Title

Outcome predictors for maternal red blood cell alloimmunization with anti-K and anti-D managed with intrauterine blood transfusion.

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Conflicts

The authors report no conflicts of interest.

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Presentation of Results

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Abstract

Background: Red blood cell (RBC) alloimmunization with anti-D and anti-K comprise the majority of cases of fetal hemolytic disease requiring intrauterine red cell transfusion (IUT). Few studies have investigated which hematologic parameters can predict adverse fetal or neonatal outcomes.

Objective: Our aim was to identify predictors of adverse outcome, including preterm birth, intrauterine fetal demise (IUFD), neonatal death (NND) and/or neonatal transfusion.

Study Design: We reviewed the records of all pregnancies alloimmunized with anti-K and anti-D, requiring IUT over 27 years at a quaternary fetal center.

Results: We reviewed data for 128 pregnancies in 116 women undergoing 425 IUTs. The median gestational age (GA) at first IUT was significantly earlier for anti-K than for anti-D (24.3 vs 28.7 weeks, p=0.004). Women with anti-K required more IUTs than women with anti-D (3.84 vs 3.12 mean IUTs, p=0.036) and the fetal hemoglobin (Hb) at first IUT was significantly lower (5.10 vs 7.05, p=0.001). The mean estimated daily decrease in Hb did not differ between the two groups. A greater number of IUTs and a slower daily decrease in Hb (g/L/day) between first and second IUTs were predictive of a longer period *in-utero*. Earlier GA at first IUT and a shorter interval from the first IUT until delivery predicted IUFD/NND. Earlier GA and lower Hb at first IUT significantly predicted need for phototherapy and/or blood product use in the neonate. In the anti-K group, a greater number of IUTs was required in women with a higher titre. Furthermore, the higher the titre, the earlier the GA at which an IUT was required in both groups. The rate of fall in fetal Hb between IUTs decreased, as the number of transfusions increased.

Conclusion: Our study identified pregnancies at considerable risk of an unfavourable outcome with anti-D and anti-K RBC alloimmunization. Identifying such patients can guide pregnancy management, facilitates patient counselling and can optimize resource use. Prospective studies can also incorporate these characteristics, in addition to laboratory markers, to further identify and improve the outcomes of these pregnancies.

Introduction

The RhD and Kell blood group systems are the most common of more than 50 different red blood cell (RBC) antigens which can cause clinically significant alloimmunization and severe fetal and/or neonatal hemolytic disease. Anti-K and anti-D antibodies are responsible for most cases of significant fetal anemia. Intra-uterine transfusion (IUT) has significantly improved neonatal survival and morbidity, although severe hydrops remains a predictor of neurodevelopmental impairment (NDI) (1). Overall, long term neurodevelopmental outcomes following IUT are considered to be favorable, with NDI occurring in less than 4.8% of cases (2). Studies investigating which hematologic parameters can predict adverse outcomes in anti-K and anti-D alloimmunization are still lacking.

The Rh blood system and alloimmunization has been extensively studied (3, 4). Widespread use of anti-D Rh immune globulin (RhIg) for RhD negative women in pregnancy has dramatically reduced the incidence of Rh(D) alloimmunization. K antigen expression begins on fetal erythrocytes at 10-11 weeks and k at 6-7 weeks' gestation (5, 6). In certain populations the incidence of K antigen ranges from 4-6% (9% among Caucasians and 2% in those of African descent) (7, 8). The incidence of K alloimmunization is estimated to be approximately 1:1,000 pregnancies (9), but strongly depends on preventative K matching for transfusion in women of childbearing age. K alloimmunization occurs secondary to previous blood transfusion (unmatched for K) but also following feto-maternal hemorrhage.

In alloimmunized women, hemolysis is caused by erythroid specific antibodies that traverse the placenta into the fetal circulation, initiating immune destruction of fetal erythroid cells, which can lead to progressive hemolytic anemia. Antibody concentration and its affinity for antigens determines the severity of fetal anemia and thus the fetal consequences. While IgM is initially detected in the maternal circulation following the primary exposure, IgG is found during the secondary response, appearing in the circulation 5-15 weeks after exposure. IgG can cross the placenta and cause fetal anemia, as fetal RBCs sensitized by maternal IgG are destroyed by the spleen (10).

The mechanism of fetal anemia in K alloimmunization differs from that in Rh-D, in that the extent of hemolysis is less (11, 12). Anti-K antibodies do cause increasing bilirubin levels in the fetal circulation, suggesting hemolyis (13), but anemia is also due to suppression of fetal erythropoiesis (14-16). Daniels (17) described that anti-K antibodies cause phagocytosis of early erythroid progenitor cells prior to their hemoglobinization, as the K glycoprotein is one of the first erythroid-specific antigens to appear on erythroid progenitors during erythropoiesis, contrary to some Rh antigens, which appear much later. Decreased laboratory indices of hemolysis (amniotic fluid bilirubin, fetal reticulocytes and nucleated RBCs) and fetal erythropoiesis have been described in pregnancies complicated by K alloimmunization (16, 18). A dose-dependent suppression of hematopoietic progenitor cells by serum from women with circulating anti-K antibodies has also been demonstrated *in-vitro* (18).

