OBSTETRICS

Suppression of compensatory erythropoiesis in hemolytic disease of the fetus and newborn due to intrauterine transfusions

Isabelle M. C. Ree, MD; Enrico Lopriore, MD, PhD; Carolien Zwiers, MD, PhD; Stefan Böhringer, PhD; Marleen W. M. Janssen, BSc Medicine; Dick Oepkes, MD, PhD; Masja De Haas, MD, PhD

BACKGROUND: Infants with severe hemolytic disease of the fetus and newborn often require 1 or multiple intrauterine transfusions to treat fetal anemia. Intrauterine transfusions may have an inhibiting effect on fetal and neonatal erythropoiesis.

OBJECTIVE: To quantify the effect of 1 or multiple intrauterine transfusions on the fetal erythropoiesis by assessing the fetal reticulocyte counts in a population with severe hemolytic disease of the fetus and newborn.

STUDY DESIGN: This was an observational cohort study in infants admitted to the Leiden University Medical Center who received 1 or multiple intrauterine transfusions for hemolytic disease of the fetus and newborn caused by (Rh)D or Kell antibodies and were born between January 2005 and December 2018.

RESULTS: A total of 235 patients were included, of whom 189 were patients with D-mediated hemolytic disease of the fetus and newborn and 46 with Kell-mediated hemolytic disease of the fetus and newborn. Absolute fetal reticulocyte count in D-mediated hemolytic disease of the fetus and newborn declined exponentially over the course of consecutive intrauterine transfusions, with a 62% decline after 1 intrauterine transfusion (95% confidence interval, 56—67). A similar exponential decline was observed in Kell-mediated hemolytic disease of the fetus and newborn, with 32% (95% confidence interval, 19—45) decline after 1 intrauterine transfusion. This decline was not associated with the varying gestational age at the time of the first intrauterine transfusion or the total number of intrauterine transfusions. The number of red blood cell transfusions for postnatal anemia was greater for infants with D and Kell-mediated hemolytic disease of the fetus and newborn with >2 intrauterine transfusions (median of 3 [interquartile range, 2—3] vs 2 [interquartile range, 1—3], P=.035, in D-mediated disease and median of 2 [interquartile range, 1—2] vs 1 [interquartile range, 1—1], P<.001, in Kell-mediated disease). Infants born after >2 intrauterine transfusions less often required exchange transfusion in D-mediated hemolytic disease of the fetus and newborn (19/89 [21%] vs 31/100 [31%], P=.039), compared with infants with 1—2 intrauterine transfusions.

CONCLUSION: Treatment with intrauterine transfusions causes an exponential decrease in fetal reticulocyte counts in both D- and Kell-mediated hemolytic disease of the fetus and newborn. Suppression of the compensatory erythropoiesis leads to prolonged postnatal anemia and an increased requirement of red blood cell transfusions after birth.

Key words: alloimmunization, erythropoiesis, hemolytic disease of the fetus and newborn, intrauterine transfusion, reticulocytes

Hemolytic disease of the fetus and newborn (HDFN) is caused by an incompatibility between maternal and fetal erythrocyte antigens. HDFN is characterized by fetal and neonatal erythroid cell destruction due to maternal alloantibodies, which will induce compensatory erythropoiesis. In case of insufficient compensation, fetal or neonatal anemia may occur and intrauterine treatment with 1 or more red blood cell (RBC) transfusions may be indicated, as well as transfusions for persistent anemia after birth.[1,2]

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The exact effects of intrauterine transfusions (IUTs) with adult donor RBCs, which carry a different type of hemoglobin and have different oxygen binding and release characteristics compared with fetal red cells, is not known. In small populations, the effect of IUT(s) on various hematologic parameters such as fetal hematocrit, hemoglobin, leukocytes, and bilirubin has been studied.[3—5] Reticulocyte counts at birth appeared to be lower in infants with HDFN who received 1 or multiple IUTs compared with infants who were not treated with IUTs, irrespective of the hemoglobin level at birth.[3,6] Treatment with IUTs is also associated with a greater number of transfusions after birth compared with infants with HDFN not treated with IUT(s).[7] An inhibiting effect of donor blood on the fetal and neonatal erythropoiesis has been postulated before.[3] The total number of IUTs per infant may be of clinical relevance to select infants at increased risk for a complicated postnatal course.

A wider understanding of the effects of IUTs on fetal and neonatal erythropoiesis is necessary to clarify the pathophysiologic mechanisms underlying both the intrauterine and postnatal course of HDFN. In this study, we specifically aimed to quantify the effect of 1 or multiple IUTs on fetal erythropoiesis by assessing the reticulocyte counts in a large population of fetuses and infants with severe HDFN.

Methods

Study population

All infants admitted to the Leiden University Medical Center (LUMC) who received treatment with 1 or multiple IUT(s) for the treatment of HDFN caused by (Rh)D or Kell antibodies and who were born between January 2005

AJOG at a Glance

Why was this study conducted?

This study aimed to quantify the effect of intrauterine transfusions on fetal erythropoiesis in hemolytic disease of the fetus and newborn.

Key Findings

Treatment with intrauterine transfusions causes an exponential suppression of the compensatory erythropoiesis in hemolytic disease of the fetus and newborn and an increasing number of intrauterine transfusions is associated with a higher postnatal transfusion dependency.

What does this add to what is known?

In both D- and Kell-mediated hemolytic disease of the fetus and newborn, infants treated with multiple intrauterine transfusions are more likely to have a high postnatal transfusion dependency.

and December 2018 were eligible for the study. In the Netherlands, all pregnant women are routinely screened for the presence of alloantibodies in pregnancy, and maternal blood samples with a positive screening result are sent to 1 of the 2 national referral laboratories (Sanquin Diagnostic Services or the Special Institute for Blood group Investigations). Thereafter, the clinical relevance of the antibody is evaluated by assessing the antibody specificity and by assessing whether the fetus is antigen-positive. If the fetus is positive, the risk on fetal hemolysis is assessed by serially determining the antibody titer and antibody-dependent cell-mediated cytotoxicity.

