Hemolytic disease of the fetus and newborn

Zwiers, C.

HEMOLYTIC DISEASE

OF THE FETUS AND NEWBORN

CAROLIEN ZWIERS

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GENERAL INTRODUCTION

Adapted from 'Zwiers C., van Kamp I.L., Oepkes D. (in press). Management of red cell alloimmunization. In M. Kilby, A. Johnson, & D. Oepkes (Eds.), Fetal Therapy(2nd edition): Scientific Basis and Critical Appraisal of Clinical Benefits. Cambridge: Cambridge University Press.'

Hemolytic disease of the fetus and newborn (HDFN), caused by maternal red cell alloimmunization, has long been a major cause of perinatal morbidity and mortality. No antenatal treatment was available up to the 1960s. The only option was thus (preterm) induction, to enable neonatal treatment. This changed with the introduction of intrauterine intraperitoneal transfusion in 1963 by professor William Liley. However, in the early years, the complication risk of this X-ray guided intraperitoneal procedure was substantial. Outcomes gradually improved with more experience, the introduction of ultrasound-guided intravascular transfusions in the late 1980s, and advances in neonatal care.

Nowadays, both the incidence and risks of (antenatal treatment of) HDFN seem to have reached an 'as good as it gets' state in developed countries. In this thesis we aimed to summarize and evaluate current best practice, but often expert-based, management of HDFN. Furthermore, we assessed factors influencing the immunization risk and the severity of disease and evaluated standard and alternative treatment options.

In this chapter, an introduction to red cell alloimmunization and hemolytic disease of the fetus and newborn is provided.

RED CELL ALLOIMMUNIZATION

The origin of red cell alloantibodies

Red cell alloimmunization results from fetal-maternal blood group incompatibility: the fetus carries a red cell antigen, inherited from the father, that is unknown to the maternal immune system. Over 300 blood groups have been discovered so far, contributable to over 30 blood group systems[1] and incompatibilities between mother and child can exist in every single one of these blood groups. Apart from the ABO blood group system, the Rh(esus) system is the most well-known. It was discovered in the Rhesus macaque in the 1940s by Landsteiner and Wiener. This scientific milestone marks a major breakthrough in HDFN research and is therefore memorialized by the schematic illustration of a Rhesus macaque on the cover of this thesis.[2]

Fetal-maternal blood group incompatibility may lead to alloimmunization when fetomaternal hemorrhage (FMH) occurs during pregnancy or delivery (Figure 1). It is

known that FMH (often microtransfusions) can be detected in 45% of uncomplicated pregnancies during the third trimester and after 60% of deliveries.[3]

ABO incompatibility between mother and child is known to have a protective effect, reducing the chance of the alloimmunization. In the 1940s, Levine et al. already postulated this preventive effect when they noted that ABO incompatibility was less common among couples with D (formerly known as RhD) immunization in pregnancy, when compared to uncomplicated pregnancies.[4] In this thesis, we aimed to evaluate whether ABO incompatibility is also preventive for non-D immunizations (other Rh such as C, c, E or e, non-Rh such as K (Kell), Fy (Duffy), Jk (Kidd), etc.).

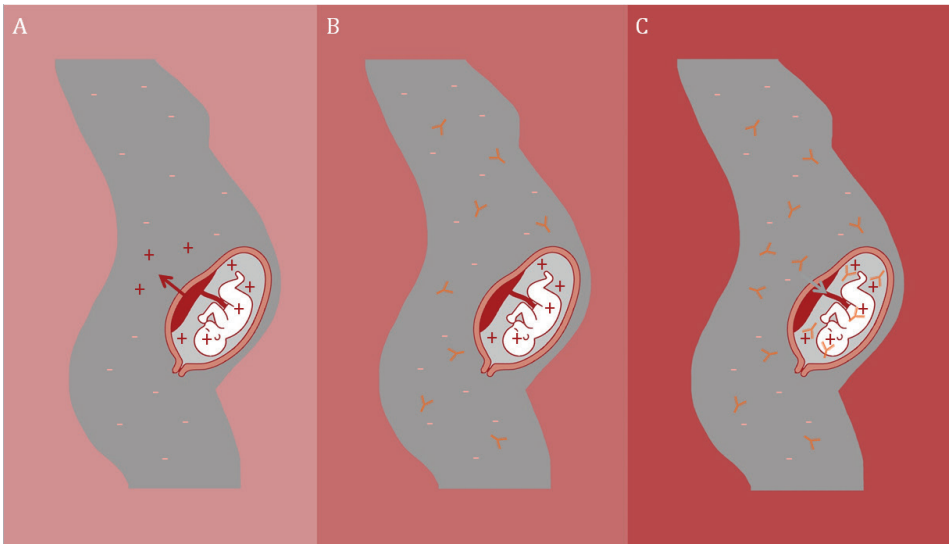

Figure 1. The process of red cell alloimmunization. A: the mother is negative for a blood group antigen, the fetus is antigen-positive. During pregnancy or after delivery, fetal red cells enter the maternal circulation. B: maternal antibodies are formed against the fetal antigen. C: in the same or a next pregnancy, the antibodies are transported across the placenta and bind fetal red cells.

Prevention, screening and incidence

In order to prevent D immunization, D-negative women carrying a D-positive child receive both antenatal (at 28-30 weeks) and postnatal anti-D prophylaxis (RhIg) in most developed countries.[5-7] Targeting the antenatal administration only to women with D-positive fetuses became possible with the introduction of cell-free fetal genotyping in maternal plasma.[8] This test can prevent that 40% of D-negative women unnecessarily receive antenatal RhIg, and is therefore progressively implemented throughout the developed world.[9,10]

Another common measure to prevent alloimmunization is matching blood transfusions for women of reproductive age for the D antigen, and, in the Netherlands, additionally for Kell, c and E.[11,12]

To date, the working mechanisms of RhIg remain partially unclear. To evaluate whether RhIg might function in a non-epitope specific manner, and because no prophylactic agent is available to prevent non-D immunization, the effect of RhIg on the occurrence of non-D antibodies was assessed in this thesis (Chapter 2).

As a result of the abovementioned prophylactic measures, the D immunization rate after delivering one D-positive child has decreased dramatically over the past decades, from 5.0% in the 1960s to 0.3% in 2008.[5,13] The prevalence of D immunization amongst all pregnant women was 0.09% in 2016, based on our nationwide prospective study (this thesis, unpublished data) and the total number of screened pregnant women.[14] To timely identify these pregnancies in which alloantibodies associated with HDFN (mainly Rh and K antibodies) do occur, all pregnant women are screened at the time of the first antenatal visit (preferably before 13 weeks' gestation)[15] as part of our free national population screening programme carried out by the National Institute for Public Health and the Environment (RIVM). Within this programme, D-negative women are again screened at 27 weeks, before the prophylactic administration of RhIg. In addition, in the Netherlands, c-negative pregnant women are also screened for relevant antibodies at 27 weeks.

RISK OF HEMOLYSIS

Clinically relevant antibodies

The clinical relevance of alloantibodies in pregnancy depends on whether the fetus carries the antigen against which the antibodies are directed, whether it concerns IgG or IgM antibodies (IgM is not transported across the placenta) and on the ability of the antibody to induce hemolysis.[16] In general, severe antenatal HDFN is most frequently caused by anti-D, anti-K or anti-c and only exceptionally by other Rh (E, C) and non-Rh antibodies such as anti-Fy or anti-Jk.[17,18]

Fetal phenotype

The first step to predict whether a pregnancy with alloantibodies is at risk for fetal hemolytic disease, is to determine whether the fetus carries the antigen against which the

antibodies are directed. If the father is found or known to be antigen-negative, no further fetal assessment is advised.[19,20] Otherwise, non-invasive fetal genotyping by polymerase chain reaction (PCR) on cell-free fetal DNA in maternal plasma is nowadays usually the next step. This test is available with a high sensitivity (>95%) for D, C, c, E, (e) and K[21] and can reliably be performed from the first trimester onwards(slightly later in gestation for K-typing). Therefore, the Royal College of Obstetricians and Gynaecologist (RCOG, United Kingdom) nowadays proposes that paternal testing might even be omitted.[22]

Serological testing

Assessment of the risk of fetal anemia in pregnancies with a positive antibody screening and an antigen-positive fetus is usually based on obstetric history and antibody titers, which is the highest dilution with positive agglutination test.[17,23] In the Netherlands, an antibody-dependent cell-mediated cytotoxity assay (ADCC) is additionally performed for risk assessment. This bioassay measures the percentage of hemolysis that the maternal antibodies induce in vitro and shows a higher specificity (and equal sensitivity) compared to antibody titer for predicting fetal anemia in D immunization.[24]

The 'critical titer' to identify pregnancies at risk for hemolysis is set on 1:16 for most (clinically relevant) antibodies in the Netherlands, the ADCC on 10% for D and 30% for non-D antibodies.[20] The ideal titer cut-off for K antibodies was long unclear and therefore set at 1:2. This was evaluated in a recent large study on K immunization in pregnancy, resulting in the recommendation to clinically monitor Kell-positive fetuses if antibody titer rises to or above 1:4.[25]

Recent studies have indicated that antibodies differ in their effector functions, as a result of differences in subclasses and glycovariants.[26-28] Furthermore, IgG-Fc receptor polymorphisms, influencing the clearance of anti-D sensitized fetal red cells, are found to be associated with HDFN severity.[29] These differences might be applicable in the future to predict which pregnancies are at risk for severe HDFN and which will only be subject to mild hemolysis.

