# Optimising neonatal management of haemolytic disease of the foetus and newborn

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# Chapter three

## Suppression of compensatory erythropoiesis in haemolytic disease of the foetus and newborn due to intrauterine transfusions

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# Abstract

## Background

Infants with severe haemolytic disease of the foetus and newborn (HDFN) often require one or multiple intrauterine transfusions (IUTs) to treat foetal anaemia. IUTs may have an inhibiting effect on foetal and neonatal erythropoiesis.

## Objective

To quantify the effect of one or multiple IUTs on the foetal erythropoiesis by assessing the foetal reticulocyte counts in a population with severe HDFN.

## Study design

This was an observational cohort study in infants admitted to the Leiden University Medical Centre (LUMC) who received one or multiple IUT(s) for HDFN caused by (Rh)D or K antibodies that were born between January 2005 and December 2018.

## Results

A total of 235 patients were included, of whom 189 patients with D-mediated HDFN and 46 with K-mediated HDFN. Absolute foetal reticulocyte count in D-mediated HDFN declined exponentially over the course of consecutive IUTs, with a 62% decline after one IUT (95%-confidence interval 56-67). A similar exponential decline was observed in K-mediated HDFN, with 32% (95%-confidence interval 19-45) decline after one IUT. This decline was not associated with the varying gestational age at the time of the first IUT or the total number of IUTs. The number of red blood cell transfusions for postnatal anaemia was greater for infants with D- and K-mediated HDFN with >2 IUTs (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 K-mediated disease). Infants born after >2 IUTs less often required exchange transfusion in D-mediated HDFN (19/89 [21%] vs 31/100 [31%], $p$=.039), compared to infants with 1-2 IUTs.

## Conclusion

Treatment with IUTs causes an exponential decrease in foetal reticulocyte counts in both D- and K-mediated HDFN. Suppression of the compensatory erythropoiesis leads to prolonged postnatal anaemia and an increased requirement of red blood cell transfusions after birth.

# Introduction

Haemolytic disease of the foetus and newborn (HDFN) is caused by an incompatibility between maternal and foetal erythrocyte antigens. HDFN is characterised by foetal and neonatal erythroid cell destruction due to maternal alloantibodies, which will induce compensatory erythropoiesis. In case of insufficient compensation, foetal or neonatal anaemia may occur and intrauterine treatment with one or more red blood cell (RBC) transfusions may be indicated, as well as transfusions for persistent anaemia after birth.[1,2] The exact effects of intrauterine transfusions (IUTs) with adult donor RBCs, which carry a different type of haemoglobin and have different oxygen binding and release characteristics compared to foetal red cells, is not known. In small populations, the effect of IUT(s) on various haematologic parameters such as foetal haematocrit, haemoglobin, leukocytes and bilirubin has been studied.[3-5] Reticulocyte counts at birth appeared to be lower in infants with HDFN who received one or multiple IUTs, compared with infants that were not treated with IUTs, irrespective of the haemoglobin level at birth.[3,6] Treatment with IUTs is also associated with a higher number of transfusions after birth compared to infants with HDFN not treated with IUT(s).[7] An inhibiting effect of donor blood on the foetal 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 foetal and neonatal erythropoiesis is necessary in order to clarify the pathophysiological mechanisms underlying both the intrauterine and postnatal course of HDFN. In this study, we specifically aimed to quantify the effect of one or multiple IUTs on foetal erythropoiesis by assessing the reticulocyte counts in a large population of foetuses and infants with severe HDFN.

# Methods

## Study population

All infants admitted to the Leiden University Medical Centre (LUMC) who received treatment with one or multiple IUT(s) for the treatment of HDFN caused by (Rh)D or K antibodies and who were born between January 2005 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 one of the two 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 foetus is antigen-positive. If the foetus is positive, the risk on foetal haemolysis is assessed by serially determining the antibody titre and antibody-dependent cell-mediated cytotoxicity (ADCC). Referral to the LUMC as national specialised centre is indicated if laboratory parameters are above determined cut-offs. These cut-offs are antibody titres tested in maternal serum ≥16 in D alloimmunisation and ≥2 in K alloimmunisation, or in case of an ADCC assay ≥50% in D alloimmunisation and ≥30% In Kell alloimmunisation. Subsequently, these high-risk pregnancies are monitored by serial Doppler measurements to assess the velocity of the blood flow in the middle cerebral artery (MCA). If MCA Doppler 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' 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 haematocrit of 0.80-0.85 L/L to minimise the risk of volume overload in the foetus. 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 foetal anaemia, a foetal blood sample is taken prior to 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 foetal data were excluded from analyses in case of HDFN caused by other alloantibodies than D or K, 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 K antibodies, IUTs are generally needed earlier in gestation compared to D-mediated disease and erythroid suppression seems to be the predominant mechanism in producing foetal anaemia rather than haemolysis.[9-11] The results of infants with D- and K-mediated HDFN were therefore reported separately.