Referral to the LUMC as national specialized center is indicated if laboratory parameters are above determined cut-offs. These cut-offs are antibody titers tested in maternal serum $\geq$16 in D alloimmunization and $\geq$2 in Kell alloimmunization, or in case of an antibody-dependent cell-mediated cytotoxicity assay $\geq$50% in D alloimmunization and $\geq$30% in Kell alloimmunization. Subsequently, these high-risk pregnancies are monitored by serial Doppler measurements to assess the velocity of the blood flow in the middle cerebral artery. If MCA Doppler of the middle cerebral artery exceeds 1.5 multiples of the median or if signs of hydrops are present, treatment with a first or subsequent IUT is indicated. One or more IUTs can be administered until 34−35 weeks of gestation, after which induced delivery is preferred to IUT treatment. The IUT technique used in the Netherlands has been previously described.[8] IUTs consist of irradiated, Parvovirus B19 and Cytomegalovirus seronegative packed erythrocytes, with an increased hematocrit of 0.80−0.85 L/L to minimize the risk of volume overload in the fetus. IUTs are preferably administered intravascularly, either into the placental cord insertion or into the intrahepatic part of the umbilical vein (often in combination with additional intraperitoneal transfusion), depending on the orientation of the placenta. To confirm the suspected fetal anemia, a fetal blood sample is taken before the procedure. Planned delivery at the LUMC and neonatal admission to the neonatal intensive care unit of the LUMC is recommended for all pregnancies after IUT.

Infant and fetal data were excluded from analyses in case of HDFN caused by other alloantibodies than D or Kell and major congenital malformations. Unsuccessful IUTs were defined as transfusion with a volume of less than 5 mL, as the pre-IUT blood sample is 5 mL, and were excluded.

In HDFN mediated by Kell antibodies, IUTs are generally needed earlier in gestation compared with D-mediated disease and erythroid suppression seems to be the predominant mechanism in producing fetal anemia rather than hemolysis.[9−11] The results of infants with D and Kell-mediated HDFN were therefore reported separately.

Data collection

Data were extracted from the hospital's patient database, including maternal and neonatal medical files and laboratory outcomes. Follow-up data on transfusions after discharge from the LUMC were collected from referral hospitals. Written consent was obtained from the parents or caregivers, and all personal data were coded before analysis. The following maternal and fetal data were recorded: number of previous births, antenatal intravenous immunoglobulin administration, maximum antibody titer, maternal age at first IUT, fetal gestational age at each IUT, total number of IUTs, volume dosage of IUT, IUT procedure access site (placental cord insertion or intrahepatic transfusion with or without intraperitoneal transfusion), fetal hemoglobin levels and leukocyte, platelet, and reticulocyte counts before every IUT. The following infant data were recorded: sex, gestational age at birth, birth weight, hemoglobin level at birth, reticulocyte count at birth, bilirubin level at birth, number of days of phototherapy, treatment with exchange transfusion (ET), treatment with postnatal RBC transfusion(s) the first 3 months after birth, and the number of postnatal transfusion(s) per infant (also known as "top-up" transfusions).

Referral hospitals receive the protocol for postnatal transfusions of the LUMC after discharge to their center to unify neonatal management. The current transfusion guideline of the department recommends a transfusion in term infants with HDFN when hemoglobin levels fall below 10.5 g/dL (6.5 mmol/L) for day 0−6, below 8.9 g/dL (5.5 mmol/L) for day 7−13, and below 7.2 g/dL (4.5 mmol/L) from day 14 onwards. A transfusion of 15 mL/kg irradiated packed erythrocytes less than 5 days old was advised throughout the study period, with a hematocrit of 0.50−0.65 L/L.

Exchange transfusion in the Netherlands is indicated within 24 hours after birth if the serum bilirubin level is above the cut-off values for exchange transfusion and proceeds to rise despite adequate intensive phototherapy (consisting of 4 phototherapy lamps), or if after 24 hours the bilirubin is above the cut-off values for exchange transfusion.

The study protocol and analysis plan were approved by the ethics committee of the LUMC (G19.041) and scientific committee of our department.

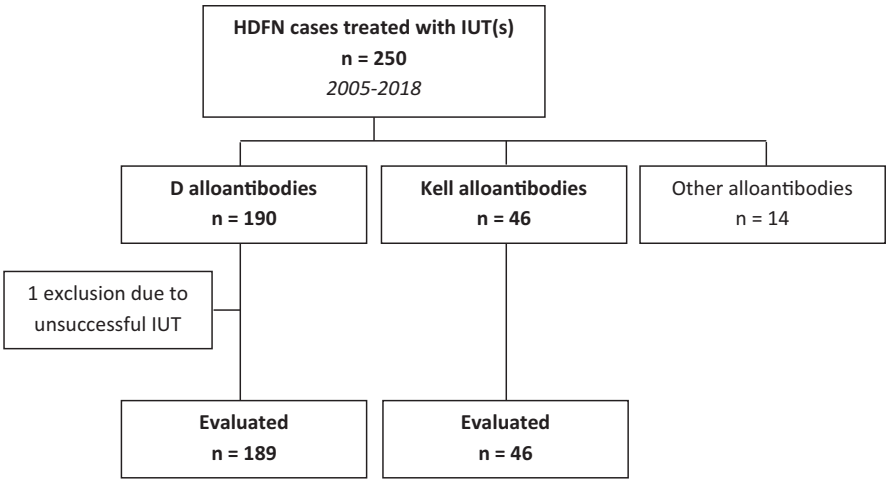
Primary and secondary outcomes

The primary outcome in this study was the suppression of fetal erythropoiesis, as defined by the decline (%) in absolute reticulocyte count ($\cdot 10^9$/L) per consecutive IUT. The primary outcome was adjusted for the total number of IUTs per infant (as indicator of disease severity) and gestational age in weeks at time of the first IUT as measure for suppression of fetal erythropoiesis. The relative reticulocyte count, as expressed per thousand RBCs (‰), also was reported. Secondary outcomes were the change in leukocytes and platelets per IUT, the reticulocyte counts at birth (absolute and relative counts), ferritin levels at birth, the proportion of neonates requiring ET, and the proportion of neonates requiring RBC transfusion(s) after birth.