Although most authors agree that HDFN severity increases in every subsequent pregnancy at risk, it is not well studied if and how obstetric history predicts outcome. We aimed to evaluate this by performing a nationwide cohort study amongst pregnant women with D immunization (Chapter 3).

MONITORING OF PREGNANCIES AT RISK FOR FETAL ANEMIA

If the results of serological risk assessment tests rise above a set cut-off value, weekly fetal monitoring is indicated. From the late 1990s onwards, peak systolic velocity measurement of the middle cerebral artery (MCA-PSV) blood flow by Doppler is the golden standard for predicting fetal anemia.[30] If MCA-PSV values exceed 1.5 multiples of the median (MoM) for gestational age, intrauterine transfusion (IUT) should urgently be performed, as measurements in this range strongly correlate with moderate to severe fetal anemia (sensitivity 100%, 12% false positive rate).

In the past, hydrops was often the first sign of fetal anemia due to red cell alloimmunization. This was alarming, as hydrops was associated with poor outcome on both the short and the long term.[31,32] The current well-organized health care system of routine early alloantibody screening, national guidelines for management and referral of cases and pooling of expertise in national reference laboratories and a referral center for fetal therapy aims to enable intervention before the development of hydrops. In this thesis, we evaluated how this system influenced hydrops rates and outcome of hydropic fetuses (Chapter 4).

INTRAUTERINE TRANSFUSIONS

The cornerstone in antenatal therapy for fetal anemia is the ultrasound guided intravascular intrauterine transfusion (IUT), replacing intraperitoneal transfusion in the 1980s.[33] In Chapter 1 of this thesis, we summarize the literature on the preparations, setting and (long-term) outcome of this treatment.

Whereas outcome after treatment with IUT(s) was fairly poor in the early years after implementation in 1980s, it is now considered relatively safe in experienced hands. Transfusion techniques and complication rates however vary between fetal therapy centers, although it is know that transfusion into the intrahepatic part of the umbilical vein or the placental cord insertion are safest (Figure 2 and Chapter 5). The current transfusion techniques and risk factors for complications are assessed in this thesis (Chapter 5). Overall, the survival after IUT in experienced centers has now reached approximately 96% (range 89-100 %, see Chapter 1).

We hypothesize that this increase could grossly be the result of three important changes: a reduction in complication rates, 2) more timely referral and therefore prevention of hydrops and 3) improvements in neonatal care. Both the complication rates and the prevention of hydrops were evaluated in this thesis (Chapters 4 and 5).

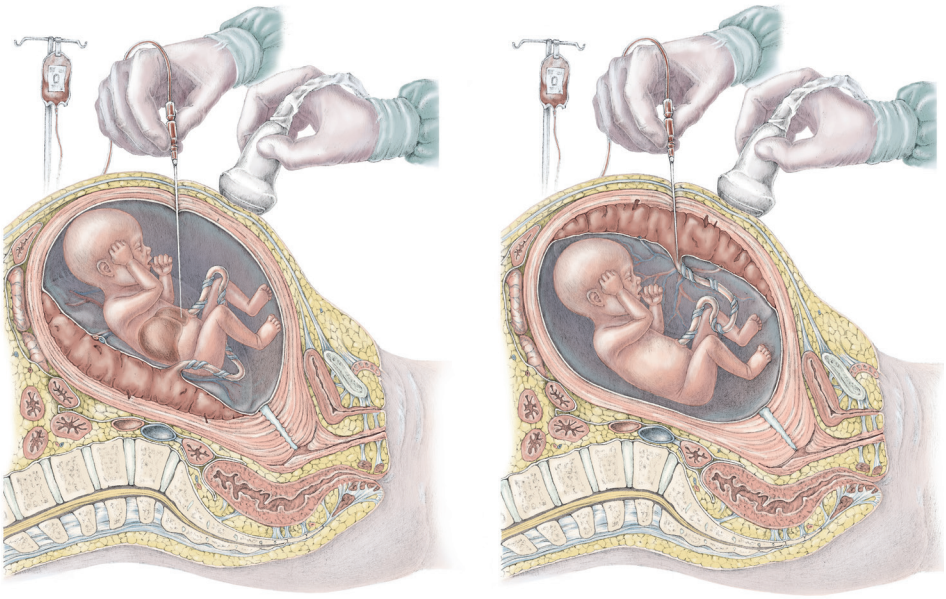

Figure 2. Intrauterine transfusion. Left: intrahepatic transfusion. Right: transfusion into the placental cord insertion (anterior placenta).

ALTERNATIVE TREATMENT

Several attempts have been made to assess the efficacy of non-invasive maternal treatment in pregnancies with severe (and early) HDFN. Most is known about therapeutic plasma exchange and/or intravenous immunoglobulins (IVIg). The literature on both of these alternative treatments is summarized in this thesis. Furthermore, we assessed the efficacy of IVIg in the multinational PETIT study. In this study, a cohort of patients with a history of early severe HDFN that was treated with IVIg, was compared to an equally severe non-IVIg group. The results are presented in this thesis (Chapter 6).

AIM AND OUTLINE OF THIS THESIS

The first attempts for intrauterine treatment by Liley et al. arose from a back-against-the-wall position: fetuses often died in-utero or suffered from extreme prematurity.[34] The founder of intrauterine transfusion in the Netherlands, professor Jack Bennebroek Gravenhorst, empirically introduced this treatment and stated:

'Hoofdzaak is het bestrijden van de anemie. Bij het voortschrijden van de technische mogelijkheden en door uitgebreidere toepassing van de laatstgenoemde methode zal ongetwijfeld een elegantere methode gevonden worden voor de toediening van het bloed, waardoor bezwaren, die thans ongetwijfeld bestaan, uit de weg geruimd zullen worden.'

In English, this quote would be: *'Main issue is to counter the anemia. The broadening of technical possibilities and more extensive application of the abovementioned method will undoubtedly lead to a more distinguished method for the administration of blood. Hereby the objections, that at present surely exist, will be eliminated.'*[35]

This thesis aims to describe the gradual disappearance of these objections by addressing our unique care system for alloimmunized mothers, assessing transfusion techniques and complications and evaluating alternative treatment. Thereby, we wish to contribute to converting the management of and intrauterine transfusion for hemolytic diseae of the fetus from expert-based to evidence based.

PART 1: OVERVIEW

General introduction

Chapter 1 – Review of the available literature on antenatal management and outcome of HDFN. This includes a detailed description of important aspects of intrauterine transfusion, the golden standard treatment for fetal anemia, and evaluates the evidence on alternative therapeutic options.

PART 2: PATHOGENESIS AND SEVERITY OF HDFN

Chapter 2 – Case-control study including pregnant women with newly detected non-D antibodies, assessing whether ABO incompatibility has a preventive effect on formation of non-D alloantibodies. Subsequently, we evaluated a possible protective effect of RhIg in a subgroup with non-Rh antibodies only.

Chapter 3 – National cohort study amongst all pregnant women with D immunization, in which we assessed if, and how disease severity increases in subsequent pregnancies complicated by HDFN. Furthermore, we aimed to identify factors from the first pregnancy with D antibodies that predict severe disease in the subsequent affected pregnancy.

Chapter 4 - 30-year cohort study evaluating trends in the condition of fetuses treated with intrauterine transfusion for red-cell alloimmunization, at the time of first transfusion and at birth. This, in relation to our well-organized health care system for alloimmunized mothers.

