respiration. Furthermore, the higher concentration of hemoglobin F in the neonates is also responsible for the decreased life span of the neonatal erythrocyte, as this type of hemoglobin tends to denature and promote damage to the erythrocyte membrane from the inside [10].

The hemodilution due to the increased blood volume following the rapid weight gain in the first months of life may also contribute to the physiologic anemia, although it is not clearly understood the impact of this factor in the decreased hemoglobin levels and RBC counts [6].

4. Anemia in preterm infants

Although all neonates develop the physiologic anemia of the newborn, the preterm infants experience a greater degree of anemia, the anemia of prematurity, which results from a combination of physiologic and pathological factors.

The postnatal drop in hemoglobin levels occurs more rapidly in preterm infants, reaching the nadir before the term ones at 4 to 6 weeks, with approximately 7 to 8 g/dL, in contrast with hemoglobin levels of 10 to 11 g/dL at the nadir of those born in term, which generally occurs at 10 to 12 weeks of life. The hemoglobin levels are gestational age dependent, thus, the more premature neonates, and the low-birth-weight ones, usually present deeper degrees of anemia [11].

The diminished production of EPO in response to anemia in premature infants is believed to exert a key role in the pathophysiology of anemia of prematurity. This is partly explained by the fact that the liver is responsible for EPO production in intrauterine life. The switch to kidneys as the main source of EPO does not occur right after birth as in the term infants. Thus, because the development of the

preterm infant is not properly completed, after birth the liver continues to be the principal site of EPO production, which is known to be less responsive to hypoxia than the kidneys. An accelerated metabolism by clearance of EPO, which is from two to fourfold in preterm infants in comparison to adults, is also responsible for the reduced levels observed in preterm infants. Therefore, the reduced EPO levels in preterm infants are a result of both diminished production and increased metabolism [12].

The iatrogenic blood loss due to phlebotomy for laboratory testing in the intensive care units is described by many authors as the main responsible for anemia of prematurity [13–15]. The practice of intensive care neonatology requires serial laboratory testing such as blood gases, electrolytes, hemocultures, hemogram. Because small premature infants usually need close monitoring, they suffer the loss of great amounts of blood.

In neonates, the total blood volume varies from 80 to 115 mL/kg, thus, a 10 mL blood sample can correspond to more than 10% of the total blood volume of a premature infant [2]. The first weeks of intensive care usually are the period when the greater amounts of blood are withdrawn [16]. In the NICUs, approximately 15 to 30% of the total blood volume per week are required for laboratory tests. For extremely preterm infants, the iatrogenic blood loss is even greater, representing up to one-third of the total blood volume in the first month of life [13]. Also, there is a so-called "hidden" blood loss due to the sampling for laboratory tests, present in cotton pads, gauze and the dead space of syringes, or tubing of butterfly needles [17].

There is a significant correlation between the amounts of blood collected for laboratory testing and the volume of blood transfused in NICU patients, where the amount of blood transfused can be equal to or greater than the blood drawn, reinforcing the evidence that the iatrogenic blood loss is a major contributor to anemia in premature infants [15].

Besides the iatrogenic blood loss, other non-physiologic conditions can aggravate the development of anemia in the neonatal period, such as blood loss, hemolysis, infections, nutrient deficiencies and infection.