Statistical analysis

Data are presented as mean (± standard deviation) or median (interquartile range [IQR]) depending on the underlying distribution. The primary outcome is visualized in a boxplot. A linear mixed model was performed to account for the fact of repeated measurements and allow for covariate adjustment. Effect sizes are reported together with 95% confidence intervals (95% CI). The outcome variable of the model was the absolute reticulocyte count and the predicting variable the IUT number. The total number of IUTs per infant and gestational age in weeks at time of the first IUT were included as potential confounders. Data were log10-transformed to unskew the distribution. Changes on the linear scale for the log10-transformed values correspond to percentage changes on the raw scale. The change in leukocytes and platelets was assessed with a linear mixed model using a random intercept per individual. The reticulocyte counts at birth between the groups after a varying number of IUTs were tested with a Kruskal–Wallis test. The secondary outcomes of proportion of neonates requiring ET and RBC transfusion after birth were tested with a χ^2 test after categorizing the infants in 2 groups: with 1–2 IUTs and >2 IUTs. Statistical analyses were performed using IBM SPSS Statistics 25.0 (IBM Corp, Armonk, NY).

FIGURE 1
Flowchart of study participants



HDFN, hemolytic disease of the fetus and newborn; *IUT*, intrauterine transfusion.

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Results

During the studied period, 250 infants treated with IUT(s) for HDFN were born and admitted to the neonatal intensive care unit of the LUMC (Figure 1). HDFN was caused by D alloimmunization (isolated or in combination with other antibodies) in 190 infants and by Kell alloimmunization in 46 infants. One infant with D alloimmunization was excluded due to a single unsuccessful IUT (<5 mL transfused, followed immediately by premature birth at 35 weeks of gestation). In total, 14 infants were excluded because of alloimmunization due to other alloantibodies.

Baseline characteristics

Baseline characteristics of the cohort are presented in Table 1. The median gestational age at the first IUT was 29.0 weeks (IQR, 24.6–32.1) in D-mediated HDFN and 25.8 weeks (IQR, 22.9–28.6) in Kell-mediated HDFN. The median number of IUTs per fetus was 2 (IQR, 2–4) and 3 (IQR, 2–4).

Hematologic parameters per IUT

Table 2 shows the pooled and unadjusted data of relevant hematologic parameters per consecutive IUT in D-mediated HDFN. IUTs were started at a median gestational age of 29 weeks (IQR, 24.6–32.1). The median transfusion volume was 56 mL (IQR, 37–80) at the first transfusion and increased with gestational age (ie, fetal weight).

The median hemoglobin level was low before every IUT but showed a gradual increase from the first IUT (hemoglobin 6.9 g/dL, IQR, 5.3–8.5) to consecutive IUTs, as is seen in fetal hemoglobin with increasing gestational age. The median absolute reticulocyte count before the first IUT was $297 \cdot 10^9$/L (IQR, 239–390) and showed an exponential decline with 70% to $92 \cdot 10^9$/L (IQR, 5–176) toward the second IUT and with an additional 28% decline to $7 \cdot 10^9$/L (IQR, 5–143) toward the third IUT (Figure 2). The

TABLE 1
Baseline characteristics

Variable	Study population, n=235	D-mediated HDFN, n=189	Kell-mediated HDFN, n=46
Number of previous births, median (IQR)	1 (1–2)	2 (1–3)	1 (1–1)
Antenatal IVIg administration, n (%)	8 (3)	6 (3)	2 (4)
Maternal age at first IUT, y, mean ± SD	32.0 ± 4.7	31.9 ± 4.7	32.3 ± 4.6
Gestational age at first IUT, wk, median (IQR)	28.0 (24.2–31.7)	29.0 (24.6–32.1)	25.8 (22.9–28.6)
Number of IUTs per fetus, median (IQR)	3 (2–4)	2 (2–4)	3 (2–4)
Maximum antibody titer, median (IQR)	256 (128–512)	256 (128–512)	128 (64–256)

HDFN, hemolytic disease of the fetus and newborn; *IQR*, interquartile range; *IUT*, intrauterine transfusion; *IVIg*, intravenous immunoglobulin; *SD*, standard deviation.
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relative reticulocyte counts showed a similar decline from 177‰ (IQR, 121–242) before the first IUT to 34‰ (IQR, 2–72) before the second IUT and further declined with consecutive IUTs.

The data per consecutive IUT in Kell-mediated HDFN are presented in Table 3. IUTs were started at a median gestational age of 26 weeks (IQR, 22.9–28.6). The median transfusion volume started at 50 mL (IQR, 30–67) at the first IUT.

The median absolute reticulocyte count before the first IUT was $133 \cdot 10^9$/L (IQR, 29–274) and declined with 86% to $19 \cdot 10^9$/L (IQR, 9–61) toward the second IUT and with an additional 8% decline to $11 \cdot 10^9$/L (IQR, 5–80) toward the third IUT (Figure 2). The relative reticulocyte counts showed a similar decline from 73‰ (IQR, 29–274) before the first IUT to 10‰ (IQR, 4–22) before the second IUT and further declined with consecutive IUTs.