PART 3: INTRAUTERINE TRANSFUSION AND OTHER TREATMENT OPTIONS

Chapter 5 – 27-year cohort study, assessing trends in complication and fetal death rates after intrauterine transfusion. We evaluated how IUT is most safely performed and aimed to identify factors leading to improved outcome.

Chapter 6 – International cohort study comparing pregnancies of women with a history of severe HDFN that are treated with or without intravenous immunoglobulins (IVIg) in a subsequent pregnancy. We aimed to assess whether IVIg could be a non-invasive alternative for (hazardous) early intrauterine transfusions.

Chapter 7 – Cochrane systematic review evaluating neonatal treatment with IVIg to reduce the need for neonatal exchange transfusions.

PART 4: SUMMARY AND DISCUSSION

This section summarizes the findings of the abovementioned studies, discusses their implications and addresses future perspectives in the field.

CHAPTER 1

INTRAUTERINE TRANSFUSION AND NON-INVASIVE TREATMENT OPTIONS FOR HEMOLYTIC DISEASE OF THE FETUS AND NEWBORN – REVIEW ON CURRENT MANAGEMENT AND OUTCOME

Carolien Zwiers
Inge van Kamp
Dick Oepkes
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Expert review of Hematology, Apr 2017; 10 (4): 337-344.

ABSTRACT

Introduction

Hemolytic disease of the fetus and newborn (HDFN) remains a serious pregnancy complication, which can lead to severe fetal anemia, hydrops and perinatal death.

Areas covered

This review focusses on the current prenatal management, treatment with intrauterine transfusion (IUT) and promising non-invasive treatment options for HDFN.

Expert commentary

IUTs are the cornerstone in prenatal management of HDFN and have significantly improved perinatal outcome in the past decades. IUT is now a relatively safe procedure, however the risk of complications is still high when performed early in the second trimester. Non-invasive management using intravenous immunoglobulin may be a safe alternative and requires further investigation.

INTRODUCTION

Hemolytic disease of the fetus and newborn (HDFN) is still a serious complication in pregnancy. The condition is caused by maternal alloimmunization to fetal red cell antigens, inherited from the father, leading to fetal hemolysis and anemia. Untreated, progressing fetal anemia may result in hepatosplenomegaly, cardiomegaly, cardiac decompensation and eventually in fetal hydrops and perinatal death. If the fetus survives, persistent hemolysis may lead to severe neonatal hyperbilirubinemia and brain injury, an irreversible condition known as 'kernicterus'. [36] Antibodies associated with severe HDFN are mostly of the anti-Rh(D) type, and to a lesser extent of the anti-Kell (anti-K1) or anti-Rh(c) type. Severe HDFN is occasionally caused by other Rh-antibodies, and only very rarely by non-Rh antibodies (Duffy, Kidd, or S).[37]

Prenatal screening for red cell antibodies and several preventive measures, such as matched blood transfusions for Rh- and K antigens and the antenatal and postnatal administration of anti-D immunoprophylaxis, have significantly reduced the incidence and severity of HDFN.[5,38] Nowadays, approximately 1/300-1/600 pregnancies ending in live births are complicated by red cell immunization.[39]

If antibodies are detected in pregnancy, the risk of HDFN is estimated using maternal serum testing for antibody levels (quantification or titers) and, mainly in the Netherlands, antibody-dependent cell-mediated cytotoxicity (ADCC) assays for RhD immunizations.[24,40-42] In most countries, a critical titer around 16, varying from 8 to 32, is used as a cut off for fetal monitoring,[23,41] although this value has a false-positive rate of 77% for predicting fetal anemia.[24] Recent studies on antibody characteristics showed that lower core fucosylation of RhD-antibodies significantly correlated with increased disease severity[28] and in anti-c immunizations, antibody galactosylation and sialylation best predicted fetal/neonatal disease.[27] Furthermore, IgG1 anti-D subtypes are associated with increased severity, in contrast to IgG3.[26] To our best knowledge, these interesting novel insights are not yet implemented in general practice. If either of these serum tests suggest an increased risk on fetal hemolysis, the patient will be monitored by serial Doppler measurements, as the peak systolic flow velocity (PSV) of the middle cerebral artery (MCA) is considered the most accurate noninvasive predictor of fetal anemia.[30,43-48]

Until the 1960s, no prenatal treatment options for severe HDFN were available. The only possible intervention in case of suspected fetal anemia was to deliver the baby

prematurely, to enable neonatal treatment. HDFN was until then a major cause of perinatal mortality. In 1963, Liley described the intrauterine intraperitoneal blood transfusion (IPT), which considerably reduced mortality rates.[34] However, the outcome of fetuses with alloimmune anemia <26 weeks' gestation and of those with hydrops remained poor (32 and 42%, respectively).[49] In 1981, direct intravascular intrauterine transfusions by fetoscopy (IUT) were first described,[50] with initial survival rates around 85%.[51] In the years that followed, ultrasound guidance gradually replaced fetoscopy[52] and since then, intravascular IUT has been the cornerstone of treatment for fetal anemia due to red-cell alloimmunization.[50,51,53] This review focusses on the current transfusion techniques, complications and promising non-invasive treatment options for HDFN.

PRENATAL TREATMENT OF HDFN

Intravascular intrauterine blood transfusion (IUT)

Transfusion preparation and details

Indication

IUT should be urgently performed if MCA-PSV Doppler exceeds 1.5 multiples of the median (MoM) and/or if signs of hydrops are present, as both correlate strongly with moderate to severe fetal anemia.[30,39,46] Timing of subsequent IUTs can be done by calculating the expected decline in hematocrit and by MCA-PSV Doppler measurements.[54] Nowadays, since the prediction using the MCA Doppler is highly reliable, fetal blood sampling is preferably directly followed by IUT and not performed as a diagnostic tool without blood available for immediate transfusion.[55,56] However, the degree of fetal anemia, assessed by the hemoglobin concentration in the pre-transfusion fetal blood sample, finally sets the conclusive IUT indication. The cut offs used for this decision differ amongst the various fetal therapy centers. However, authors agree that IUT should only be performed in case of moderate to severe anemia, usually defined as hemoglobin concentrations of four to five standard deviations below mean/median for gestational age[23,57-59] or a hemoglobin deficit of 5 g/dL or more.[60,61]

Setting

In the Leiden University Medical Center (LUMC), the Dutch national referral center for fetal therapy, the operating team performing IUTs is composed of a staff-perinatologist, an experienced ultrasonographer and one or two operating nurses.[55] This corresponds

to the approach of other centers, although in some centers an additional perinatologist or pediatrician is present.[62-64]

Authors agree that IUTs need to be performed under aseptic conditions, guided by continuous ultrasound/Doppler, using a 20-22 gauge needle [55,62,63,65,66]. No data are available on the influence of needle size on procedure complications.

Premedication

Maternal premedication varies from local anesthetics only to routine indomethacin and/or pethidine/promethazine to combined spinal epidural analgesia,[55,58,63] the latter being used to facilitate an emergency caesarian section if needed. There is no expert uniformity or scientific evidence supporting routine use of prophylactic antibiotics or corticosteroids[60,63,67,68] at IUT and these prophylactic measures are not routinely used at our center.[55]

Fetal premedication consists of an intramuscularly (or intravenously) administered paralytic agent and/or fetal pain medication. Because of the reported lower risk of complications following IUT when applying fetal paralysis in all cases, routine use is advocated.[55,57] For fetal paralysis, atracurium (0.4 mg/kg), vecuronium (0.1 mg/kg) or pancuronium (0.1 mg/kg) are the most commonly used products.[69-72] Atracurium or vecuronium are often used as first-line premedication option due to the fact that these short-lasting agents give sufficient paralysis for IUT completion. Furthermore, pancuronium is associated with several cardiovascular side-effects.[73]

As the neurologic basis for nociception is present from 24-28 weeks' gestation and hormonal and circulatory stress responses have been reported from as early as 18-20 weeks', fetal analgesia should be considered when performing invasive fetal procedures.[74] Authors advocate 10 µg/kg fentanyl to reduce the fetal stress response and possible fetal pain sensation.[74] However, other authors have found that these fetal hemodynamic and stress hormone changes are more likely to be caused by volume expansion than by fetal stress, as the response was independent of insertion site.[75,76]

Transfusion volume

The transfusion volume is calculated by the method described by Rodeck in 1984,[51] making use of estimated fetoplacental volume (V), fetal hematocrit in pre-transfusion

sample (Ht_1), donor blood hematocrit (Ht_2) and the aimed fetal hematocrit post-transfusion (Ht_3):

$$Transfusion\ volume = V(Ht_3 - Ht_1)/Ht_2$$

Examples of used calculations for computing the fetoplacental volume (V) are:

- *0.1 mL volume/g of estimated fetal weight,*[77] or
- *0.15 mL volume/g of estimated fetal weight,*[78] or
- *1.046 + (fetal weight in grams) x 0.14.*[79]

In order to simplify these formulas, Giannina et al.[77] introduced a simplified equation and compared this to previously described methods:[79,80]

Transfusion volume = 0.02 x target increase in fetal Ht per 10% x g of estimated fetal weight,

assuming that donor blood hematocrit is approximately 75%. This equation was shown to be equally accurate as the formula introduced by Rodeck and is therefore very useful as a simplified calculation method for transfusion volume.[51,77] Target hematocrit should be around 45%.[55,68,72,78,80]. Furthermore, fetal hemoglobin (Hb) testing prior to IUT is nowadays often used to precisely calculate the volume to transfuse.