The severity of hemolytic disease of the fetus and newborn varies. For example, K alloimmunization ranges from 11-50% (19, 20) . Laboratory variables that have been associated with disease severity include ABO maternal fetal mismatches and characteristics of the antibody. Clinical characteristics associated with severity have not been well defined (21). We thus aim to

investigate predictors of poor fetal outcome, to provide further guidance in the optimal management of anti-D and anti-K alloimmunization. Our primary objective was to identify factors which could prolong pregnancy, by reducing the need for iatrogenic pre-term birth. Our secondary objective was to investigate any predictors of intrauterine fetal demise (IUFD), neonatal death (NND) or the postnatal need for (a composite outcome of) neonatal transfusion or exchange transfusion, phototherapy and/or use of intravenous immunoglobulin (IVIG). Furthermore, we sought to determine the role and contribution of anti-K antibody titration.

Methods

This retrospective single-center study was conducted at Mount Sinai Hospital, Toronto, Canada, the referral center for RBC alloimmunization and IUT for most of Ontario and several other Canadian provinces. We included all pregnancies alloimmunized with anti-K and anti-D as a single antibody, between 1991 and 2018 that underwent at least 1 fetal transfusion at our centre. Fetuses undergoing transfusion for anemia unrelated to anti-D or anti-K or patients with multiple antibodies were excluded from this study. (Research Ethics Board approval 12-0113-C).

In all pregnancies with RBC alloimmunization, antibody titers were determined and other causes of fetal anemia were excluded by ultrasound (US). Fetal blood sampling (FBS) was performed if the MCA-PSV was ≥ 1.5 Multiples of the Median (MoM). Prior to 2006, FBS was conducted when the amniotic fluid optical index at 450nm (ΔOD_{450}) fell in zone III of the Liley graph (22).

Fetal blood transfusion

If the fetal hemoglobin was < 100 g/L or > two standard deviations (SD) below the normal value for that gestation, RBCs were transfused. We used O D negative, and *(as of 2015)* K-negative donor RBCs, which were phenotyped, irradiated, leuko-reduced/Buffy coat and serologically negative for cytomegalovirus and cross-matched to a maternal sample, packed to a hematocrit of 75–80%. At our centre, we perform approximately 70% of IUT's via the fetal intrahepatic vein (IHV) and most of the remainder via the placental cord insertion (PCI), occasionally resorting to a fetal intra-cardiac or intra-peritoneal approach at very early gestational ages. The transfusion volume is based on the estimated fetal weight, donor and fetal pre-transfusion Hb and target Hbs, presence of hydrops and the feto-placental blood volume(23).

Data collection

We extracted data from medical records, US reports and the transfusion medicine laboratory. Data included maternal characteristics, pregnancy history, antibody and titer level using tube titration, number of IUTs and Hb concentration at the beginning and end of all IUTs. Neonatal outcomes included survival, mode of delivery, GA at delivery, birth weight, Hb level at birth, IUFD, NND and the need for neonatal transfusion or phototherapy or IVIG. Data were collected until the time of the baby's discharge from hospital. Indications for phototherapy, simple or exchange transfusion in neonates were previously described (Garabedian et al, 2015). In brief, an exchange transfusion was performed when bilirubin levels were above a certain threshold and phototherapy alone was ineffective in reducing them. Indications for a neonatal RBC transfusion were an Hb < 100g/L or if anemia was symptomatic (pallor, poor feeding, tachycardia,

tachypnea). The overall survival rate was determined as the number of infants discharged alive from hospital, divided by the total number of fetuses undergoing IUT.

Antibody titre determination

Antibody titration was done using the saline indirect antiglobulin test and R_2R_2 homozygous cells for D (double dose) and heterozygous K-positive cells (single dose) for K. Titre was expressed as the reciprocal of the highest plasma dilution that showed 1+ agglutination. Titres were determined by two registered technologists to ensure agreement. All titres were tested in parallel with the plasma/serum from the previous frozen sample.

Statistics

Descriptive, univariate and bivariate analysis: Means and standard deviations were reported for normally distributed, and median and inter-quartile ranges for continuous variables which were not normally distributed. Categorical variables were summarized as frequencies and proportions. Baseline characteristics of pregnant women with a single anti-K or anti-D undergoing IUT were compared using Student's *t*-test for normally distributed variables. The equality of variances between the two samples was estimated using the folded f-test. When variances were equal between samples, the pooled method was used to estimate the t-statistic and in the event of inequality, the *Satterhwaite* approximation was used. Wilcoxon's rank sum test was used to compare variables which were not normally distributed. *Chi-square* test was used for categorical variables and *Fisher's* exact test for < 5 observations.