<table>
<tr><th colspan="3">Non-physiological causes of anemia in the neonatal period</th></tr>
<tr><th>Blood loss</th><th>Decreased erythrocyte production</th><th>Increased erythrocyte destruction</th></tr>
<tr><td>• Fetomaternal hemorrhage</td><td rowspan="2">• Infections:
Parvovirus B-19
Cytomegalovirus
Syphilis
Toxoplasmosis</td><td>• Immune-mediated hemolysis</td></tr>
<tr><td>• Twin-twin transfusion</td><td rowspan="3">• Red cell membrane deffects:
Hereditary Spherocytosis
Hereditary elliptocytosis
Hereditary pyropoikilocytosis</td></tr>
<tr><td>Fetoplacental hemorrhage</td><td rowspan="3">Nutritional deficiencies:
Iron
Vitamin B12
Folate
Copper
Vitamins : A, C, E.</td></tr>
<tr><td>• Cord and placental malformations</td><td>• Red cell enzymatic deficiencies:
G6PD deficiency
Piruvate kinase deficiency</td></tr>
<tr><td>• Internal hemorrhage: head, brains, lungs, liver</td><td rowspan="2">Congenital disorders of erythrocyte production:
Diamond-BlackFan Anemia
Fanconi Anemia</td><td rowspan="2">Hemoglobinopathies:
α and β Thalassemia</td></tr>
<tr><td>• Iatrogenic blood loss</td></tr>
</table>

Figure 2.
Non-physiologic causes of neonatal anemia.

In the neonatal period, the causes of anemia are classified into 3 groups: blood loss, decreased erythrocyte production and increased erythrocyte destruction. The causes of neonatal anemia are resumed in **Figure 2**.

5. Blood loss

Blood loss in the neonates can occur before, during or after the delivery, by several mechanisms, associated with 5–10% of the cases of severe neonatal anemia [18].

During pregnancy, the passage of fetal blood cells to maternal circulation, which is called fetomaternal hemorrhage, occurs in 50% of all pregnancies without promoting any harm to the mother or the fetus. However, in about 1 to 10% of pregnancies, fetomaternal hemorrhage can be substantial, when the blood loss reaches 40 to 100 mL. The fragile separation between the maternal and fetal circulation is described as the main mechanism behind fetomaternal hemorrhage before delivery. Fetomaternal hemorrhage also occurs during delivery, as a consequence of trauma and erosion to placental villi during labour. Other causes of fetomaternal hemorrhage include diagnostic amniocentesis, percutaneous umbilical blood sampling, and traumatic injury to the mother during pregnancy [11, 19].

Another cause of fetal hemorrhage before delivery is the twin-twin transfusion, which occurs in approximately one-third of all monozygotic, monochorionic twin pregnancies, with a mortality rate of approximately 15%. Twin-twin transfusion is responsible for considerable discrepancies of hemoglobin levels between the twins, usually greater than 5 g/dL, leading to simultaneous anemia and polycythemia. The donor twin usually presents low birth weight and may manifest congestive heart failure and shock. The receptor twin is at risk of hyperviscosity syndrome, disseminated intravascular coagulation, hyperbilirubinemia and respiratory distress [11, 20, 21].

In the intrapartum period, fetoplacental hemorrhage can occur, commonly when the infant is held above the placenta, as in cesarean section, before the cord clamping [11].

Cord malformations, such as velamentous insertion are associated with an increased risk of rupture of the blood cord vessels during labour, leading to significant intrapartum hemorrhage. Placental abnormalities such as placenta previa and abruptio placentae can also result in significant maternal and fetal blood loss during delivery [17, 18, 20].

Internal hemorrhage in the newborn can be caused by trauma during birth, bleeding disorders, vitamin K deficiency, and congenital vascular malformations. The most common sites of internal hemorrhage in the neonate are the head, brain, lungs, liver, and less frequently spleen, and adrenals. Traumatic deliveries are frequently associated with subdural/subarachnoid hemorrhage and cephalhematoma [11, 18, 22, 23].

6. Hemolysis

An important contributor to neonatal anemia is the increased erythrocyte destruction by hemolysis, which is associated with congenital and acquired conditions. In the neonatal period, hemolytic disorders can be classified as immune-mediated hemolysis, red cell membrane defects, red cell enzymatic deficiencies and hemoglobinopathies.