Blood source

Intrauterine transfusions are usually carried out with O-negative, washed, irradiated, leukocyte depleted blood, negative for the antigens against which the mother is immunized.[55,81] In the Netherlands, donor blood for IUTs is additionally matched with the maternal Duffy, Kidd and S blood group, to reduce the high risk on the formation of new antibodies.[82] Donations are usually from an allogenic donor, as multiple maternal blood donations have been associated with adverse pregnancy outcome,[83,84] although a direct cause–effect relation seems unlikely.[85] Altogether, proposed advantages[69,81,85,86] usually do not outweigh these possible adverse effects of autologous donations.

Simple vs. exchange

Intrauterine exchange transfusion (IUET) has been proposed as an alternative to simple IUT as exchange transfusions may result in a more stable hematocrit[87], potentially decrease the risks of (temporary) volume overload and increase the interval between

procedures. However, the risk of procedure-related complications associated with IUETs may be higher, due to longer duration and needle movements.[88] Furthermore, the excess volume after simple IUT is thought to exit the intravascular compartment, decreasing the risk of volume overload and fetuses seem to tolerate single IUT quite well.[89,90] In a recent (relatively small) cohort study in which IUT and IUET were compared,[62] no differences in benefits or complications were found. However, data on the duration of the procedures were not available. Nowadays, most fetal therapy centers opt for simple transfusions rather than exchange transfusions.

Puncture site

Possible puncture sites or procedure access sites are: intrahepatic, placental cord insertion, transamniotic 'free loop' needling, intraperitoneal and (exceptionally) sites as the fetal heart or chorionic plate vein.[57] All transfusions should be aimed intravenously, as arterial punctures are associated with high complication rates.[57,91] The fetal liver and placental cord insertion are shown to be the safest puncture sites, whereas free loop needling is a higher risk procedure and should in our view best be avoided.[57,58,66-68]

Authors have postulated a beneficial effect of combined intravascular and intraperitoneal transfusion on the inter-procedure interval.[89] In our center, the liver gained more and more popularity as a puncture site in the last decades and currently 57.1% of transfusions are performed intrahepatic, frequently combined with intraperitoneal transfusion. Analysis of the effect of this combined technique on transfusion interval is planned. Our recent cohort study showed that transplacental cord punctures were nowadays performed in 41.3% of procedures and a free loop of cord was the chosen puncture site in only 1.1% of IUTs. In 0.5% of procedures, blood was transfused intraperitoneally only.[57]

Outcome

Many fetal therapy centers have reported on their IUT results in recent years. Table 1 contains a summary of survival after intravascular IUT from a selection of studies published in the last 10 years. Reported live birth rates after IUT vary from 81.9-100%.[57,58,64-68,92-94]

(Procedure-related) complications

Possible complications during or following IUT are: bleeding from the puncture site, cord occlusion, brady- or tachycardia and PPROM or preterm (emergency) delivery.[91,92] Furthermore, an intrauterine infection might be diagnosed following any invasive

procedure. These IUT complications may lead to maternal morbidity, an emergency cesarean section (CS) or even fetal death.

Table 1. Overall survival after intrauterine transfusion

Author, year	N	Hydrops (%)	GA at first IUT[a]	Technique	Preferred puncture site	Overall survival (%)
Somserset, 2006	221	26.9	25 (16-32)	IUST	Liver	91
Weisz, 2009	154	11.1	*26 (-)*	IUET	-	88,9
Tiblad, 2011	284	11.8	-	IUST	Liver	91.8
Johnstone-Ayliffe, 2012	114	13	26 (17-35)	IUST	Liver	93.5
Birchenall, 2013	256	-	30 (16-35.4)	-	Liver or PCI	95.3
Walsh, 2013	242	16	29.1 (19.2-34.4)	-	PCI	95.1
Pasman, 2015	135	14	-	IUST	PCI	100
Sainio, 2015	339	11.5	29 (18-36)	-	Free loop	96.2
Deka, 2016	303	21.6	*26.9 (19.7-33.8)*	-	PCI	96.1
Zwiers, 2016[b]	937	12.9	27 (16-36)	IUST	Liver	97
Overall						***95.2***

N: number of transfusions; GA: gestational age; IUT: intrauterine transfusion; Overall survival: live birth rate; IUST: intrauterine single transfusion; IUET: intrauterine exchange transfusion; PCI: placental cord insertion.
[a]weeks, median (range) or *mean (range).*
[b]result of cohort since 2001 shown.

We recently reported the largest cohort study on procedure-related complications after 1678 IUTs (741 unto 2000 and 937 from 2001 onwards).[57] We found a 1.2% procedure-related complication rate per procedure in the new cohort (3.3% per fetus), compared to 3.4% per procedure (9.8% per fetus) before 2001 (P=0.003 and 0.001, respectively). In experienced hands, 1.8% of fetuses died as a direct result of the procedure, indicating a 0.6% procedure-related fetal demise rate per procedure. Refraining from fetal paralysis, arterial and free loop needling were found to be important risk factors for adverse outcome.[57] Furthermore, operators should perform at least 10 IUTs per year to retain their competence.[95]

A summary of (procedure-related) complications in recent studies is shown in Table 2.[57,58,65-68,92,93]

IUTs performed early in the second trimester, before 20-22 weeks of gestation, carry substantially higher risks on (procedure-related) complications and fetal loss compared to later IUTs, due to technical challenges of early procedures and severity of disease

[57,66,91,96]. Reported survival rates for IUT series started before 20-22 weeks lie around 76-88%.[96-99] Lindenburg et al. found a fourfold risk of perinatal death after IUTs <20 weeks' gestation, compared to IUTs later in gestation.[98]

Table 2. Procedure-related complications and fetal loss

Author, year	N	PR complications (%)	Fetal loss (%)	PR fetal loss[a] (%)
Somserset, 2006	67/221	-	2.1	-
Weisz, 2009	54/154	-	11.1	-
Tiblad, 2011	85/284	16.5/4.9	5.9	4.7/1.4
Johnstone-Ayliff, 2012	46/114	13/5.2	6.5	2.1/0.9
Pasman, 2015	56/135	3.6/1.5	0	0
Sainio, 2015	104/339	23.1/7.1	3.8	3.8/1.2
Deka, 2016	102/303	8.8/3	3.9	4.9/1.65
Zwiers, 2016[b]	334/937	3.3/1.2	3	1.8/0.6
Overall	***848/2487***	***7.8/2.7***	***3.9***	***1.9/0.8***

N: number of fetuses/transfusions; PR: procedure related.
PR complications: infection, PPROM or preterm delivery within 7 days, emergency cesarean section, fetal loss. Numbers shown per fetus/per procedure.
[a]per fetus/per procedure.
[b]result of cohort since 2001 shown.