## Data collection

Data was 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 was coded prior to analysis. The following maternal and foetal data were recorded: number of previous births, antenatal intravenous immunoglobulins administration, maximum antibody titre, maternal age at first IUT, foetal 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), foetal haemoglobin levels and leukocyte, platelet, and reticulocyte counts before every IUT. The following infant data were recorded: sex, gestational age at birth, birth weight, haemoglobin 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 three 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 centre to unify neonatal management. The current transfusion guideline of the department recommends a transfusion in term infants with HDFN when haemoglobin 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 haematocrit 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 outcome

The primary outcome in this study was the suppression of foetal 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 foetal erythropoiesis. The relative reticulocyte count, as expressed per thousand RBCs (‰), was also 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 is presented as mean (± standard deviation, SD) or median (interquartile range, IQR) depending on the underlying distribution. The primary outcome is visualised 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 $\chi 2$ test after categorising the infants in two groups: with 1-2 IUTs and $>2$ IUTs. Statistical analyses were performed using IBM SPSS Statistics (version 25.0; SPSS Inc, Chicago, IL).

# 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 alloimmunisation (isolated or in combination with other antibodies) in 190 infants, and by K alloimmunisation in 46 infants. One infant with D alloimmunisation 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 alloimmunisation due to other alloantibodies.

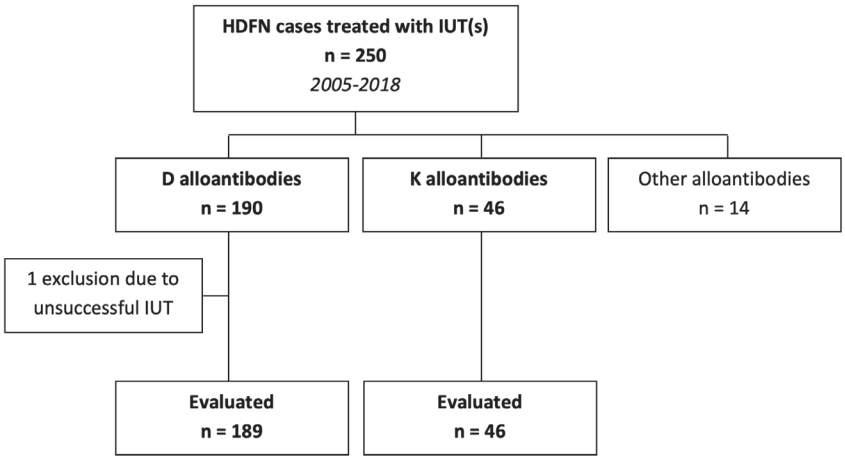


**Figure 1.** Flowchart of study population
*HDFN,* haemolytic disease of the foetus and newborn; *IUT*, intratuterine transfusion.

## 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 K-mediated HDFN. The median number of IUTs per foetus was 2 (IQR 2-4) and 3 (IQR 2-4).

**Table 1.** Baseline characteristic

| Variable | Study population n = 235 | D-mediated HDFN n = 189 | K-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 (years) - mean ± SD | 32.0 ± 4.7 | 31.9 ± 4.7 | 32.3 ± 4.6 |
| Gestational age at first IUT (weeks) - median (IQR) | 28.0 (24.2-31.7) | 29.0 (24.6-32.1) | 25.8 (22.9-28.6) |
| Number of IUTs per foetus - median (IQR) | 3 (2-4) | 2 (2-4) | 3 (2-4) |
| Maximum antibody titre - median (IQR) | 256 (128-512) | 256 (128-512) | 128 (64-256) |

*HDFN*, haemolytic disease of the foetus and newborn; *IQR*, interquartile range; *IUT*, intrauterine transfusion; *IVIg*, intravenous immunoglobulin; *n*, number; *SD*, standard deviation.

## Haematologic parameters per IUT

Table 2 shows the pooled and unadjusted data of relevant haematologic 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 (i.e., foetal weight).