6.1 Immune mediated-hemolysis

Immune-mediate hemolysis occurs when fetal erythrocytes enter the maternal circulation, physiologically, by fetomaternal hemorrhage or amniocentesis. Surface antigens present in fetal red blood cells stimulates the maternal immune system to produce antibodies against them. Those antibodies are usually IgG, which can cross the placental barrier and enter the fetal circulation, marking the fetal erythrocytes for removal by the reticuloendothelial system [18]. The majority of cases of immune-mediated hemolysis in the neonatal period are result of ABO and Rh incompatibilities, where the Rh system is associated with more severe anemia. Other blood group systems like Kell and Duffy can also result in clinically relevant hemolysis [24].

6.2 Red cell membrane defects

Quantitative or qualitative disorders in red blood cell membrane proteins can impair erythrocyte deformability and stability, therefore leading to hemolysis.

Hereditary spherocytosis (HS) is the most common hemolytic anemia due to red blood cell membrane defect, which is usually transmitted as an autosomal dominant trait. The central event in HS is the loss of membrane surface area, leading to reduced deformability, due to defects of membrane proteins ankyrin and spectrin, band 3 or protein 4.2. Abnormal spherocytes with impaired deformability are frequently trapped and destroyed in the spleen leading to hemolysis and thus to anemia and hyperbilirubinemia. In the neonatal period, hemolysis can be enhanced because hemoglobin F poorly binds 2,3 diphosphoglycerate, which remains in the free form, contributing to the destabilization of protein interactions in the erythrocyte membrane. Anemia is the most frequent complication of HS in the neonatal period. Other consequences include cholelithiasis, hemolytic episodes, and aplastic crises [10, 18, 25, 26].

Hereditary elliptocytosis (HE) is a group of diseases that are characterized by the presence of elliptical erythrocytes in the peripheral blood, the main defects in this disease concern the alpha and beta spectrin, protein 4.1, band 3 and rarely glycophorin A. Although is more frequent than HS in the general population, HE is less likely to cause significant hemolysis and anemia. HE is usually asymptomatic until 4–6 months of life, although neonatal hemolytic anemia, jaundice and fetal hydrops are also described [10, 27, 28]. Hereditary pyropoikilocytosis is a subtype of HE, where the erythrocyte morphology resembles those seen in the blood smear of patients after severe burns. It is more frequent in individuals of African descendance and causes severe anemia and hemolysis in the neonatal period [9].

6.3 Red cell enzymatic deficiencies

Defects in red blood cells enzymes production can induce damage and decrease the lifespan of erythrocytes. The most common is Glucose-6-pyruvate-kinase (G6PD) deficiency, a hereditary X linked recessive disorder that affects over 400 million people in the world. G6PD catalyze the first reaction in the pentose phosphate pathway, responsible for NADPH production, which is essential to maintain adequate levels of reduced glutathione for protecting the erythrocyte from oxidative stress. G6PD deficient erythrocytes are vulnerable to oxidative damage and hemolysis In the neonatal period, G6pD deficiency can result in significant hemolytic anemia and hyperbilirubinemia that may require phototherapy and exchange transfusion [9, 10, 29, 30].

The second enzymatic deficiency associated with neonatal hemolytic anemia is for pyruvate kinase (PK), an enzyme from the glycolytic pathway of the erythrocyte, which is responsible for the generation of adenosine triphosphate (ATP). Therefore, PK deficiency reduces the amount of energy available for the erythrocytes, which cannot maintain their content of potassium and water, resulting in subsequent premature cell death. Most newborns with PK deficiency develop severe jaundice, hemolytic anemia and less frequently, liver dysfunction [10, 18, 31].

6.4 Hemoglobinopathies

Erythrocyte hemoglobin defects are also responsible for clinically relevant cases of neonatal anemia. Thalassemia is a group of genetic disorders of globin-chain production, that results in an imbalance between the α-globin and β-globin chain production. The α-thalassemia syndromes result from the deletion of one or more genes of the four α-globin genes, and its clinical manifestation will depend on the number of genes deleted. A single α-globin gene deletion results in a carrier state which is asymptomatic. Deletion of two genes results in α-thalassemia trait, which usually presents as microcytosis and mild anemia. Hemoglobin H disease results from the deletion of 3 α-globin genes. This leads to a significant imbalance between α and β chain production and the formation of hemoglobin H (β4) and hemoglobin Barts (γ4). Patients with hemoglobin H disease are often born with hypochromic, hemolytic anemia, and are at risk for significant neonatal hyperbilirubinemia. The deletion of all 4 α-globin genes results in homozygous α-thalassemia, with a high risk of intrauterine death, severe hemolytic anemia and hydrops fetalis [9, 10, 32].