To improve the perinatal outcome in this specific group with fetal anemia early in the second trimester, several alternative strategies have been proposed. The intrahepatic transfusion route is probably the preferred route, as the surrounding tissue makes it easier to keep the needle in place, despite the small vessel size in early pregnancy (<3-5 mm before 20 weeks' gestation).[66,78] Some authors promote intraperitoneal transfusions instead[100] or noninvasive treatment options to postpone early intravascular transfusions.[98]

Non-invasive options

Several non-invasive treatment options have been proposed to postpone IUT in early severe HDFN, not as a sole treatment if fetal anemia is already present.[78]

Therapeutic plasma exchange (TPE)

In TPE, the patient's plasma is removed and replaced with albumin-rich fluid by passing the patient's blood through a cell separator.[101] The maternal antibodies directed against fetal red cell antigens are then removed. TPE may cause a decrease in antibody titers of as much as 75%, resulting in a reduction of the risk of fetal hemolysis.[101,102] However,

the beneficial value of TPE alone to postpone IUT in early severe HDFN was found to be disappointing.[78,98,101-103] The deficiency of therapeutic plasma exchange as a single treatment is possibly due to a rebound effect, causing a rapid rise in antibody levels to amounts equal as or higher than before TPE was performed, even if the pheresis is continued.[98,102]

Although the use of TPE is considered safe in pregnancy,[101] side and adverse effects do occur. For example, an increase in antibody-dependent cell-mediated cytotoxicity after TPE has been described, apart from the above-mentioned rebound phenomenon of antibody concentrations.[104] Second, placental blood flow might be altered during TPE, as fluctuations in pressure or electrolyte levels may cause variations in maternal blood pressure. Furthermore, with maternal serum extraction, coagulation factors and immunoglobulin levels in maternal blood fall, causing increased risks on postpartum hemorrhage and maternal and neonatal infections.[103,105]

As all available knowledge regarding TPE for severe HDFN is derived from observational case series, only category III recommendations can be made and decision-making should be individualized.[101] The decision to apply this technique for postponing an early IUT could be made in cases with a history of severe HDFN and should be individualized. Authors agree that, if at all, it is best used combined with intravenous immunoglobulins (IVIG), as described below.[105,106]

Intravenous immunoglobulins (IVIG)

The effect of IVIG in HDFN may result from various mechanisms including (1) inhibition of Fc-mediated antibody passage across the placenta, (2) negative feedback on maternal antibody production and/or (3) reticuloendothelial Fc-receptor saturation/blockage, amongst others resulting in decreased uptake of opsonized fetal cells by macrophages.[102,107-111] Although IVIG may prevent or reduce fetal hemolysis, it does not treat fetal anemia.[112]

In most fetal therapy centers applying IVIG, it is started at 400 mg/kg maternal weight/day for 5 consecutive days, repeated every 2-3 weeks.[108,110,112,113] An alternative regime could be 500 mg-1 g/kg maternal body weight weekly.[106,114] At our center, the first administration of IVIG is preferably planned in the 12th week of pregnancy in an outpatient setting usually at a dose of 500 mg/kg, followed by weekly infusions of the same dose. These follow-up infusions are offered as a home service by Sanquin Diagnostics, the national

blood and plasma product supply organization in the Netherlands, reducing the burden of frequent hospital visits.

Few fetal therapy centers administrate IVIG directly to the fetus after gaining intraperitoneal or intravascular access.[115-117] The increased volume given to the fetus could lead to cardiac compromise.

IVIG as a sole treatment to postpone early IUTs has shown promising results in several case series.[111,113] The only prospective study on maternal IVIG administration was performed by Margulies et al. and reported on 24 severely Rh-sensitized patients. In total, three fetal demises occurred (12.5%), in hydropic fetuses. IVIG treatment caused a significant decline in anti-D titers and hemolysis rate and even averted invasive IUT in this severely affected group if started before 28 weeks' gestation. Nevertheless, they concluded that for hydropic fetuses and for fetuses with advanced fetal anemia, IUT is inevitable.[118] In a retrospective study of the same group, patients receiving IVIG before 20 weeks had significantly less hydropic fetuses and a lower fetal mortality rate compared to patients treated with IUT alone.[112]

However, in another small series of four cases of severe RhD immunization, IVIG did not seem to have any effect on transfusion frequency, maternal antibody titers or hydrops.[119] Recently, a prospective case-control study in 34 women compared IUT with fetal IVIG infusion to IUT alone and described a slower hematocrit decline after IUT in the IVIG group.[117] Similar results were found before, but no impact on perinatal outcome was reported.[115,116]

An occasionally used strategy is the combination of IVIG and TPE. This treatment strategy is thought to oppose the previously mentioned rebound effect of TPE alone and might intensify the effect of IVIG and TPE on perinatal outcome. IVIG transfer to the fetus in HDFN is thought to start from 10–12 weeks' gestation, supporting treatment schedules starting from this gestational age.[102] A possible schedule could be: 3 serial TPE treatments in the 12th week of pregnancy, followed by weekly IVIG administration,[106] although evidence for this schedule is scarce.[120] Authors agree that this combined technique should be reserved for the most severe cases. The largest series reports on 9 severe HDFN cases, in which a combined regimen of three TPE procedures and weekly IVIG was used. Maternal antibody titers were reduced, IUT seemed to be postponed and all babies

were alive and well at birth.[106] Several case reports have been published with favorable outcome following this combined approach.[114,121-125]

Reported side effects of IVIG are rare but may include: headache, fever, myalgia and low back pain, rush or chills, urticaria, nausea and vomiting, tachycardia, chest tightness, hypotension and shortness of breath.[72,78,126,127] These events usually occur 30-60 min after admission and especially the headache could be prevented by 1000 mg acetaminophen before infusion.[72] Although very rare, renal failure, aseptic meningitis, anaphylaxis (mainly in case of IgA deficiency), hemolytic anemia, thromboembolism and pulmonary edema are described.[78,126,127]

Last, IVIG is an expensive treatment, with reported prices around $6000/week.[128] The costs and benefits of IVIG should be weighed against those of early IUT. Importantly, early IUT may result in more complications, which also carries along additional costs.[98] Furthermore, IVIG treatment might even prevent perinatal deaths to occur, a situation associated with a high psychological burden and of which the costs are difficult to quantify.

Recommendations are based on observational studies only and are therefore weak. However, there seems to be a beneficial effect of IVIG (with or without TPE) to postpone early transfusions and therefore it should be considered in patients with a history of severe HDFN.

EXPERT COMMENTARY

In the past decades, improved prevention and screening strategies have greatly reduced the incidence of severe HDFN and it is nowadays considered a rare disease. Consequently, yearly IUT numbers per fetal therapy center decrease and centralization of this highly specialized care becomes more and more important. Fetal therapy centers from all over the world collaborate and invest in high-quality research to deliver the best possible patient care in this field. Not only did the incidence of HDFN decline, a dramatic improvement in prognosis of HDFN was achieved. With the introduction of intravascular IUTs and the availability of valuable noninvasive diagnostics for fetal anemia, survival increased remarkably. Several adjustments in transfusion technique, such as transfusing through the intrahepatic vein or placental cord insertion instead of using a free loop of cord and applying routine fetal paralysis, have further improved outcome. Procedure-

related complication rates are currently as low as 3.3% per fetus and 1.2% per procedure in experienced hands.[57] Nonetheless, preventable fetal losses do occur, especially in fetuses in need for IUT before 20–22 weeks.[98] A truly evidence -based noninvasive approach to further reduce fetal loss rates is however not yet available. Nevertheless, the use of intravenous immunoglobulins to postpone these hazardous early IUTs, potentially combined with TPE, shows promising results in case series and single center case-control studies.[112,118] Therapeutic plasma exchange alone seems unable to create similar results.

FIVE-YEAR VIEW

Ideally a randomized controlled trial should be performed to assess the efficacy and benefits of IVIG in early severe HDFN. However, the auspicious results of IVIG published so far have possibly made it unethical to randomly assign patients with a history of severe HDFN to a 'non-IVIG' study group. Therefore, an international multicenter cohort study on the effect of IVIG is currently performed, in which IVIG cases are compared to a reference group with similar disease severity but without IVIG treatment. Depending on the results of this study, a randomized trial could still be considered, taking above-mentioned ethical dilemmas into account. If IVIG treatment proves to be disappointing, other options such as stem cell treatment and gene therapy will be investigated.

KEY ISSUES

- Although prophylaxis has strongly reduced the incidence of maternal immunization against fetal red cell antigens, HDFN is still a serious pregnancy complication which may lead to severe fetal anemia, hydrops and perinatal death.
- Intravascular intrauterine transfusions are the cornerstone in prenatal management and have significantly improved perinatal outcome in the past decades.
- Several risk factors for adverse outcome after IUT have been defined, such as refraining from fetal paralysis, arterial puncture and free loop needling. Avoiding these hazardous techniques causes continuously rising survival rates worldwide.
- Performing IUTs before 20-22 weeks' gestation greatly increases the risk on complications.
- The use of IVIG is an emerging non-invasive strategy to postpone these early IUTs. More research is needed to support its assets.