Linear mixed models

The primary outcome was expressed as the decline (%) in absolute reticulocyte count per consecutive IUT and was analyzed by fitting a linear mixed model to account for the fact of repeated measurements and allow for covariate adjustment. The data were logarithmically transformed data (log10), the results are shown in Tables 4 and 5. In D-mediated HDFN, an adjusted decline after 1 IUT in absolute reticulocyte count of (1−0.38) 62% (95% CI, 56–67) was calculated, which is a back-calculation from the logistically transformed data reported in Table 4. After 2 IUTs, the absolute reticulocyte count was reduced by an adjusted percentage of ($1-0.38^2$) 85% compared with the initial reticulocyte count (95% CI, 81–89), by ($1-0.38^3$) 94% after 3 IUTs (95% CI, 91–96), 99% after 4 IUTs (95% CI, 96–99), and 100% after 5 IUTs (95% CI, 99–100). The gestational age at the time of the first IUT per week and total number of IUTs per infant were not statistically significant in this model ($P=.628$ and $P=.200$, respectively).

In Kell-mediated disease, an adjusted decline after 1 IUT in absolute reticulocyte count of (1−0.67) 32% (95% CI, 19–45) was calculated (Table 5). After 2 IUTs, the absolute reticulocyte count is reduced by ($1-0.67^2$) 54% compared with the initial reticulocyte count (95% CI, 34–70), by ($1-0.67^3$) 70% after 3 IUTs (95% CI, 47–83), 80% after 4 IUTs (95% CI, 57–91), and 86% after 5 IUTs (95% CI, 65–95). The gestational age at the time of the first IUT per week and total number of IUTs per infant were not statistically significant in this model ($P=.208$ and $P=.196$, respectively).

Hematologic parameters at birth and clinical outcomes

Table 6 shows the pooled data of various hematologic parameters at birth and clinical outcomes of D-mediated HDFN. Infants were born at a median gestational age of 36 weeks, irrespective of the total amount of IUTs. The absolute and relative reticulocyte counts at birth were lower if infants received multiple IUTs, falling from $171 \cdot 10^9$/L (IQR, 89–284) in infants born after 1 IUT to $10 \cdot 10^9$/L (IQR, 3–22) in infants born after 5 IUTs ($P<.001$) and from 58‰ (IQR, 25–83) in infants born after one IUT to 2‰ (IQR, 1–5) in infants born after 5 IUTs. Fewer infants required ET after >2 IUTs (19/89, 21%), compared with infants with 1–2 IUTs (31/100, 31%), $P=.039$. Infants after >2 IUTs needed more postnatal transfusions compared with infants after 1–2 IUTs (median of 3 [IQR, 2–3] vs 2 [IQR, 1–3], $P=.035$). Ferritin levels increased with subsequent IUTs, from 609 μg/L after 1 IUT to 745 μg/L (IQR, 481–2289) after 4 IUTs.

Table 7 shows the same data in Kell-mediated HDFN. These infants were also born at a median gestational age of 36 weeks. The absolute and relative reticulocyte counts at birth were lower if infants received multiple IUTs, falling from $120 \cdot 10^9$/L (IQR, 46–232) in infants born after 1 IUT to $15 \cdot 10^9$/L (no IQR due to n=5, with 2 missing values) in infants born after 5 IUTs ($P=.065$) and from 35‰ (IQR, 13–57) in infants born after 1 IUT to 3‰ (no IQR due to n=5, with 2 missing values) in infants born after 5 IUTs. No infants required ET after birth. Infants needed more postnatal transfusions after >2 IUTs compared with infants after 1–2 IUTs (median of 2 [IQR, 1–2] vs 1 [IQR, 1–1], $P<.001$). Ferritin levels increased with subsequent IUTs, from 609 μg/L (IQR, 414–845) after one IUT to 776 μg/L (IQR, 565–860) after 4 IUTs.

TABLE 2
Pooled data of consecutive intrauterine transfusions in D-mediated HDFN

IUT number	1 (n=189)	2 (n=148)	3 (n=89)	4 (n=48)	5 (n=16)	6 (n=3)
Gestational age, wk, median (IQR)	29.0 (24.6–32.1)	30.0 (26.4–33.1)	31.0 (28.7–33.3)	33.0 (30.9–34.0)	33.6 (31.1–34.3)	34.1[a]
Volume transfused blood, mL, median (IQR)	56 (37–80)[b]	63 (44–77)	74 (57–89)	77 (64–90)	77 (59–85)	94[a]
IUT administration route[c]						
Placental cord, n (%)	127 (67)	102 (69)	61 (69)	34 (71)	11 (69)	1 (33)
Intrahepatical, n (%)	2 (1)	1 (1)	1 (1)	0 (0)	0 (0)	0 (0)
Combination, n (%)	55 (29)	41 (28)	27 (30)	12 (25)	5 (31)	2 (67)
Hemoglobin level before IUT, g/dL, median (IQR)[d]	6.9 (5.3–8.5)	7.4 (6.1–8.9)	7.4 (6.5–8.5)	8.1 (6.8–8.9)	8.5 (7.4–9.7)	8.7[a]
Reticulocyte count before IUT (‰), median (IQR)[e]	177 (121–242)	34 (2–72)	3 (2–56)	2 (2–4)	2 (1–2)	2[a]
Reticulocyte count before IUT ($\cdot 10^9$/L) - median (IQR)[f]	297 (239–390)	92 (5–176)	7 (5–143)	5 (4–12)	5 (4–7)	5[a]

HDFN, hemolytic disease of the fetus and newborn; *IQR*, interquartile range; *IUT*, intrauterine transfusion.