The median haemoglobin level was low before every IUT, but showed a gradual increase from the first IUT (haemoglobin 6.9 g/dL, IQR 5.3-8.5) to consecutive IUTs, as is seen in foetal haemoglobin 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) towards the second IUT and with an additional 28% decline to $7 \cdot 10^9$/L (IQR 5-143) towards the third IUT (Figure 2). The 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 K-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) towards the second IUT and with an additional 8% decline to $11 \cdot 10^9$/L (IQR 5-80) towards 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) beforethe second IUT and further declined with consecutive IUTs.

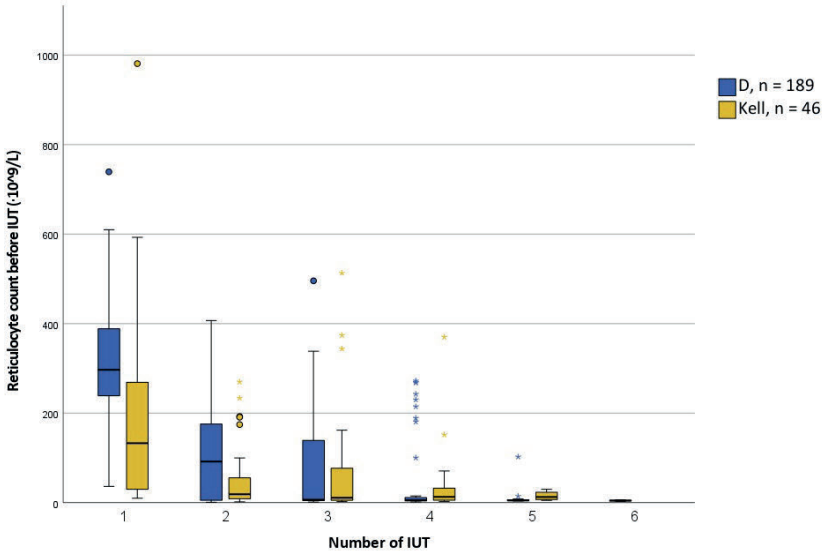


**Figure 2.** Boxplot of foetal reticulocyte course in D- and K-mediated HDFN
*IUT*, intrauterine transfusion.
*Points* are outliers, defined as values 1.5-3 times the interquartile range, *asterisks* are extreme outliers, defined as values >3 times the interquartile range.

**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 (weeks) - 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) |
| Haemoglobin 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*, haemolytic disease of the foetus and newborn; *IQR*, interquartile range; *IUT*, intrauterine transfusion; *n*, number.
[a] No interquartile range due to n=3; [b] 2 missing values (187/189), 2 missing values (146/148), 0 missing values, 0 missing values, 0 missing values, 0 missing values; [c] groups do not add up to 100% due to 9 missing values; [d] 2 missing values (187/189), 1 missing value (147/148), 1 missing value (88/89), 0 missing values, 0 missing values, 0 missing values; [e] 12 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] 6 missing values (183/189), 2 missing values (146/148), 2 missing values (87/89), 0 missing values, 0 missing values.

**Table 3**. Pooled data of consecutive intrauterine transfusions in K-mediated HDFN

| IUT number | 1 (n = 46) | 2 (n = 42) | 3 (n = 32) | 4 (n = 20) | 5 (n = 5) |
|---|---|---|---|---|---|
| Gestational age (weeks) - 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) |
| Haemoglobin 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*, haemolytic disease of the foetus and newborn; *IQR*, interquartile range; *IUT*, intrauterine transfusion; *n*, number.
[a] 5 missing values (41/46), 3 missing values (39/42), no missing values (32/32), 1 missing value (19/20), 1 missing value (4/5).

## Linear mixed models

The primary outcome was expressed as the decline (%) in absolute reticulocyte count per consecutive IUT, and was analysed 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 Table 4 and 5. In D-mediated HDFN, an adjusted decline after one 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 two IUTs the absolute reticulocyte count was reduced by an adjusted percentage of ($1-0.38^2$) 85% compared to the initial reticulocyte count (95% CI 81-89), by ($1-0.38^3$) 94% after three IUTs (95% CI 91-96), 99% after four IUTs (95% CI 96-99), and 100% after five 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 K-mediated disease, an adjusted decline after one IUT in absolute reticulocyte count of (1-0.67) 32% (95% CI 19-45) was calculated, Table 5. After two IUTs, the absolute reticulocyte count is reduced by ($1-0.67^2$) 54% compared to the initial reticulocyte count (95% CI 34-70), by ($1-0.67^3$) 70% after three IUTs (95% CI 47-83), 80% after four IUTs (95% CI 57-91), and 86% after five 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).