CHAPTER 2

ABO INCOMPATIBILITY AND RHIG IMMUNOPROPHYLAXIS PROTECT AGAINST NON-D ALLOIMMUNIZATION BY PREGNANCY

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ABSTRACT

Background

Hemolytic disease of the fetus and newborn (HDFN) is caused by maternal antibodies against fetal red blood cell antigens, most often anti-D, -K or -c. ABO incompatibility between mother and child and anti-D immunoprophylaxis (RhIG) are known to reduce the risk of D immunization and subsequent HDFN. However, no immunoprophylaxis has been developed to prevent non-D immunizations.

Study design and methods

We evaluated whether ABO incompatibility has a preventive effect on formation of non-D alloantibodies, by performing a case-control study including pregnant women with newly detected non-D antibodies, identified within a nationwide data set, immunized during their first pregnancy and/or delivery. Subsequently, we assessed a possible protective effect of RhIG in a subgroup with non-Rh antibodies only. The proportions of previous ABO incompatibility and of RhIG administrations of these women were compared to the known rate of 19.4% ABO incompatibility and 9.9% RhIG administrations (D- women carrying a D+ child) in the general population of pregnant women.

Results

A total of 11.9% of the 232 included immunized women had a possible ABO incompatibility in their first pregnancy (vs. expected 19.4%; 95% confidence interval [CI], 7.3-18.8; *P*=0.036). Furthermore, 1.0% of women with non-Rh antibodies were D-, delivered a D+ child and had therefore received RhIG, whereas 9.9% was expected (95% CI 0.18-5.50; *P*=.003).

Conclusion

We found that ABO incompatibility and RhIG reduce the risks not only for D, but also for non-Rh immunizations, suggesting that antibody-mediated immune suppression in this condition is not antigen specific.

INTRODUCTION

Hemolytic disease of the fetus and newborn (HDFN) is a serious pregnancy complication, caused by maternal antibodies against fetal red blood cell (RBC) antigens. These antibodies may provoke fetal hemolysis, resulting in fetal anemia, hydrops, and even death if left untreated.[17,37] HDFN is most frequently caused by antibodies with anti-D specificity, followed by anti-K, anti-c, anti-E, other Rh antibodies, or exceptionally, anti-Fy (Duffy) or anti-Jk (Kidd).[16,17,37,129]

Already in 1943, Levine et al. did the pivotal observation that ABO incompatibility occurred less in patients with D immunization during pregnancy compared to couples without D immunization, indicating a preventive effect of ABO incompatibility on the formation of D antibodies.[4] This observation was confirmed by others, of which Nevannlina and Vainio most widely studied the effect of mother-child ABO incompatibility on D immunization.[130] These observations eventually led to the hypothesis that the development of anti-D immunoglobulin prophylaxis (RhIG) could prevent D immunization.[131]

Indeed, postnatal prophylaxis with RhIG, introduced in the 1960s, and additional antenatal prophylaxis in the 1990s, have drastically reduced the risk for D immunizations by pregnancy or birth.[5] As a consequence, RhIG is a very effective measure to prevent D immunizations. Several possible pathways have been hypothesized and thoroughly studied in the past decades, although the exact mechanisms of action of RhIG still remain unclear.[132-136]

Clinically relevant RBC alloantibodies directed against other RBC antigens (non-D RBC alloantibodies), in the absence of D antibodies, were found at screening in the first trimester of pregnancy in 0.33% of all pregnancies in the Netherlands between 2002 and 2004.[16] As mentioned, non-D antibodies might also cause HDFN, although to a lesser extent than anti-D.[16] To prevent non-D alloimmunization, women of reproductive age (<45 years) in need for RBC transfusions receive K-matched (from 2004 onward) and c- and E-matched (2011 onward) blood units in the Netherlands.[11] So far, no immunoprophylaxis has been developed to prevent non-D alloimmunization, although the clinical relevance of implementing anti-KEL[137] and anti-HPA-1a[138] immunoglobulin has been investigated in murine models.

It is not known whether the immunization against non-D RBC antigens might be preventable by administration of an immunoprophylaxis, like in D immunization. Therefore, we first assessed whether ABO mismatch in pregnancy also reduces the risk of immunization toward non-D RBC antigens. Subsequently, we investigated if the administration of RhIG to D- mothers protects for alloimmunization against non-Rh antigens.

MATERIALS AND METHODS

Study design

We performed a case-control study, comparing pregnant women with one previous delivery and non-D alloantibodies detected at first trimester screening that most likely were immunized by RBC antigens of their first child (cases), to the Dutch population of pregnant women (control population).

Study population

Previously, all women with non-D alloantibodies, but no D antibodies, found at first trimester screening in the Netherlands between September 1, 2002 and June 1, 2003, and between October 1, 2003 and July 1, 2004, were included in the prospective OPZI (Opsporing en Preventie Zwangerschapsimmunisatie/Detection and Prevention of Pregnancy Immunisation) study.[16] These cases were identified at Sanquin Diagnostics, the Dutch national reference laboratory, or BIBO (Bijzonder Instituut voor Bloedgroepen Onderzoek/Special Institute for Blood Group Investigation), where the specificity of all RBC alloantibodies found at first trimester screening in regional laboratories is determined. All women with non-D alloantibodies from the OPZI study were initially included in this study as cases. To facilitate subgroup analyses of different antibody specificities, additionally, women with newly detected anti-E, anti-K, anti-Fy or anti-Jk, and without D antibodies, identified at the laboratory of Sanquin Diagnostics between July 2012 and September 2015 and between January and September 2016 were included. Subsequently, to compose a group of women that was most likely immunized by one previous pregnancy or delivery, multiparous or nulliparous women were excluded, as well as women with blood transfusions after a negative antibody screen in their previous pregnancy, and women with partners negative for the antigen against which the maternal antibodies were directed. The likelihood that part of the population was not immunized by their previous delivery, but by a miscarriage or abortion in between was considered

nihil, as we previously found that these factors are not associated with an increased risk of alloimmunization.[129]

Cases were compared to the general pregnant Dutch population. If ABO incompatibility or RhIG administration would have a protective effect on any type of immunization, this would be indicated by a low incidence of ABO incompatibility or RhIG administrations in our case group compared to the general population. Therefore, we compared the probability of ABO incompatibility of the cases with the calculated proportion in the general population, based on the distribution of AB antigens in a Caucasian population.[1] Second, the proportion of cases that previously received RhIG was compared to the proportion of D negative women with D+ fetuses in the general Caucasian population, assuming a 100% coverage of the national prevention program for pregnancy immunization. [139] We hypothesize that the preventive effect of RhIG on non-Rh immunizations is limited to D+ fetal RBCs and would be less profound or absent in pregnancies of D- women carrying a D- child. Therefore, we considered D- women with D- fetuses, who received untargeted antenatal prophylaxis before the introduction of fetal D typing in maternal blood in 2011, as not having received RhIG.

Data collection

From the OPZI database we collected laboratory data (antibody type; paternal antigen phenotype; blood group of mother, father and second child), data on the obstetric history and data on blood transfusions after a previous negative antibody screen in the first pregnancy.[16] Laboratory data (antibody type, paternal antigen phenotype, ABO blood group of mother and father) concerning the additional cases were collected from the Sanquin database. After written informed consent from the women, additional clinical data were obtained from the patients' midwife, gynecologist or general practitioner.

Ethical considerations

As patients were not subjected to additional interventions due to this study, formal ethical approval was not mandatory in the Netherlands and was therefore not obtained. All participants gave informed consent.

Statistics

To compare proportions, 95% confidence intervals (CI) and concordant p values were obtained using the Wilson score, where a p value less than 0.05 was considered significant.

The probability of ABO incompatibility in the first pregnancy in cases was estimated twice: 1) based on the ABO blood group of the cases and their partners and, more accurately, 2) based on the ABO blood group of the cases and their partners, as well as the ABO blood group of the children born from the pregnancy with alloantibodies, and compared to population probabilities on incompatibility using the same variables. All calculations are shown in Tables S1 through S6 (available as supporting information in the online version of this paper).

We assessed the comparability of cases and general population in respect to RhIG administrations by comparing the number of D negative mothers in both groups. Subsequently, to compare the number of RhIG administrations in the cases and the general population, we planned to analyze two separate subgroups, the Rh (non-D, anti-C/Cw and anti-E) and non-Rh antibodies, as the risk to develop anti-Rh antibody specificities is dependent on the D phenotype of the mother. Since there is a strong linkage between RHCE (e.g., RHce) and D, almost all D- women are c- and e+. As a consequence, women who develop anti-c, anti-e or anti-f are virtually always D+ and never receive RhIG. Therefore, these antibody specificities were excluded in the planned Rh subgroup analyses.