[a] No IQR due to n=3; [b] Two missing values (187/189), 2 missing values (146/148), 0 missing values, 0 missing values, 0 missing values; [c] groups do not add up to 100% due to 9 missing values; [d] Two missing values (187/189), 1 missing value (147/148), 1 missing value (88/89), 0 missing values, 0 missing values, 0 missing values; [e] Twelve missing values (177/189), 10 missing values (138/148), 9 missing values (80/89), 4 missing values (44/48), 1 missing value (15/16), 0 missing values; [f] Six missing values (183/189), 2 missing values (146/148), 2 missing values (87/89), 0 missing values, 0 missing values.

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Discussion

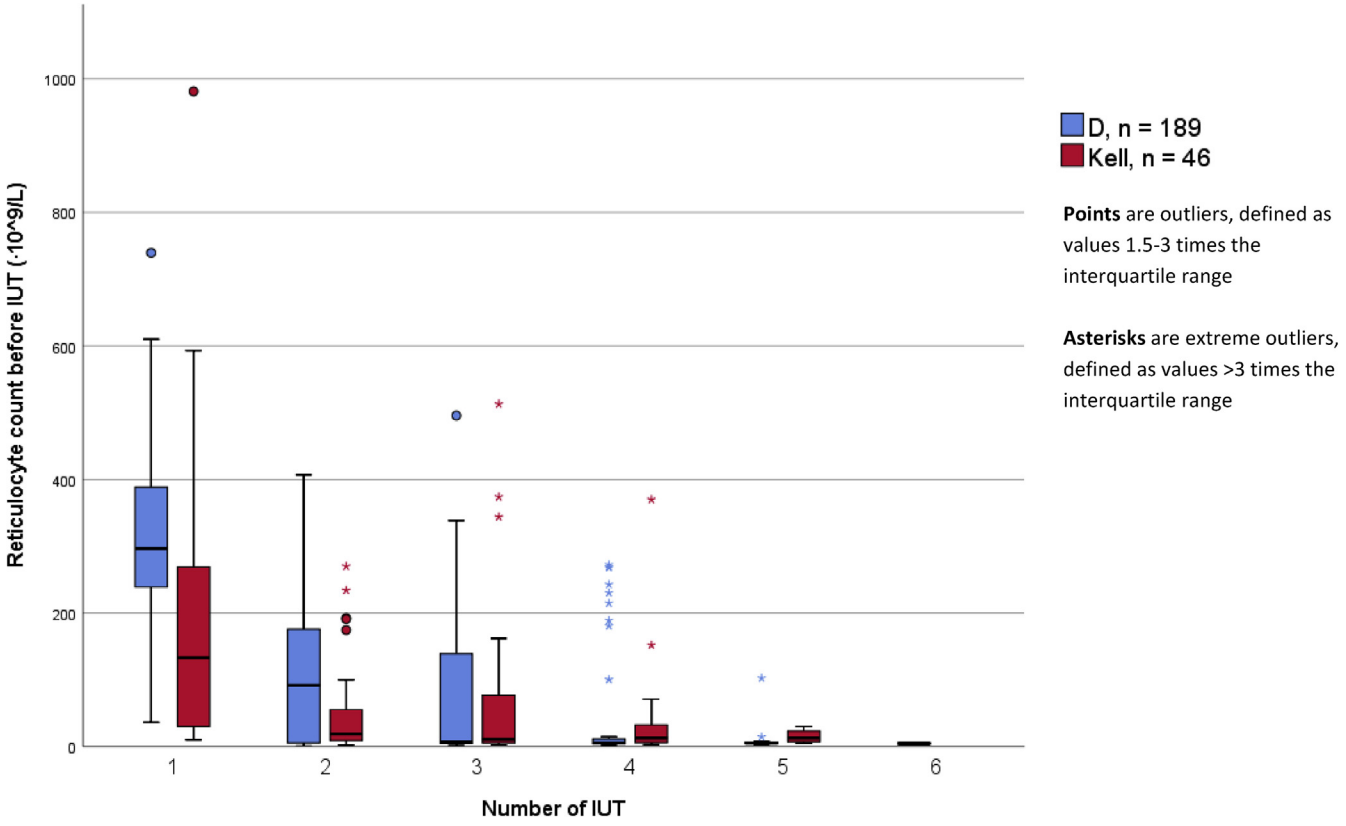
Principal findings

In this study, we assessed the suppressive effect of 1 or multiple IUTs on the compensatory fetal erythropoiesis in severe HDFN. The fetal reticulocyte count showed an exponential decline over the course of consecutive IUTs in both D- and Kell-mediated HDFN, with near disappearance of fetal reticulocytes after 2 IUTs. This suppressive effect was seen regardless of type of alloimmunization and has important clinical consequences for infants after birth. The exponential decrease in fetal reticulocyte counts and prolonged suppression of erythropoiesis leads to prolonged postnatal anemia and an increased requirement of RBC transfusions after birth. A previous study performed in our center already identified a low reticulocyte count at birth as a potential risk factor for postnatal RBC transfusions in this population,[7] which we now confirmed as directly related to the number of IUTs. In addition, since infants born after multiple IUTs have less erythrocyte production and more donor blood in their circulation, the hemolysis is reduced, resulting in a lower requirement of exchange transfusions in D-mediated HDFN. No infants with Kell antibodies required exchange transfusion after birth, in line with previous findings.[12] The strong suppressive effect was limited to the erythropoiesis, as a similar decline was not observed in fetal leukocytes and platelets.