Association between Anti-K antibodies and time spent in-utero after the first IUT: A multi-variable linear logistic regression model was constructed. The model included *a priori* selected variables: antibody type (Anti-K/Anti-D), titre, maternal age, history of IUFD/NND, number of IUTs, Hb at first IUT, daily decrease of Hb between first and second IUTs and presence of fetal hydrops. Predictors were deemed to exhibit multi-collinearity if the variance inflation factor (VIF) was >4. The multi-variable linear regression model was evaluated using the Omnibus F-test to determine if there was a significant association between any characteristics and the time spent *in-utero* after the first IUT. We assessed model fit to the data with the R^2 test.

Multivariable logistic regression for determination of composite adverse pregnancy outcomes: We used backward variable selection for binary multiple logistic regression. The overall logistic regression model significance was determined by the Likelihood Ratio (LR) Omnibus test. Effect estimates were expressed as odds ratios (OR) and their 95% confidence intervals (CI). The model fit was assessed using Hosmer-Lemeshow's goodness of fit test (calibration), as well as using discrimination (c-statistic). As the Hosmer-Lemeshow test was non-significant, the model was assumed to be appropriate.

As some women had multiple pregnancies during the study period, we investigated for patient level clustering using marginal generalized estimating equation (GEE). Marginal model plots were used to assess and confirm model fit over the co-variate patterns. No influential outliers were found and there was no suggestion of overspecification. As ORs did not differ between this model and our binary multiple logistic regression model, adjusting for clustering was not necessary for this analysis.

All analyses were performed using SPSS software (IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp)

Results

In total, 116 women with 128 pregnancies and 425 IUTs with anti-K or anti-D as a single antibody were identified. Median maternal age at first transfusion was 31 years (27.0-35.0) for women with anti-K and 32 years (23.6-40.6) for women with anti-D antibodies. Median GA at first IUT differed significantly between anti-K and anti-D antibody groups (24.3 (14.4-34.2) vs 28.7 (24.4-33.0) weeks, respectively, p=0.004). Fetuses affected by anti-K antibodies required more IUTs than those affected by anti-D antibodies (3.84±1.7 *vs* 3.12±1.62 IUTs, p=0.036), and Hb at first IUT was significantly lower in the anti-K group (51.0 (34.8-67.3) *vs* 70.5 (56.2-84.9) g/L, p=0.001) (**Table 1**). The interval between the first IUT and delivery was 69.6±41 days in the anti-K group *vs* 54.6± 35.6 in the anti-D group, but this did not reach statistical significance (*p*=0.061). The daily decrease in Hb between the first and second IUT (as a marker of severity of hemolytic disease of the fetus and newborn), development of fetal hydrops and the number of infants born less than 32 or 28 weeks gestation did not differ significantly between the two groups (**Table 1**). Mean GA at delivery was 35.0±5.5 weeks in the anti-K and 36.0±3.8 weeks in the anti-D group, with 87.1% and 93.9% of babies born alive respectively. The proportion of neonates requiring phototherapy, IVIG and exchange or simple transfusion was comparable across the two groups (**Table 1**).

With each additional IUT, 22.5 days were gained *in-utero*. (**Table 2**). Regression analysis showed that delivery occurred, on average, one week sooner after the first IUT for each 10g/L/day Hb reduction between the first two IUTs (p=0.01), accounting for the number of IUTs. The average number of days gained *in-utero* after the second IUT for fetuses with an average decrease in Hb of 6.05g/L/day was four weeks. GA at first IUT and 'interval from the first IUT until delivery' predicted an IUFD/NND outcome in anti-D/anti-K alloimmunization. Regression analysis of the anti-D group alone showed similar levels of significance for these variables, however analysis of the anti-K group did not show significance. Combination of the two groups in the model, presented in **Table 3**, strengthened significance**.** With the first IUT at 23 weeks, the OR of IUFD or NND were 1:12 vs 1:39 at 28 weeks gestation and 1:300 at 36.7 weeks gestation, respectively (**Table 3**). There were two IUFDs in the anti-K group and three in the anti-D group. There were no NNDs in the anti-K cohort, and two in the anti-D group.

Earlier gestational age at first IUT and a lower Hb at first IUT were significant predictors of a composite blood product requirement postnatally (**Table 4**). These variables were significant predictors when analyzing the anti-D group alone, but not when analyzing the anti-K group alone. Amongst all women with anti-D/anti-K alloimmunization, the odds of a neonatal blood product being required was 43% if the first IUT was at 28 weeks GA with a median neonatal Hb of 66g/L at the time. In the anti-K group, 9.7% delivered <28 weeks GA compared to 4.1% of the anti-D group. In the anti-K cohort, 32% required phototherapy, 12.9% IVIG and 16.1% either

an exchange or simple transfusion, compared to 44.9%, 11.2% and 32.6 % respectively in the anti-D cohort.