RESULTS

In total, 1326 women with new non-D antibodies were included (Figure 1). After excluding women in their first ongoing pregnancy or with more than one previous birth, women with an antigen-negative partner, or with a history of RBC transfusion after a negative antibody screen in their previous pregnancy, 232 women remained and were included in the analysis. E antibodies were most frequently found, followed by anti-K, anti-c, anti-C, and anti-Jk. The median maternal age at first alloantibody detection was 32 (range 19-40) years.

ABO incompatibility

Data on ABO blood group of all women with non-D antibodies were complete and for 201 of 232 partners (Table S5). Based on these data, we determined that the first pregnancy was surely compatible in at least 74.6% of the cases, whereas in the total population this is only in 66.5% (p=0.015). The probability of an ABO incompatible first pregnancy was 14.1% in cases (28/201) versus 19.4% in the general population (95% CI, 9.9-19.6; p=0.058; Tables S1, S2, and S5). The accuracy to estimate whether the first pregnancy was ABO incompatible was increased by taking the blood group of the second child into account.

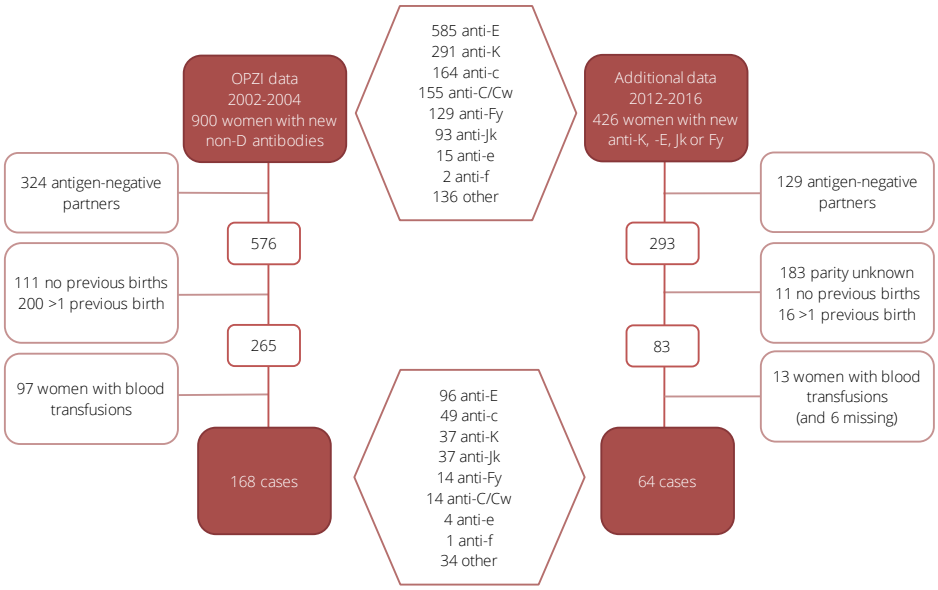

Figure 1. Selection of cases. The total number of antibodies may differ from 232 as women may have developed more than one antibody.

These data were available for 124 of the 232 cases (Table S6) and this more accurate estimation showed that the first pregnancy had surely been compatible in 79.0% of the cases, compared to 66.5% in the general population (p=0.003; Tables S3, S4, and S6). The probability of an ABO-incompatible first pregnancy in this specific group is shown in Table 1. In total, the first pregnancy might have been ABO incompatible in 11.9% of cases, significantly less than the 19.4% in the Dutch population (95% CI, 7.3-18.8; p=0.036; Tables

Table 1. ABO incompatibility in previous pregnancy per maternal ABO blood group[a]

	Probability of incompatible first pregnancy[b]			
Maternal blood group	**Cases, n/n[c]**	**Cases, % (95% CI)**	**Population, %**	***P***
O	11.7/50	23.34 (13.78-36.70)	30.72	.26
A	2/54	3.70 (1.02-12.53)	6.09	.46
B	1.1/12	9.43 (1.83-36.68)	24.60	.22
AB	0/8	0	0	1
All blood groups	14.8/124	11.94 (7.34-18.81)	19.36	.04

[a]Wilson score used for 95% CI and concordant *P*-values.
[b]Based on combination of ABO blood group of mother, father and second child. See supplemental tables 3,4 and 6 for calculations.
[c]Possible number of incompatible cases/total number of cases per blood group with complete ABO data.

S3, S4, and S6). The group was too small to calculate a potential difference in protective effect between anti-A and anti-B.

RhIG administrations

In total, four of 232 cases received RhIG in their previous pregnancy and/or after their first delivery. In the general (Caucasian) population, this is 9.9%.[1] One D– woman received untargeted antenatal prophylaxis while carrying a D– child and was therefore considered as not having received RhIG prophylaxis. We planned to analyze Rh and non-Rh specificities separately. We found that, in the subgroup of women with anti-E or anti-C, the proportion of women being D– was far lower than expected (2/83 [2.4%] vs. 22.3% and 1/9 [11.1%] vs. 78.4%, respectively).[1] Therefore, we did not continue the planned separate analysis for Rh antibodies.

In cases with non-Rh antibodies, the percentage of D– women without necessity of RhIG prophylaxis was approximately as expected (5/99, 5.1% vs. 7.0% expected [16.9% D– women of whom 41.2% were carrying a D– child and therefore without an indication for prophylaxis[1]]). Table 2 therefore shows the results of this subgroup of cases with non-Rh antibodies and separate analyses for different non-Rh antibody specificities. Only one of 99 (1%) women with non-Rh antibodies received RhIG in her previous pregnancy and/or after her first delivery, significantly less often than the expected number of 10 women based on calculations for the general population (16.9% D– women of whom 58.8% were carrying a D+ child).

Table 2. Subgroup analyses of RhIG administrations in 99 cases with non-Rh antibodies compared to the population[a]

	Number/proportion of RhIG administrations		**95% CI**	***P***
Antibody specificity	Cases, n/total	Cases, %[b]		
All non-Rh	1/99[c]	1.0	0.18-5.50	.003
Anti-K	1/37	2.7	0.48-13.82	.14
Anti-Jk	0/37	0	0-9.41	.04
Anti-Fy	0/14	0	0-21.53	.21
Other	0/34	0	0-10.15	.05

[a]Wilson score used for 95% CI and concordant *P*-values.
[b]Compared to 9.9%, the calculated probability of D- women carrying a D+ fetus.[1]
[c]The sum of different antibodies may differ from 99 as women may have developed more than one antibody.

DISCUSSION

In this study, we assessed whether ABO mismatch in pregnancy may reduce the risk of immunization towards non-D RBC antigens. In 232 women with non-D alloantibodies due to their first ongoing pregnancy or delivery, we found a significantly smaller proportion of possible ABO incompatible first pregnancies in cases than in general population, implicating a preventive effect of ABO incompatibility on non-D antibody formation.

Subsequently, we evaluated whether RhIG also prevents non-Rh immunizations and found that only 1% had previously received RhIG prophylaxis, whereas approximately 10% was expected. This underrepresentation of D– pregnant women with previous RhIG prophylaxis indicates a possible protective effect of RhIG on formation of non-Rh alloantibodies. These findings also suggest that, in general, for all pregnant women, non-Rh immunizations might be preventable via a mechanism similar to prevention of D immunizations. The prophylactic effect of both ABO mismatch and RhIG is not absolute, as is also not the case for RhIG and ABO incompatibility in D immunization.[5,130,140]

Our finding that ABO incompatibility also protects against non-D immunizations is in line with early studies of Levine, reporting on a protective effect on c and K immunizations.[140,141] Later, Stern[142] also postulated an effect of ABO incompatibility on other types of immunization, although the possible influence of a previous blood transfusion was not completely clear in this study.

The found preventive effect on non-D immunizations may be clinically relevant, as severe HDFN may also be caused by anti-K (prevalence, 1.02/1000), anti-c (0.71/ 1000 pregnancies), and (rarely) by other Rh and non-Rh antibodies.[16,143] If anti-K or anti-c is present, this can lead to severe HDFN in 26%[16] to 53% of pregnancies with K+[25] and in 10% of pregnancies with c+ children.[16] It was not possible to determine whether anti-D immunoprophylaxis might prevent c immunizations, as women at risk for development of c antibodies are virtually always D+ and therefore never receive RhIG.