Hemoglobin H is unstable and has an extremely high oxygen affinity and is thus unable to effectively deliver oxygen. It is also relatively unstable and causes ineffective erythropoiesis and hemolytic anemia, due to membrane injury from oxidative damage resulting in shortened red cell survival [33].

The β-globin chain defects, including sickle cell disease and β-thalassemia, are not usually associated with anemia or hemolysis during the neonatal period, because the erythrocytes of newborn infants contain large amounts of fetal hemoglobin (α2γ2) [9].

7. Decreased erythrocyte production

7.1 Infections

Congenital infections are an important cause of neonatal anemia by decreased erythrocyte production. Parvovirus B19, a DNA virus with an affinity for erythroid progenitor cells can be vertically transmitted to the fetus during pregnancy in susceptible women, who represent approximately 35% of all pregnancies, leading to severe anemia, miscarriage, non-immune hydrops and stillbirth, although the majority of the cases is asymptomatic [20, 22].

Other congenital infections associated with anemia in the neonatal period are cytomegalovirus, syphilis and toxoplasmosis [34–36].

7.2 Nutritional deficiencies

Although iron deficiency is not a common issue in the neonatal period for full-term infants, as the neonatal iron stores are obtained from maternal blood at the last weeks of the third trimester, the preterm neonates have lower iron stores. In consequence, these individuals are more prone to iron deficiency anemia in the

postnatal period, concomitantly to physiologic anemia [37]. Also, low birth-weight infants and those with perinatal blood loss are at risk of developing iron deficiency anemia. Other nutritional deficiencies that can imply the development of anemia in the newborn include folate, copper, vitamin B12, vitamin B6, vitamin A, vitamin C and vitamin E [18, 38].

7.3 Congenital disorders of erythrocyte production

Diamond-Blackfan anemia (DFA) is a rare congenital syndrome that affects mostly the erythroid precursor in the bone marrow. DFA is characterized by severe anemia in infants, that is usually macrocytic or normocytic, with reticulocytopenia. Approximately 25% of patients are anemic at birth, and hydrops fetalis occurs rarely. Associated congenital abnormalities include triphalangeal or duplicated digits, abnormal facies, genitourinary, musculoskeletal, cardiac and ophthalmological abnormalities. Various mutations in genes encoding ribosomal proteins are responsible for the pathogenesis of DFA [3, 39].

Fanconi's anemia (FA) is a rare autosomal recessive disorder associated with bone marrow failure and increased susceptibility to leukemia and other types of cancer. Patients with FA usually present congenital malformations and have high sensitivity to alkylating agents and radiation. Various mutations are associated with FA, and all FA genes code for proteins that play roles in various cellular pathways, especially in DNA cross linking and repair. In the neonatal period, FA patients may present with cytopenias, congenital malformations, or both [9, 40].

8. Diagnosis of anemia

The diagnosis of anemia in the newborn can be challenging, especially for those in intensive care. However, the accurate diagnosis and the determination of the underlining cause of the anemia is essential for adequate clinical management.

8.1 Clinical diagnosis

The diagnostic approach usually includes the patient history (familial, maternal, patient), physical examination and laboratory tests.

The patient history is based on familial and maternal medical history, pregnancy, delivery and postpartum period.

Familial history must be checked for the presence of any relatives with history of chronic anemia because genetic causes for neonatal anemia could be present such as Diamond-Blackfan and Fanconi anemia, enzymatic deficiencies and erythrocyte membrane defects.