The higher the antibody titre, the greater the number of IUTs required in the anti-K group **(Figure 1A).** There was a similar trend in anti-D alloimmunized patients, however the correlation was not significant **(Figure 1B).** Earlier GA at first IUT significantly correlated with a higher K-titre **(Figure 2A)** and D-titre **(Figure 2B)** at that time point, suggesting that IUTs were required at an earlier GA when titres were higher. No pregnancy in either the K or D groups with a titre < 1:32 required an IUT. **Figure 3** shows the reduction in the average daily Hb drop between each subsequent IUT in the anti-K and in the anti-D groups.

Discussion

These findings emphasize that the evaluation of pregnancy parameters such as GA or Hb at first IUT, number of IUTs and daily decrease of Hb between the first and second IUTs predict adverse pregnancy outcomes and need for neonatal transfusion. Our multi-variable linear regression analysis demonstrated that a greater number of IUTs predicted more days spent *in-utero*, with an additional 22.5 days gained *per* transfusion, highlighting the importance of IUTs to adequately suppress fetal erythropoiesis and maintain adequate fetal Hb levels, allowing the pregnancy to advance, thus reducing the likelihood of complications from prematurity. Prematurity is the leading cause of infant mortality and lifelong NDI effects such as cerebral palsy, mental retardation, visual and hearing impairment. A more marked daily decrease in Hb between first and second IUT (g/L/day), also predicted a shorter time spent *in-utero*. Delivery can be estimated to occur 1 week earlier (from the first IUT) for each 0.1g/L/day Hb reduction between the first two IUTs, which can aid IUT and delivery timing. Pregnancy prolongation and prenatal anemia optimization are pivotal in reducing neonatal morbidity. Neonatal anemia directly correlated with severe intra/peri-ventricular haemorrhage, with more frequent occurrence of an haemodynamically significant patent ductus arteriosus and **a** longer period of mechanical ventilation and ionotropic support in low-birth-weight premature infants (24).

Furthermore, a more rapid fall in Hb level between the first and second IUT was associated with an increased risk of an adverse outcome, such as IUFD or NND. We have also established that the earlier in gestation that IUTs commence, the higher the odds of either an IUFD or NND, which may reflect the impacts of prematurity and worse disease, as evidenced by the increased cumulative transfusion requirements. Pregnancies complicated by anti-D and anti-K antibodies have previously been shown to require phototherapy in 67% *vs* 50% of cases respectively and RBC simple/exchange transfusion in 23% *vs* 50% respectively (3). In our cohort, these interventions were considerably reduced, with phototherapy being required in 32% and 44%, and RBC simple or exchange transfusion in 19% and 33% in the anti-D and anti-K cohorts respectively. IVIG was used in 13% and 11% of cases (**Table 1**). This likely represents the effect of close US monitoring and procedural experience due to the large volume of IUTs performed at our center, which result in optimized neonatal blood parameters and adequate suppression of haematopoiesis at delivery. However, later GA at delivery was associated with a need for more neonatal blood transfusions (**Table 4**), likely due to the degree of suppression of neonatal erythropoiesis, secondary to RBC transfusion. Over the last decade at our institution, IVIG has

been more frequently used in the most severe cases, with the goal of deferring the initial RBC transfusion. Our data underline the need for a longer period of neonatal follow-up and support until the baby's baseline marrow function recovers.

Our data support a correlation between the number of IUTs required and the K-titre. Maternal K-titre can be used to predict IUT requirement, and to counsel patients on the mean number of IUTs they are likely to require during pregnancy using the following formula $y=0.43*\log_2$ titre + 0.49. For example the mean predicted number of IUTs in a patient with a titre of 1:32 = 0.43 *(5) +0.49 = 2.6. Furthermore, we have established a correlation between the mean GA at first IUT and the K titre ($y= -2.15 * \log_2$ titre +41.81). A patient with a titre of 1:32 would be predicted to need the first IUT at a (mean) GA of 31.1 weeks (y=-2.15*(5) + 41.81). The average daily Hb drop between the first and second IUT was similar in the anti-K and anti-D groups. The rate of decrease in Hb dropped with subsequent IUTs as expected, due to fetal bone marrow suppression and replacement of fetal with adult red blood cells.

In this series, no fetus with an anti-K antibody titre of < 1:32 by tube testing needed an IUT. Previous studies have suggested lower cut-offs such as 1:16 (25) or 1:4 (26). The tube tire method has also proven accurate in predicting HDFN at titres >1:16 in non-D and non-K alloimmunized pregnancies (27). Titre identification might alleviate maternal anxiety and could minimize the need for travel, often entailing work absence and/or family inconvenience, if Doppler evaluation of the MCA-PSV cannot be reliably assessed locally.