**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 | <0.001 | 1.71-3.70 | 51-5011 |
| Absolute reticulocyte count[a] | -0.42 | 0.38 | 0.03 | <0.001 | (-0.48)-(-0.36) | 0.33-0.44 |
| Gestational age at first IUT (per week) | 0.01 | 1.02 | 0.01 | 0.628 | (-0.02)-0.03 | 0.95-1.07 |
| Total number of IUTs (per IUT) | -0.07 | 0.85 | 0.52 | 0.200 | (-0.17)-0.04 | 0.68-1.10 |

*HDFN*, haemolytic disease of the foetus and newborn; *IUT*, intrauterine transfusion.
[a] Log10 transformed to unskew data.

**Table 5.** Linear mixed model of the decline in reticulocyte count per consecutive IUT in K-mediated HDF

| Parameter | B | $10^B$ | Std. error | *p*-value | 95% CI | $10^{95\%\ CI}$ |
|---|---|---|---|---|---|---|
| Intercept | 1.60 | 39 | 0.92 | 0.084 | (-0.22)-3.41 | 0.60-2570 |
| Absolute reticulocyte count[a] | -0.17 | 0.67 | 0.04 | <0.001 | (-0.26)-(-0.09) | 0.55-0.81 |
| Gestational age at first IUT (per week) | 0.03 | 1.07 | 0.02 | 0.208 | (-0.02)-0.08 | 0.95-1.20 |
| Total number of IUTs (per IUT) | -0.13 | 0.75 | 0.10 | 0.196 | (-0.32)-0.07 | 0.48-1.17 |

*HDFN*, haemolytic disease of the foetus and newborn; *IUT*, intrauterine transfusion.
[a] Log10 transformed to unskew data.

## Haematologic parameters at birth and clinical outcomes

Table 6 shows the pooled data of various haematologic 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 one IUT to $10 \cdot 10^9$/L (IQR 3-22) in infants born after five IUTs ($p<.001$) and from 58‰ (IQR 25-83) in infants born after one IUT to 2‰ (IQR 1-5) in infants born after five IUTs. Less infants required ET after >2 IUTs (19/89, 21%), compared to infants with 1-2 IUTs (31/100, 31%), $p=.039$. Infants after >2 IUTs needed more postnatal transfusions compared to 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 one IUT to 745 µg/L (IQR 481-2289) after four IUTs.

Table 7 shows the same data in K-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 one IUT to $15 \cdot 10^9$/L (no IQR due to n=5, with 2 missing values) in infants born after five IUTs ($p=.065$) and from 35‰ (IQR 13-57) in infants born after one IUT to 3‰ (no IQR due to n=5, with 2 missing values) in infants born after five 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 four IUTs.

**Table 6.** Haematologic 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 (weeks) - median (IQR) | 36 (36-37) | 36 (36-37) | 36 (35-37) | 36 (36-37) | 36 (35-37) | 37[a] |
| Birth weight (grams) - median (IQR) | 2815 (2653-3045) | 2945 (2610-3170) | 2705 (2480-3001) | 2860 (2590-3074) | 2800 (2504-3053) | 2542[a] |
| Haemoglobin 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 (·$10^9$/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 (days) - 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] |

*HDFN*, haemolytic disease of the foetus and newborn; *ET*, exchange transfusion; *IQR*, interquartile range; *IUT*, intrauterine transfusion; *n*, number; *RBC*, red blood cell.

[a] No interquartile range due to n=3; [b] 0 missing values, 1 missing value (58/59), 0 missing values, 0 missing values, 0 missing values, 0 missing values; [c] 8 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] 9 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] 1 missing value (40/41), 0 missing values, 1 missing value, (40/41), 0 missing values, 0 missing values, 1 missing value (2/3); [f] 4 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).

**Table 7.** Haematologic parameters at birth and clinical outcomes (infant) in K-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 (weeks) - median (IQR) | 36 (36-37) | 36 (35-37) | 36 (36-37) | 37 (36-37) | 36 (35-37) |
| Birth weight (grams) - median (IQR) | 2867 (2493-3256) | 2810 (2566-3120) | 3053 (2736-3340) | 3230 (2935-3500) | 2890 (2385-3041) |
| Haemoglobin 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 (·$10^9$/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 (days) - 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) |

*HDFN*, haemolytic disease of the foetus and newborn; *ET*, exchange transfusion; *IQR*, interquartile range; *IUT*, intrauterine transfusion; *n*, number; *RBC*, red blood cell.