Maternal medical history concerns the before pregnancy, pregnancy and postpartum. Bleeding disorders, history of chronic anemia, infections, medication use, trauma or vaginal bleeding, must be included on maternal medical history. Furthermore, maternal laboratory tests such as blood type, screening for antibodies and hemogram can provide useful information for the diagnostic approach of the anemia in the newborn.

As the twin-twin hemorrhage is a substantial cause of neonatal anemia due to blood loss, the type of pregnancy (multiple or singleton) is also relevant for diagnosis [11].

The method of delivery and the presence of intrapartum intercurrences like maternal hemorrhage or newborn distress should also be considered.

The patient history contains information concerning age, gestational age at the delivery, sex, birthweight, history of infections, ventilatory support, medication and transfusions.

Physical examination of the anemic newborn must verify diminished activity, feeding difficulties and dyspnea at rest. Skin examination is necessary for check the presence of pallor, which is present generally in various types of anemia, and jaundice, which is associated with hemolytic anemias. Additionally, hepatosplenomegaly presence is an important finding in physical examination, which is also an indicative of hemolytic anemia [22].

9. Laboratory diagnosis

For the laboratory diagnosis of anemia, a stepwise diagnostic approach is recommended to avoid unnecessary tests. This approach is benefic for preventing iatrogenic blood loss of the newborn and other consequences of repeated blood withdrawals, and also reduces the costs for diagnosis. The laboratory tests for the diagnosis of neonatal anemia are presented in **Figure 3**.

The first step on the laboratory diagnosis is the establishment of the anemia, which concerns an adequate interpretation of a complete blood count considering proper reference values for the newborn infant age. Once there is a diagnosis of anemia, the second step is to determine the cause, which can be challenging, especially for preterm infants under intensive care. There are numerous laboratory tests that can be helpful to establish the cause of anemia. However, its choice will depend on the clinical examination, and also on the availability of tests where the NICU is located.

Reticulocyte count will provide useful information concerning the bone marrow capacity to produce new erythrocytes. Automated reticulocyte counts are more accurate than manual techniques because of the standardization of cell size and DNA content, which enables the identification of the number of reticulocytes per microliter of blood [41].