Limitations

Limitations to this study are predominantly due to retrospective analyses such as data availability in medical records. The study interval spanned many years where identification of need of IUT changed from invasive to non-invasive approaches using Doppler ultrasonography of the MCA PSV. Titration was performed using tube testing and not column agglutination or methods determining antibody concentration thus may not be generalizable to those laboratories.

Conclusions

Our study identified the pregnancies with anti-D and anti-K alloimmunization which are at an increased risk of an unfavourable outcome. Identifying these patients can inform pregnancy management, facilitates counselling of patients and can direct resource use. Prospective studies can also incorporate these characteristics, in addition to laboratory markers, to further identify **such** at risk pregnancies and improve their perinatal outcomes.

Principal Findings

1. Gestational age (GA) at first intrauterine blood transfusion (IUT) and interval from first IUT until delivery predicted fetal/neonatal death. GA and Hb at first IUT were significant predictors of need for phototherapy and/or blood product use in the neonate.

2. No pregnancy with an anti-K antibody titre <1:32 (by tube testing) required an IUT.

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Authorship Contributions

E. Vlachodimitropoulou, N. Shehata and G. Ryan conceived and designed the study, aided with data analysis and wrote the manuscript; M. Garbowski aided with the data analysis and interpretation and manuscript review and Shelly Solomon aided with data collection and manuscript review. PGR. Seaward, R. Windrim, N Abbasi, J. Keunen, E. Kelly and T. Van Mieghem contributed the data and reviewed the manuscript.

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Tables

Table 1. Baseline characteristics of anti-K and anti-D alloimmunized pregnancies receiving IUTs between 1991 and 2018 at Mount Sinai Hospital, Toronto, Canada.

Characteristics	**Anti-K** n = 31	**Anti-D** n = 97	**p-value**
Demographics at Study Entry			

Maternal Age (years), Median (IQR)	31.00 (27.0-35.0)	32 (23.6-40.6)	0.76
Antibody titre, n (%) 1:32 1:64 1:128 1:256 1:512 1:1,024 1:2,048	 3 (9.7) 5 (16.1) 3 (9.7) 10 (32.3) 6 (19.4) 2 (6.5) 2 (6.5)	 2 (2.1) 9 (9.3) 16 (16.5) 24 (24.7) 28 (28.9) 14 (14.4) 5 (5.2)	
History of HDFN, n (%)	4 (12.9)	49 (50.5)	0.001
History of IUFD, n (%)	4 (14.8)	11 (12.6)	0.51*
History of NND, n (%)	1 (3.2)	2 (2.1)	0.57*
GA at first IUT (weeks), (median, IQR)	24.3 (14.4-34.2)	28.7 (24.4-33.0)	0.004
Number of IUTs, Mean ± SD (range)	3.84 ±1.7 (1-6)	3.12 ±1.62 (1-9)	0.036
Hemoglobin characteristics			
Hb at first IUT (g/L), Median (IQR)	51.0 (34.8-67.3)	70.5 (56.2-84.9)	0.001
Daily decrease of Hb between first and second IUT (g/L/day), Mean (SD)	0.038 (0.057-0.095)	0.039 (0.022-0.056)	0.075
Daily decrease of Hb between 2nd and 3rd IUTs (g/L/day), Mean (SD)	0.035 (0.028- 0.043)	0.033 (0.027-0.039)	0.31
MCA PSV prior to first IUT (cm/s), Median (IQR)	58.2 (46.8-69.6)	66.9 (53.9-79.9)	0.20
Pregnancy outcomes			
Twins, n (%)	0 (0.0)	1 (2.0)	0.58
Hydrops fetalis in this pregnancy, n (%)	4 (12.9)	14 (14.3)	0.56*
Time between first IUT and delivery (days), Mean (SD)	69.57 (40.91)	54.60 (35.57)	0.061
GA at delivery (weeks), Mean (SD)	35.01 (5.5)	36.02 (3.78)	0.28
Delivery outcome Live birth, n (%) Miscarriage, n (%) IUFD, n (%) NND, n (%) Termination of pregnancy, n (%)	 27 (87.1) 1 (3.2) 2 (6.5) 0 (0.0) 1 (3.2)	 92 (93.9) 1 (1.0) 3 (3.0) 2 (2.0) 0 (0.0)	 0.37 0.42* 0.67* 0.57* 0.24
Delivery < 32 weeks, n (%)	3 (9.7)	8 (8.2)	0.73*
Delivery < 28 weeks, n (%)	3 (9.7)	4 (4.1)	0.36*
Birth weight percentile, median (IQR)	70 (54)	47.5 (59.25)	0.73
Apgar at 1 minute, Mean (SD)	7.04 (2.92)	7.18 (2.97)	0.83
Apgar at 5 minutes, Mean (SD)	7.88 (2.62)	7.93 (2.71)	0.93
Neonatal phototherapy, n (%)	10 (32.3)	44 (44.9)	0.07

Neonatal IVIG, n (%)	4 (12.9)	11 (11.2)	0.75
Neonatal exchange transfusion, n (%)	1 (3.2)	11 (11.2)	0.28*
Neonatal "top-up" transfusion, n (%)	5 (16.2)	21 (21.4)	1.00*
Hb g/L at delivery, median (IQR)	136 (49.5)	137 (45.25)	0.96
Bilirubin (highest recorded within 72 hours of age), Mean (SD)	80.77 (57.96)	111.38 (86.40)	0.25

*Fisher's Exact Test. Chi-square tests were used for all other categorical variables and two-sample t-tests for all normally distributed continuous variables.