Results

Interpretation of the reticulocyte counts and hemoglobin levels as found in this study is complicated by the lack of validated fetal reference values in unaffected pregnancies. However, Nicolaides et al[13] described a linear physiologic decrease in absolute reticulocyte count from a mean of $27.5 \cdot 10^9$/L (or 100‰) at 17 weeks of gestation to $17.5 \cdot 10^9$/L (or 40‰) at 40 weeks of gestation. The hemoglobin concentration was described to increase linearly with gestation from respective means of 11.0 g/dL to 15.5 g/dL at 40 weeks. For our data, this means that before treatment with a first IUT, affected and severely anemic fetuses

FIGURE 2
Boxplot of fetal reticulocyte course in D- and Kell-mediated HDFN



HDFN, hemolytic disease of the fetus and newborn; *IUT*, intrauterine transfusion.

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TABLE 3
Pooled data of consecutive intrauterine transfusions in Kell-mediated HDFN

IUT number	1 (n=46)	2 (n=42)	3 (n=32)	4 (n=20)	5 (n=5)
Gestational age, wk, median (IQR)	25.8 (22.9–28.6)	28.0 (25.3–30.9)	30.4 (28.6–32.7)	33.3 (30.8–34.3)	33.0 (32.2–34.4)
Volume transfused blood, mL, median (IQR)	50 (30–67)	59 (40–74)	77 (66–88)	76 (70–94)	83 (73–109)
IUT administration route					
Placental cord, n (%)	28 (61)	26 (62)	21 (66)	15 (75)	4 (80)
Intrahepatical, n (%)	2 (4)	0 (0)	0 (0)	0 (0)	0 (0)
Combination, n (%)	16 (35)	16 (38)	11 (34)	5 (25)	1 (20)
Hemoglobin level before IUT, g/dL, median (IQR)	6.1 (4.1–8.2)	7.4 (6.1–8.7)	7.2 (5.9–8.2)	7.6 (7.0–9.2)	7.7 (6.9–8.2)
Reticulocyte count before IUT, ‰, median (IQR)[a]	73 (29–128)	10 (4–22)	4 (2–27)	4 (2–15)	5 (3–9)
Reticulocyte count before IUT, $\cdot 10^9$/L, median (IQR)[a]	133 (29–274)	19 (9–61)	11 (5–80)	13 (5–35)	9 (6–23)

HDFN, hemolytic disease of the fetus and newborn; *IQR*, interquartile range; *IUT*, intrauterine transfusion.

[a] Five missing values (41/46), 3 missing values (39/42), no missing values (32/32), 1 missing value (19/20), 1 missing value (4/5).

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TABLE 4
Linear mixed model of the decline in reticulocyte count per consecutive IUT in D-mediated HDFN

Parameter	B	10^{B}	Std. error	*P* value	95% CI	$10^{95\%\ CI}$
Intercept	2.70	501	0.51	<.001	1.71−3.70	51−5011
Absolute reticulocyte count[a]	−0.42	0.38	0.03	<.001	−0.48 to − 0.36	0.33−0.44
Gestational age at first IUT (per week)	0.01	1.02	0.01	.628	−0.02 to 0.03	0.95−1.07
Total number of IUTs (per IUT)	−0.07	0.85	0.52	.200	−0.17 to 0.04	0.68−1.10

CI, confidence interval; *HDFN*, hemolytic disease of the fetus and newborn; *IUT*, intrauterine transfusion.

[a] log10 transformed to unskew data.

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showed, as expected, a marked reticulocytosis before IUT in line with the ongoing process of hemolysis and compensation by extramedullary hematopoiesis. The initial reticulocytosis is less pronounced in Kell immunization compared with D (median reticulocyte count before IUT $133 \cdot 10^9$/L [IQR, 29−274] vs $297 \cdot 10^9$/L [IQR, 239−390]), although a similar exponential decrease is observed after the course of multiple IUTs. In our data, IUTs were necessary at an earlier gestational age (25.8 vs 29.0 weeks) in Kell-mediated disease, but similar differences in the degree of reticulocytosis also were found in fetal blood samples that were matched for gestational age.[9]

After 2 IUTs, the reticulocyte count can be considered as below the physiologic reticulocyte count for gestational age regardless of type of alloimmunization. The reticulocyte count at birth remains high for infants born after few IUTs and hemoglobin levels at birth are on the lower end of normal reference values regardless of the number of IUTs.

Interpretation

IUTs have previously been postulated to suppress erythropoiesis, although the pathophysiologic mechanism of this process is unclear. It is known that fetal erythrocytes have a shorter lifespan than adult erythrocytes.[14] It may be that an IUT with adult longer living erythrocytes directly corrects anemia to such extent that the hypoxic stimulus that leads to production of erythropoietin (EPO) is reduced. However, the ongoing hemolysis and physiologically declining effect of the transfusion should again impel compensatory erythropoiesis. IUTs may additionally disrupt fetal erythropoiesis due to the transfusion of adult hemoglobin, which has other oxygen dissociation characteristics. Fetal RBCs predominantly contain fetal hemoglobin. The concentration of fetal hemoglobin is gradually replaced by adult hemoglobin toward the end of pregnancy. At birth, fetal hemoglobin comprises 60%−80% of total hemoglobin in the full-term newborn.[15] Fetal hemoglobin has a greater oxygen affinity with a left-shifted oxygen dissociation curve compared with adult hemoglobin to account for the relatively hypoxic intrauterine environment.[16] Experiments in sheep showed that exchange transfusion in sheep fetuses using adult sheep blood resulted in an overall decrease in oxygen affinity and saturation and, interestingly in view of our results, an increased reticulocytosis, whereas hemoglobin levels remained constant.[17] Adult hemoglobin is, however, also known to provide better peripheral tissue oxygenation compared with fetal hemoglobin,[16] which may result in a local reduction of hypoxic stimulus, causing reduced EPO production, which might explain the observed drastic decline in reticulocytes. In our institute, fetal EPO levels are not routinely measured. If an EPO level decline indeed underlies the found reticulocyte decline, it may be useful to start EPO treatment before birth.