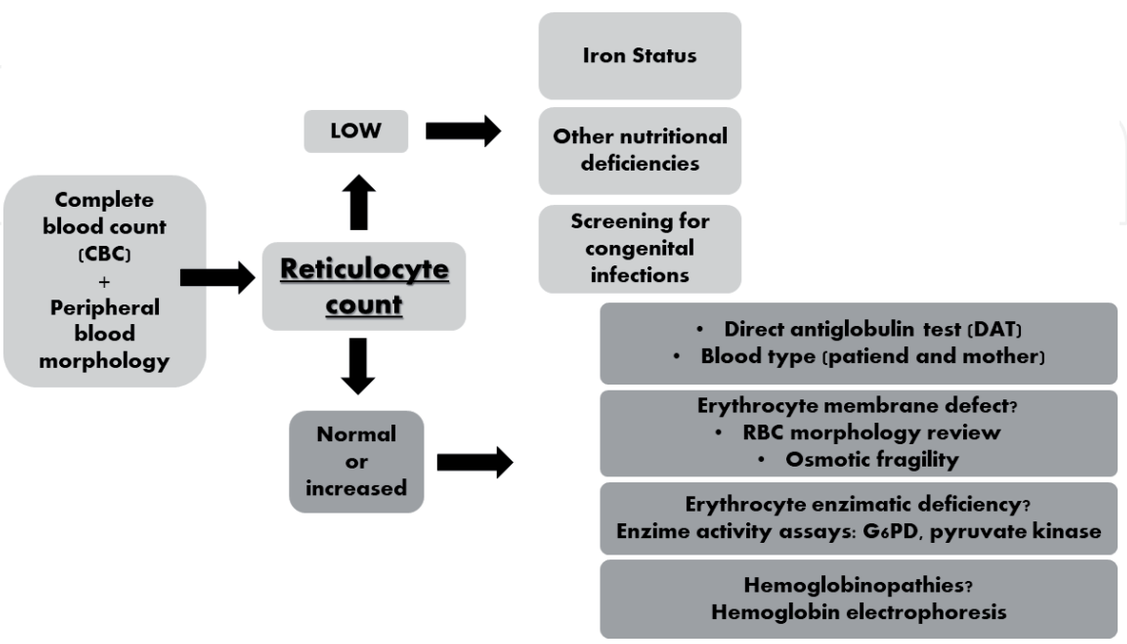


Figure 3.
Laboratory tests for diagnosis of neonatal anemia.

During the first two weeks of life, the reticulocyte count decreases gradually. In infants with anemia, however, the bone marrow will attempt to compensate through increased erythropoietic activity, characterized by an elevated reticulocyte count. If the erythropoiesis in the bone marrow production is impaired, the reticulocyte count will remain low. In the presence of anemia with low reticulocyte counts, a diagnosis of bone marrow suppression or dysfunction should be considered. The next steps may comprehend the nutritional deficiencies with a complete iron status and vitamin and other elements if necessary [18].

Besides the reticulocyte count, the automated hematologic analyzers have useful parameters for the assessment of iron status, like the reticulocyte hemoglobin content (CHr) and immature reticulocyte fraction (IRF), which reflects the actual availability of iron for erythropoiesis. These parameters are helpful in the assessment and early detection of anemia with no additional costs or need for more blood sampling [42, 43].

To identify iron deficiency anemia, the hemoglobin concentration must be confirmed by other measurements of iron status. Iron status markers include: hemoglobin concentration, mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), Chr, total iron-binding capacity, serum ferritin (SF) and transferrin saturation.

As SF is an acute-phase protein, its concentrations may be elevated in the presence of chronic inflammation, infection, malignancy, or liver disease. Because Chr is not affected by these factors or anemia of chronic disease, it is a preferable marker of iron status. It provides a measure of iron available to cells recently released from the bone marrow [41, 44].

Serology for infectious congenital diseases associated with decreased erythrocyte production such as parvovirus B19, cytomegalovirus, syphilis and toxoplasmosis should also be performed. Bone marrow aspirate/biopsy should be performed in the cases where de peripheral blood tests were not enough to establish a proper diagnosis of the cause of anemia. The congenital disorders of erythrocyte production are also characterized by anemia with low reticulocyte counts. If there is a clinical suspicion of genetic disorders such Fanconi anemia and Diamond-Blackfan, specific molecular tests must be required [39, 40].

In the anemic patient with a normal or elevated reticulocyte count, the next diagnostic steps should be focused on the hemolytic anemias.

Evaluation of the hemolytic anemia in newborn infants should include blood typing of the mother and the infant and direct antiglobulin testing (DAT), to detect immune-mediated hemolysis [29].

Examination of the peripheral blood smear is also essential for a proper diagnosis of anemia. Neonatal erythrocytes show considerable heterogeneity, with a greater number of irregularly shaped RBCs, particularly in premature infants, which difficult the morphological analysis. Nucleated RBCs are present in the blood of healthy newborns on the first day of life. Finding 0 to 10 nucleated RBCs per 100 WBCs is typical. In term infants, nucleated RBCs are rapidly cleared from the circulation after birth. However, they persist in peripheral blood in small numbers for up to 1 week of life in preterm infants [2]. Increased counts are often the result of prematurity, anemia of different etiologies, increased erythropoiesis from chronic conditions, acute stress-mediated release from the marrow stores, and postnatal hypoxia [45].