Abbreviations: GA – gestational age, IUT – intrauterine transfusion; HDFN– Hemolytic disease of the fetus and newborn; IUFD – intra-uterine fetal death; NND – neonatal death; Hb – hemoglobin; MCA PSV – Middle cerebral artery peak systolic velocity; IVIG - intravenous immunoglobulin.

Table 2. Multivariable linear regression model with *a priori* selected predictors of *the time (in days) between the first IUT and delivery* (Outcome).

Predictor Variables	Adjusted B Coefficient Estimate (95% CI)	Test Statistic	p-value
Omnibus F-test (F, p-value)		47.684	0.001
Constant	-20.21 (-47.46 to 7.04) 27.85 (22.94 to 32.76) *	-1.47 -3.01	0.14 <0.001
Antibody type (Reference = Anti-D)	-1.08 (-9.46 to 7.30)	-0.26	0.80
Titre level (Reference = 1:32)	0.002 (-0.003 to 0.008)	0.89	0.38
Maternal age	0.13 (-0.51 to 0.77)	0.40	0.69
History of IUD/ NND	-4.61 (-14.86 to 5.63)	-0.89	0.37
Number of IUTs	22.57 (19.75 to 25.38) 22.52 (20.32 to 24.72) *	15.91 20.30	<0.001 <0.001
Hemoglobin at first IUT	-0.23 (-2.00 to 1.55)	-0.25	0.80
Daily decrease of Hb between first and second IUT (g/L/day)	-6.60 (-11.72 to -1.47) -7.11 (-11.64 to -2.58) *	-2.55 -3.11	0.01 0.002
Hydrops fetalis in this pregnancy	-5.70 (-15.28 to 3.89)	-1.18	0.24

R^2= 0.80

Abbreviations: IUT – intrauterine transfusion; HDFN– Hemolytic disease of the fetus and newborn; IUD – intrauterine death; Hb – hemoglobin; MCA PSV – Middle cerebral artery peak systolic velocity; CI - confidence interval, NND – neonatal death.

*Linear regression model including the two significant predictors: 'number of IUTs' and 'Daily decrease of Hb between first and second IUT'. Model variables re-centered to the mean Hb drop of 0.605 g/dL/day between the first and second IUT (IUT=2).

Table 3. Multivariable binary logistic regression model for an IUFD/NND outcome.

Predictor	OR (95% CI)	p-value

Omnibus Likelihood Ratio chi-square = 16.28?		<0.001
Constant	0.081	<0.001
GA at first IUT	0.79 (0.67 to 0.93)	0.006
Time between the first IUT and delivery*	0.98 (0.96 to 0.99)	0.018

Abbreviations: IUFD – intra-uterine fetal death; NND – neonatal death; CI - confidence interval; OR - odds ratio; GA – gestational age, IUT – intrauterine transfusion;
Hosmer-Lemeshow goodness of fit; Chi-square = 3.61 (df8) (p =0.89)
Model variable re-centered to 23 weeks (25th percentile)
*Not included in the final model due to collinearity

Table 4. Multivariable binary logistic regression model for a composite transfusion outcome (Neonatal: "Top-up" transfusion/Exchange transfusion/ Phototherapy/ IVIG)

Predictor	OR (95% CI)	p-value
Omnibus Likelihood Ratio chi-square = 11.73		0.008
Constant	0.43	0.074
GA at delivery	1.11 (1.00 to 1.23)	0.046
Hb at first IUT	0.84 (0.72 to 0.99)	0.038

Abbreviations: CI - confidence interval; OR - odds ratio; IUT – intrauterine transfusion; GA - gestational age, IVIG - intravenous immunoglobulin;
Hosmer-Lemeshow goodness of fit; Chi-square = 5.42 (df8) (p =0.71)
Model re-centered to 28 weeks GA at delivery and the median Hb at first IUT of 6.6 g/dL

Figure Legends

Figure 1. The relationship between maternal K-titre (A) and D-titre (B) and the number of IUTs required during the pregnancy.