Interestingly, we found a similar disruption of (compensatory) fetal erythropoiesis by IUTs in fetuses and infants with D and Kell-mediated

TABLE 5
Linear mixed model of the decline in reticulocyte count per consecutive IUT in Kell-mediated HDFN

Parameter	B	10^{B}	Standard error	*P* value	95% CI	$10^{95\%\ CI}$
Intercept	1.60	39	0.92	.084	−0.22 to 3.41	0.60−2570
Absolute reticulocyte count[a]	−0.17	0.67	0.04	<.001	−0.26 to −0.09)	0.55−0.81
Gestational age at first IUT (per week)	0.03	1.07	0.02	.208	−0.02 to 0.08	0.95−1.20
Total number of IUTs (per IUT)	−0.13	0.75	0.10	.196	−0.32 to 0.07	0.48−1.17

CI, confidence interval; *HDFN*, hemolytic disease of the fetus and newborn; *IUT*, intrauterine transfusion.

[a] log10 transformed to unskew data.

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TABLE 6
Hematologic parameters at birth and clinical outcomes (infant) in D-mediated HDFN

Number of IUTs per infant	1 (n=41)	2 (n=59)	3 (n=41)	4 (n=32)	5 (n=13)	6 (n=3)
Male, n (%)	29 (71)	33 (56)	25 (61)	15 (47)	10 (77)	1 (33)
Gestational age at birth, wk, median (IQR)	36 (36–37)	36 (36–37)	36 (35–37)	36 (36–37)	36 (35–37)	37[a]
Birthweight, g, median (IQR)	2815 (2653–3045)	2945 (2610–3170)	2705 (2480–3001)	2860 (2590–3074)	2800 (2504–3053)	2542[a]
Hemoglobin level at birth, g/dL, median (IQR)[b]	11.9 (9.9–13.9)	12.1 (10.5–13.5)	12.9 (10.8–15.3)	12.7 (11.3–14.6)	11.4 (10.1–12.3)	12.7[a]
Reticulocytes at birth, ‰, median (IQR)[c]	58 (25–83)	43 (4–57)	13 (2–46)	3 (2–9)	2 (1–5)	2[a]
Reticulocytes at birth, $\cdot 10^9$/L, median (IQR)[c]	171 (89–284)	150 (16–246)	71 (12–228)	15 (10–39)	10 (3–22)	10[a]
Ferritin level at birth, μg/L, median (IQR)[d]	609 (414–845)	668 (564–858)	836 (592–1187)	736 (595–962)	745 (481–2289)	940[a]
Bilirubin level at birth, mg/dL, median (IQR)	124 (83–146)	95 (70–122)	112 (82–142)	87 (70–108)	96 (81–120)	96[a]
Phototherapy, d, median (IQR)	5 (4–6)	5 (4–5)	4 (3–5)	4 (3–5)	4 (3–5)	4[a]
Infants requiring ET, n (%)	16 (39)	15 (25)	9 (22)	6 (19)	1 (8)	3 (100)
Infants requiring RBC transfusion, n (%)[e]	36 (90)	51 (86)	37 (93)	29 (91)	13 (100)	2 (100)
Number of RBC transfusion(s), median (IQR)[f]	2 (2–3)	2 (1–3)	2 (2–3)	3 (2–3)	3 (2–4)	4[a]

ET, exchange transfusion; *HDFN*, hemolytic disease of the fetus and newborn; *IQR*, interquartile range; *IUT*, intrauterine transfusion; *RBC*, red blood cell.

[a] No IQR due to n=3; [b] Zero missing values, 1 missing value (58/59), 0 missing values, 0 missing values, 0 missing values; [c] Eight missing values (33/41), 4 missing values (55/59), 6 missing values (35/41), 3 missing values (29/32), 2 missing values (11/13), 0 missing values; [d] Nine missing values (32/41), 14 missing values (45/59), 11 missing values (30/41), 5 missing values (27/32), 6 missing values (7/13), 0 missing values; [e] One missing value (40/41), 0 missing values, 1 missing value, (40/41), 0 missing values, 0 missing value, 1 missing value (2/3); [f] Four missing values (37/41), 6 missing values (53/59), 4 missing values (37/41), 2 missing values (30/32), 0 missing values, 1 missing value (2/3).

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HDFN, whereas these are known to have a different pathophysiology and clinical course. Even low antibody titers in pregnancy can cause severe fetal anemia in Kell alloimmunization,[18] for example, although these neonates require overall less phototherapy and less exchange transfusions compared with D alloimmunization. There is in both D and Kell alloimmunization a similar high degree of neonatal anemia and transfusion dependency after birth.[12] Kell antigens appear on erythroid progenitor cells early in erythropoiesis,[19] and erythroid suppression seems to be the predominant mechanism in producing fetal anemia, rather than hemolysis.[9–11] This is reflected by our finding that the reticulocyte count before the first IUT was substantially lower in Kell than in D immunized pregnancies (133 vs $297 \cdot 10^9$/L). The difference cannot be explained by the difference in gestational age only. As erythroid suppression is already part of the pathogenesis in Kell alloimmunization, it is of particular interest that one or multiple IUTs have an added suppressive effect.

We also considered the alternative hypothesis that the disrupted erythropoiesis is related to iron load. Iron deficiency can cause and prolong anemia, but excessive iron as caused by the ongoing hemolysis and the multiple IUTs, can also be toxic to erythropoiesis.[20,21] Despite overall high ferritin levels at birth in this transfused population, these levels are not high enough to cause toxicity or explain the degree of erythropoiesis suppression observed in this study.

Research implications

Our study has several research implications for the future. More studies are needed to investigate the relationship between IUTs, HDFN, and EPO to further understand these observations. At our center, we are currently performing a randomized clinical trial to assess the effect of exogenous administered darbepoetin after birth on postnatal transfusion dependency in IUT-treated infants (NCT03104426), of which the first results are expected in 2021.