With significant hemolysis, fragmented red blood cells (schistocytes) are usually observed. Additionally, close attention should be paid to the presence of spherocytes or elliptocytes. Hereditary spherocytosis and elliptocytosis, the most commonly inherited membrane defects, can present as hemolytic anemia [25–27]. If the presence of hereditary spherocytosis is suspected, special attention should

be paid to the mean corpuscular hemoglobin content (CHCM), which is usually increased (CHCM >36 g/dL) [28].

If there is clinical suspicion of an erythrocyte membrane defect, the osmotic fragility test will be helpful to confirm the diagnosis. Glycerol lysis and Pink tests are also used as first-line laboratory tests. Other more specialized tests for erythrocyte membrane defects include: flow cytometry, ektacytometry and SDS-PAGE of erythrocyte membrane proteins [28, 29, 46].

Observation of erythrocyte morphology can be useful in cases of erythrocyte enzymatic deficiencies. In the peripheral blood of individuals with G6PD deficiency, bite cells and blister cells are usually seen, as a consequence of oxidative damage to the erythrocyte membrane [30]. The definitive diagnosis of G6PD deficiency will be determined with quantitative tests of enzyme activity. However, falsely negative tests in severe cases of enzymatic deficiency, where great amounts of erythrocytes are removed from the circulation by hemolysis. In those cases, if the clinical suspicion remains, the test for enzymatic activity must be repeated after 3 months [31].

As the hematologic features of pyruvate kinase deficiency are not different from other hemolytic anemias, the definitive diagnosis is obtained with direct quantitative tests for enzyme activity. If there is a clinical suspicion of an erythrocyte enzyme defect, both G6PD and PK tests should be performed [31].

Other laboratory tests that are usually helpful for determining the presence of hemolytic anemia are: increased indirect bilirubinemia, increased LDH and decreased haptoglobin [46].

Thalassemias are usually characterized by low mean corpuscular volume (MCV) and low mean corpuscular hemoglobin (MCH). It is also necessary to exclude iron deficiency anemia by performing a complete iron profile. Morphological features in the peripheral blood are usually helpful to sustain the suspect of thalassemia. Anisopoikilocytosis (variation in shape and size of the erythrocytes) with the presence of target cells are the most typical changes. Tear-drop cells and erythroblasts are usually seen in the most severe forms. Basophilic stippling and polychromasia are also frequent. For the investigation of thalassemia, hemoglobin electrophoresis and hemoglobin HPLC are used as the main screening tests. Confirmatory tests, if necessary, are made by DNA analysis for α and β-globin mutations [47].

10. Issues related to preanalytical condition in laboratory diagnosis

The quality of laboratory test results is affected by preanalytical variables such as sample collection, specimen handling, sample size, and analytic interference. The most important precatalytic factors that influence the laboratory results of neonates and infants are the limited blood availability, the variation of results depending on blood sampling sites, and the effect of vigorous crying or exertion on hematologic test results [2].

The total blood volume depends on the height and weight of the individual and, therefore, is markedly low in infants, especially in premature newborns. Repeatedly blood withdrawal is associated with iatrogenic anemia, thus, this practice must be avoided as much as possible [16].

As the blood collection tubes contain a fixed amount of anticoagulant, collecting an amount of blood which is smaller than the recommended by the manufacturer may result in hemodilution or clotting and cause erroneous results. In order to avoid such problems, pediatric collection tubes for reduced volumes can be used. However, as most of pediatric tubes does not follow standard dimensions, its use brings challenges to clinical laboratories, including the need for manual handling,

because the hematology analyzers are not usually adapted to process those tubes automatically. In addition, blood clotting, hemolysis, and insufficient sample volume are common issues when dealing with neonatal specimens [2, 48].

Hemolysis is the most frequent preanalytical issue, especially in neonatal patients [49] The main cause of hemolysis in those patients is the site of collection, where de arterial catheter collections are associated with greater numbers of hemolyzed samples [48].

The blood sampling site is also a relevant factor that influences the complete blood count results. Significant differences between capillary, venous and arterial sites have been reported. Thus, it is useful to concern the sample of collection for proper interpretation of the results [50, 51].

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