Figure 1A. Mean number of IUTs at each K-titre

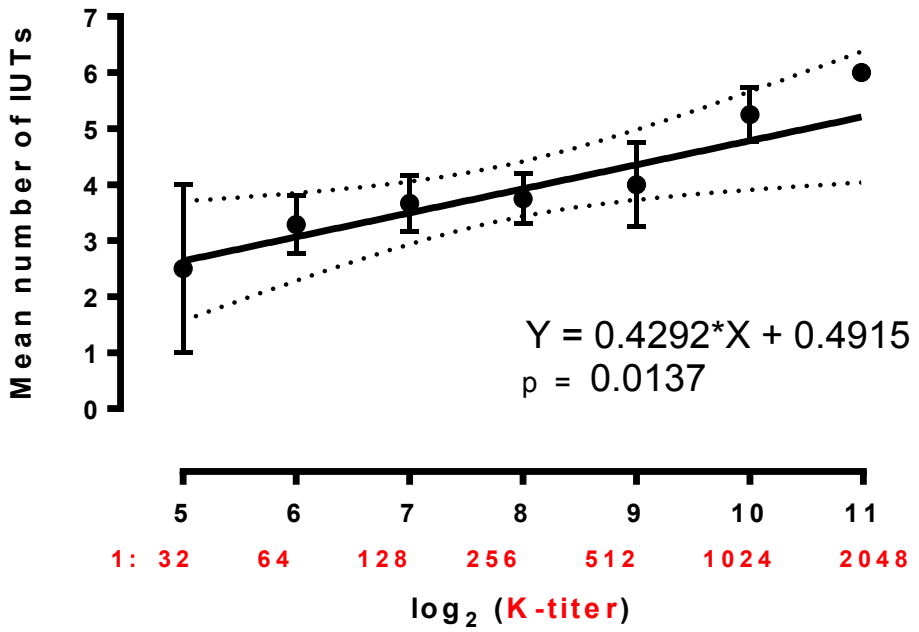

Figure 1B. Mean number of IUTs at each D-titre

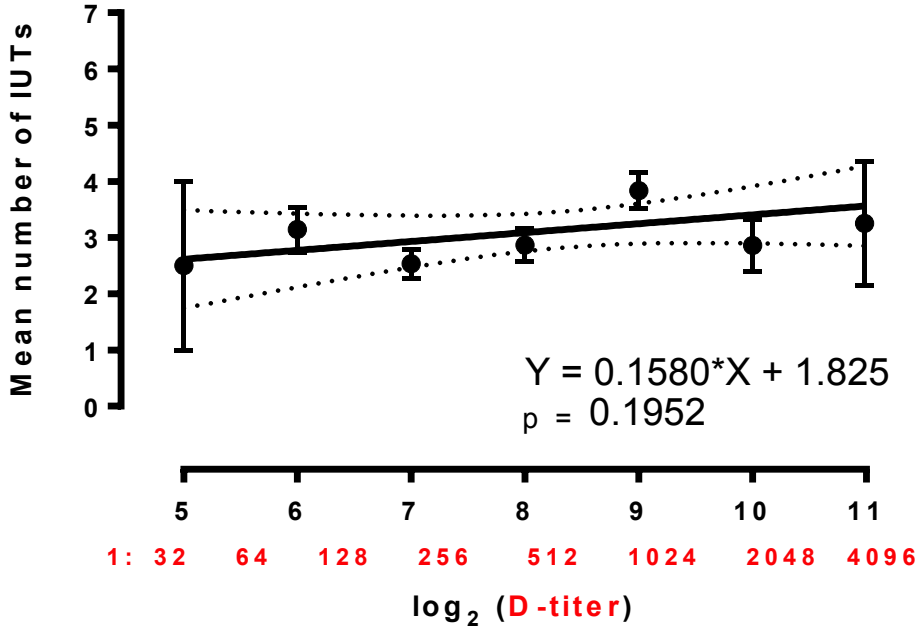

Figure 2. The relationship between maternal K-titre (2A) and D-titre (2B) and gestational age at the first IUT.