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

# Discussion

## Principal findings

In this study, we assessed the suppressive effect of one or multiple IUTs on the compensatory foetal erythropoiesis in severe HDFN. The foetal reticulocyte count showed an exponential decline over the course of consecutive IUTs in both D- and K-mediated HDFN, with near disappearance of foetal reticulocytes after two IUTs. This suppressive effect was seen regardless of type of alloimmunisation and has important clinical consequences for infants after birth. The exponential decrease in foetal reticulocyte counts and prolonged suppression of erythropoiesis leads to prolonged postnatal anaemia and an increased requirement of RBC transfusions after birth. A previous study performed in our centre 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 haemolysis is reduced, resulting in a lower requirement of exchange transfusions in D-mediated HDFN. No infants with K 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 foetal leukocytes and platelets.

## Results

Interpretation of the reticulocyte counts and haemoglobin levels as found in this study is complicated by the lack of validated foetal 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' gestation to $17.5 \cdot 10^9$/L (or 40‰) at 40 weeks' gestation. The haemoglobin 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 anaemic foetuses showed, as expected, a marked reticulocytosis before IUT in line with the ongoing process of haemolysis and compensation by extramedullary haematopoiesis. The initial reticulocytosis is less pronounced in K immunisation 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 K-mediated disease, but similar differences in the degree of reticulocytosis were also found in foetal blood samples that were matched for gestational age.[9]

After two IUTs, the reticulocyte count can be considered as below the physiologic reticulocyte count for gestational age regardless of type of alloimmunisation. The reticulocyte count at birth remains high for infants born after few IUTs and haemoglobin 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 foetal erythrocytes have a shorter life-span than adult erythrocytes.[14] It may be that an IUT with adult longer living erythrocytes directly corrects anaemia to such extent that the hypoxic stimulus that leads to production of erythropoietin (EPO) is reduced. However, the ongoing haemolysis and physiologically declining effect of the transfusion should again impel compensatory erythropoiesis. IUTs may additionally disrupt foetal erythropoiesis due to the transfusion of adult haemoglobin, which has other oxygen dissociation characteristics. Foetal RBCs predominantly contain foetal haemoglobin. The concentration of foetal haemoglobin is gradually replaced by adult haemoglobin towards the end of pregnancy. At birth, foetal haemoglobin comprises 60-80% of total haemoglobin in the full-term newborn.[15] Foetal haemoglobin has a greater oxygen affinity with a left-shifted oxygen dissociation curve compared with adult haemoglobin to account for the relatively hypoxic intrauterine environment.[16] Experiments in sheep showed that exchange transfusion in sheep foetuses 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 haemoglobin levels remained constant.[17] Adult haemoglobin is, however, also known to provide better peripheral tissue oxygenation compared with foetal haemoglobin[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, foetal 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) foetal erythropoiesis by IUTs in foetuses and infants with D- and K-mediated HDFN, whereas these are known to have a different pathophysiology and clinical course. Even low antibody titres in pregnancy can cause severe foetal anaemia in K alloimmunisation[18], for example, although these neonates require overall less phototherapy and less exchange transfusions compared with D alloimmunisation. There is in both D and K alloimmunisation a similar high degree of neonatal anaemia and transfusion dependency after birth.[12] K antigens appear on erythroid progenitor cells early

in erythropoiesis[19], and erythroid suppression seems to be the predominant mechanism in producing foetal anaemia, rather than haemolysis.[9-11] This is reflected by our finding that the reticulocyte count before the first IUT was substantially lower in K than in D immunised pregnancies (133 vs 297·$10^9$/L). The difference cannot be explained by the difference in gestational age only. As erythroid suppression is already part of the pathogenesis in K alloimmunisation, 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 anaemia, but excessive iron as caused by the ongoing haemolysis 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 centre we are currently performing a randomised clinical trial to assess the effect of exogenous administered darbepoetin alfa after birth on postnatal transfusion dependency in IUT-treated infants (NCT03104426), of which the first results are expected in 2022.

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 haemoglobin for a period of two to three 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 centre of expertise, resulting in a near-complete collection of data of an increasingly rare disease. This enabled us to further detail the haematologic effects of IUTs and HDFN and further unravel its pathophysiology. Ultimately, we are moving forward toward further individualisation 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 foetal 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 foetal 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 titres. 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, i.e., 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.

## Conclusions

From a pathophysiologic and clinical point of view, our study highlights the potential negative effect of one or multiple IUTs on erythropoiesis and the observed prolonged effects after birth. A distinction can be made between infants treated with one or two IUTs and infants treated with multiple IUTs. The latter group not only reflects more severe disease as indicated by the severe foetal anaemia prompting the higher 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 foetal reticulocyte counts is observed and infants born after multiple IUTs show a prolonged suppressed erythropoiesis with a greater transfusion need.

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