TABLE 7
Hematologic parameters at birth and clinical outcomes (infant) in Kell-mediated HDFN

Number of IUTs per infant	1 (n=4)	2 (n=10)	3 (n=12)	4 (n=15)	5 (n=5)
Male, n (%)	2 (50)	4 (40)	7 (58)	12 (80)	2 (40)
Gestational age at birth, wk, median (IQR)	36 (36–37)	36 (35–37)	36 (36–37)	37 (36–37)	36 (35–37)
Birthweight, g, median (IQR)	2867 (2493–3256)	2810 (2566–3120)	3053 (2736–3340)	3230 (2935–3500)	2890 (2385–3041)
Hemoglobin level at birth, g/dL, median (IQR)	11.4 (11.2–15.6)	13.5 (11.9–15.5)	12.8 (11.8–14.5)	13.2 (11.1–14.2)	10.6 (7.5–12.7)
Reticulocytes at birth, ‰, median (IQR)	35 (13–57)	15 (10–43)	18 (7–38)	7 (4–41)	3[a]
Reticulocytes at birth, $\cdot 10^9$/L, median (IQR)	120 (46–232)	68 (45–154)	76 (34–162)	24 (14–94)	15[a]
Ferritin level at birth, μg/L, median (IQR)	609[b]	668 (564–858)[c]	681 (547–1248)[d]	776 (565–860)[e]	1011[f]
Bilirubin level at birth, mg/dL, median (IQR)	56 (45–63)	64 (39–88)	61 (53–77)	74 (51–85)	60 (45–69)
Phototherapy, d, median (IQR)	2 (1–2)	2 (2–5)	3 (2–3)	2 (2–4)	2 (2–4)
Infants requiring ET, n (%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Infants requiring RBC transfusion, n (%)	1 (25)	7 (70)	8 (67)	13 (87)	4 (80)
Number of RBC transfusion(s), median (IQR)	1[g]	1 (1–1)	1 (1–2)	2 (1–2)	3 (2–4)

ET, exchange transfusion; *HDFN*, hemolytic disease of the fetus and newborn; *IQR*, interquartile range; *IUT*, intrauterine transfusion; *RBC*, red blood cell.

[a] No IQR due to n=5, with 2 missing values (3/5); [b] No IQR due to n=4, with 2 missing values (2/4); [c] Four missing values (6/10); [d] Five missing values (7/12); [e] Four missing values (11/15); [f] No IQR due to n=5, with 4 missing values (1/5); [g] No IQR due to n=1, with 3 missing values.

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The clinical implications of the neonatal reticulocyte count after birth were elaborated on in previous work of our research group, which identified a low reticulocyte count after birth as predictor of postnatal RBC transfusion need.[7] We recommend postnatal measurement of reticulocyte count along with postnatal hemoglobin for a period of 2–3 months as part of neonatal follow-up after birth to shed further light on the state of recovery of the erythropoiesis.

Strengths

The major strength in this study is inclusion of a large group of infants treated with the same protocol in one center of expertise, resulting in a near-complete collection of data of an increasingly rare disease. This enabled us to further detail the hematologic effects of IUTs and HDFN and further unravel its pathophysiology. Ultimately, we are moving forward toward further individualization of treatment and follow-up of infants affected by HDFN, identifying those groups of infants at the greatest risk for a complicated disease course and pinpointing treatment towards these infants.

Limitations

One of the limitations of this study is that there are no fetal blood samples in between IUTs. It is expected that the reticulocyte course has a more fluctuating course after an IUT is administered than what can be seen based on the available samples. As mentioned previously, the lack of endogenous fetal and neonatal EPO levels is also of concern and could yield additional crucial information in future studies as well as follow-up of neonatal antibody titers. Due to the nature of the IUT procedure, missing values were to be expected and are reported with the data. The small volume blood samples are susceptible to agglutination, which may be enhanced by improper handling of the sampled volume after the procedure (turning of sample tube). These missing values are, however, considered as "at random" and no further statistical measures were taken to address this. Finally, reticulocyte

counts have to be seen as so-called endogenous variables, ie, counts at a given time point depend on values observed at previous time points as they influence the clinical decision of administering IUTs. More complex interactions between IUTs and reticulocyte counts than discussed here are thinkable. We believe that our interpretation is the clinically most plausible one.

Conclusion

From a pathophysiologic and clinical point of view, our study highlights the potential negative effect of 1 or multiple IUTs on erythropoiesis and the observed prolonged effects after birth. A distinction can be made between infants treated with 1 or 2 IUTs and infants treated with multiple IUTs. The latter group not only reflects more severe disease, as indicated by the severe fetal anemia prompting the greater amount of IUTs, but has an additional more pronounced suppression of erythropoiesis. In conclusion, we state that after IUT treatment for HDFN, an exponential decrease in fetal reticulocyte counts is observed and infants born after multiple IUTs show a prolonged suppressed erythropoiesis with a greater transfusion need. ■

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Author and article information

From the Center for Clinical Transfusion Research, Sanquin Research, Leiden (Drs Ree, Zwiers, and De Haas); Department of Pediatrics, Division of Neonatology, Leiden University Medical Center, Leiden (Drs Ree, Lopriore and Ms Janssen); Department of Obstetrics, division of Fetal Medicine, Leiden University Medical Center, Leiden (Drs Zwiers and Oepkes); Department of Biomedical Data Sciences, Leiden University Medical Center, Leiden (Dr Böhringer); Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden (Dr De Haas); and Department of Immunohematology Diagnostics, Sanquin, Amsterdam (Dr De Haas), the Netherlands.

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Corresponding author: Isabelle Ree, MD. i.m.c.ree@lumc.nl