Figure 2A. Mean gestational age at 1st IUT at each K-titre

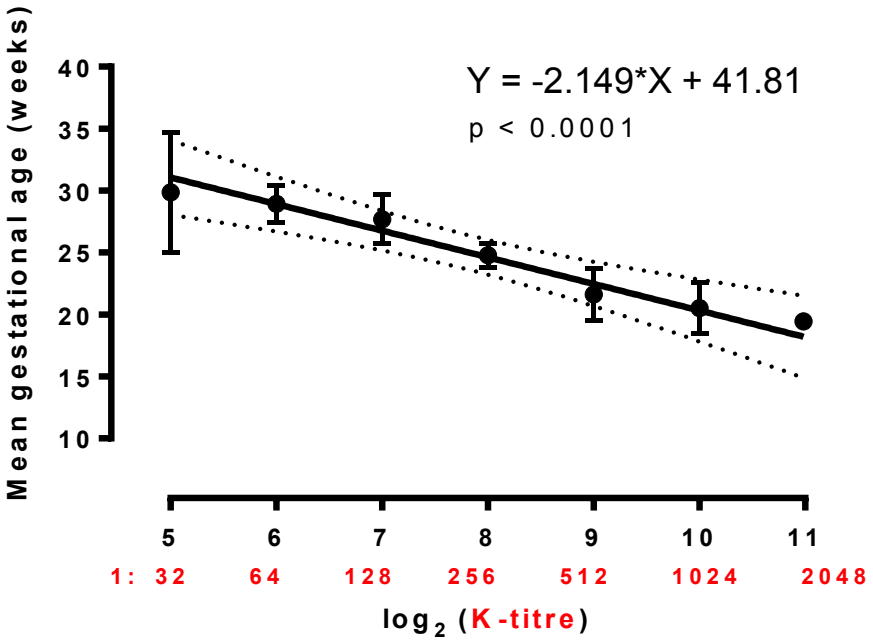

Figure 2B. Mean gestational age at 1st IUT at each D-titre

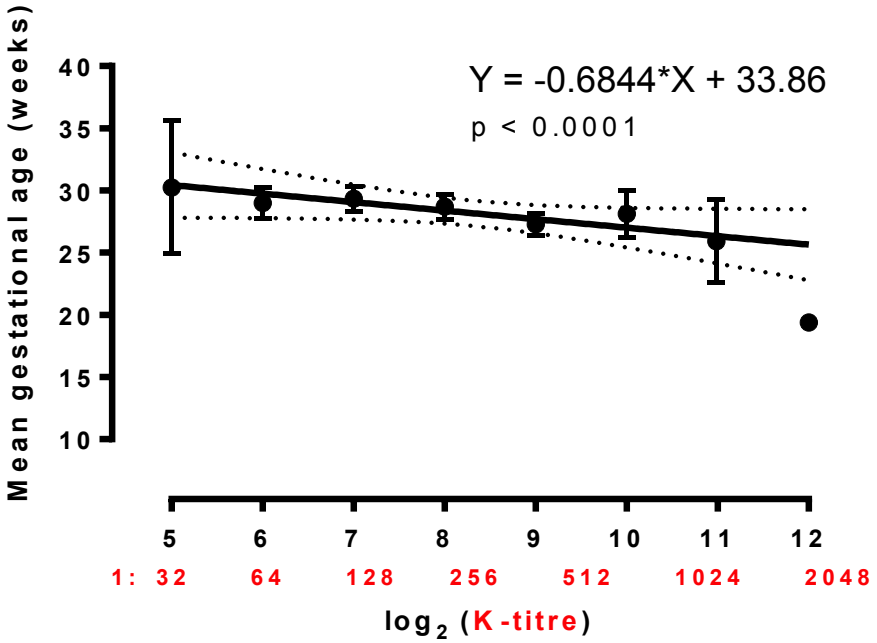

Figure 3. The relationship between the rate of change of hemoglobin following each sequential IUT in K (3A) and D (3B) alloimmunization.

Figure 3A. Rate of decrease of Hb per day following each IUT for all anti K- titres

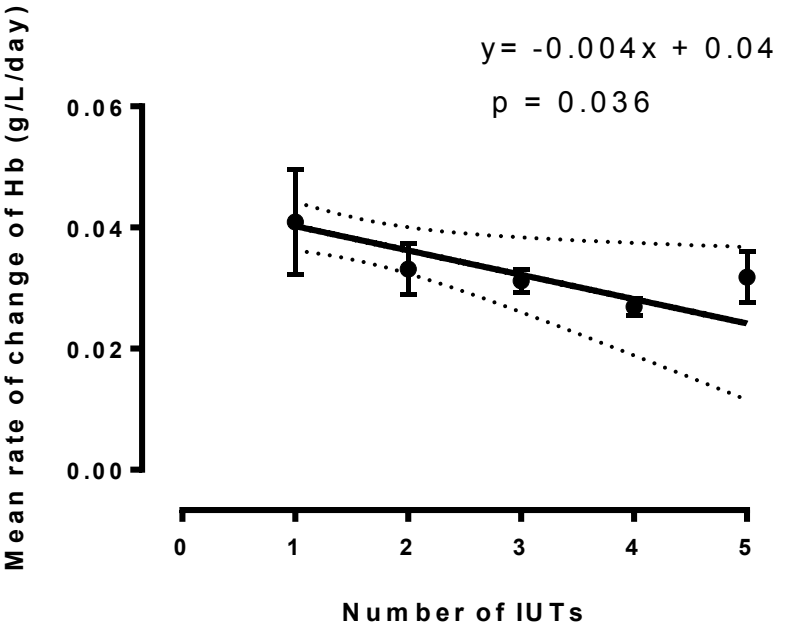

Figure 3B. Rate of decrease of Hb per day following each IUT for all anti D- titres