The strength of this study is that we assessed only women with one previous birth and thereby we selected a group of women exposed to approximately the same amount of fetal RBCs. Furthermore, in this matter we effaced the possible immunosuppressive effect of RhIG administrations in pregnancies (ending in miscarriage or termination) before the previous pregnancy. We further specified our cohort by electing women

with a high probability of being immunized by their previous pregnancy or delivery, as we excluded women with antigen-negative partners and those with blood transfusions after their first pregnancy or delivery.

We believe that the retrospective study design does not reduce this study's value, as RhIG coverage is more than 98% in the Netherlands[139] and therefore the comparison in RhIG administration between cases and Dutch population could well be made. Moreover, the cases were prospectively collected in the OPZI study. Another strong point is that, although the ABO blood group of the first child and therefore the true proportion of incompatible first pregnancies was unavailable, a distinct approximation could be made as in approximately 50% of cases, the ABO blood group of mother, father, and second child was known.

By not including women who developed D antibodies, part of the D– population (of which a considerable proportion might be 'high-responders',[134] very prone to develop additional antibodies) was excluded. This exclusion did not affect the found preventive effect of RhIG on the development of non-Rh antibodies, as we previously found that in primiparous women with newly detected D antibodies and without a previous blood transfusion, non-Rh antibodies in addition to D antibodies are rarely developed.[144]

However, a limitation of our study is that by not including women with D antibodies, we were not able to evaluate the effectiveness of RhIG in preventing the development of Rh antibodies. This is reflected by the observation that we found barely any D– women with anti-E and anti-C. Because of the linkage disequilibrium between RHD and RHCE alleles, anti-C and anti-E are mainly formed in pregnancies with D+ children. As the D antigen is a more immunogenic antigen than E or C, women in whom RhIG fails will make anti-E/C most likely in addition to anti-D. Possibly, in this manner, RhIG not only protects strongly against D immunizations, but also against anti-E or anti-C.

Furthermore, we are limited to a relatively small sample size in the subgroup analysis with non-Rh antibodies only, to assess a protective effect of RhIG. However, even in this small sample the difference between the expected (10) and observed (1) number of women who previously received RhIG is statistically significant.

Although several studies have previously addressed the possible mechanisms of action of RhIG, the exact mechanism(s) remain unclear.[134-136,145,146]

Whereas the antigen masking or steric hindrance hypothesis appears to be the prevailing mechanism in the antibody-mediated immune suppression model with sheep RBCs in mice,[147] this mechanism insufficiently explains RhIG function in humans, based on the low level of opsonization sufficient to exert suppression.[132-134] Furthermore, antigen masking is an antigen-specific mechanism and if this was the main explanation for RhIG function, it would not prevent development of other RBC alloantibody specificities as found in our study. In agreement with our findings, in a mouse model it was shown that antibodies directed against a nonimmunogenic Fy antigen could mediate immune suppression toward the immunogenic antigen (HEL), although in these studies the Fy and HEL were expressed on the same protein (HOD).[148]

Furthermore, the recently postulated antigen-specific "antigen-modulation hypothesis", in which the preventive effect of anti-KEL sera on KEL immunization was attributed to the complete removal or substantial modulation of the KEL antigen, is not in line with our findings.[137,146] A possible explanation to this discrepancy is that antibody responses might function through different mechanisms for different antigens.

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SUPPLEMENTAL MATERIAL

Table S1. Calculation of ABO incompatibility in general population based on the ABO blood group of mother and father

Maternal genotype		Paternal genotype		Maternal and paternal genotypes	Probability of genotype child based on ABO genotype mother and father					
Genotype	Genotype proability[149]	Genotype	Genotype probability[149]	Probability of combination	OO	OA	OB	AA	AB	BB
OO	0.48	OO	0.48	0.2304	1	-	-	-	-	-
OO	0.48	OA	0.34	0.1638	0.5	0.5	-	-	-	-
OO	0.48	OB	0.08	0.0405	0.5	-	0.5	-	-	-
OO	0.48	AA	0.06	0.0291	-	1	-	-	-	-
OO	0.48	AB	0.03	0.0144	-	0.5	0.5	-	-	-
OO	0.48	BB	<0.01	0.0018	-	-	1	-	-	-
OA	0.34	OO	0.48	0.1638	0.5	0.5	-	-	-	-
OA	0.34	OA	0.34	0.1164	0.25	0.5	-	0.25	-	-
OA	0.34	OB	0.08	0.0288	0.25	0.25	0.25	-	0.25	-
OA	0.34	AA	0.06	0.0207	-	0.5	-	0.5	-	-
OA	0.34	AB	0.03	0.0102	-	0.25	0.25	0.25	0.25	-
OA	0.34	BB	<0.01	0.0013	-	-	0.5	-	0.5	-
OB	0.08	OO	0.48	0.0405	0.5	-	0.5	-	-	-
OB	0.08	OA	0.34	0.0288	0.25	0.25	0.25	-	0.25	
OB	0.08	OB	0.08	0.0071	0.25	-	0.5	-	-	0.25
OB	0.08	AA	0.06	0.0051	-	0.5	-	-	0.5	-
OB	0.08	AB	0.03	0.0025	-	0.25	0.25	-	0.25	0.25
OB	0.08	BB	<0.01	0.0003	-	-	0.5	-	-	0.5
AA	0.06	OO	0.48	0.0291	-	1	-	-	-	-
AA	0.06	OA	0.34	0.0207	-	0.5	-	0.5	-	-
AA	0.06	OB	0.08	0.0051	-	0.5	-	-	0.5	-
AA	0.06	AA	0.06	0.0037	-	-	-	1	-	-
AA	0.06	AB	0.03	0.0018	-	-	-	0.5	0.5	-
AA	0.06	BB	<0.01	0.0002	-	-	-	-	1	-
AB	0.03	OO	0.48	0.0144	-	0.5	0.5	-	-	-
AB	0.03	OA	0.34	0.0102	-	0.25	0.25	0.25	0.25	-
AB	0.03	OB	0.08	0.0025	-	0.25	0.25	-	0.25	0.25
AB	0.03	AA	0.06	0.0018	-	-	-	0.5	0.5	-
AB	0.03	AB	0.03	0.0009	-	-	-	0.25	0.5	0.25
AB	0.03	BB	<0.01	0.0001	-	-	-	-	0.5	0.5
BB	<0.01	OO	0.48	0.0018	-	-	1	-	-	-
BB	<0.01	OA	0.34	0.0013	-	-	0.5	-	0.5	-
BB	<0.01	OB	0.08	0.0003	-	-	0.5	-	-	0.5
BB	<0.01	AA	0.06	0.0002	-	-	-	-	1	-
BB	<0.01	AB	0.03	0.0001	-	-	-	-	0.5	0.5
BB	<0.01	BB	<0.01	<0.0001	-	-	-	-	-	1

Table S2. Probability of incompatible pregnancy based on maternal and paternal ABO blood group in general population

Maternal phenotype	Paternal phenotype	Probability of phenotype child based on phenotype mother and father[a]				Probability of incompatible pregnancy
		O	A	B	AB	
O	O	1.00	-	-	-	-
O	A	0.42	0.58	-	-	0.58
O	B	0.48	-	0.52	-	0.52
O	AB	-	0.50	0.50	-	1.00
A	O	0.42	0.58	-	-	-
A	A	0.18	0.82	-	-	-
A	B	0.20	0.28	0.22	0.30	0.52
A	AB	-	0.50	0.21	0.29	0.50
B	O	0.48	-	0.52	-	-
B	A	0.20	0.28	0.22	0.30	0.58
B	B	0.23	-	0.77	-	-
B	AB	-	0.24	0.50	0.26	0.50
AB	O	-	0.50	0.50	-	-
AB	A	-	0.50	0.21	0.29	-
AB	B	-	0.24	0.50	0.26	-
AB	AB	-	0.25	0.25	0.50	-

Grey cells reflect possible incompatible combinations

[a]Calculated from the probabilities per ABO genotype shown in supplemental Table 1.

Table S3. Calculation of ABO incompatibility in the first pregnancy in general population based on the ABO blood group of mother, father and second child

Maternal genotype		Paternal genotype		Genotype of second child		Combination of genotypes	Probability of genotype first child based on genotype mother, father and second child					
Genotype	Genotype probability[149]	Genotype	Genotype probability[149]	Genotype	Genotype probability	Probability of combination	OO	OA	OB	AA	AB	BB
OO	0.48	OO	0.48	OO	1	0.2304	1	-	-	-	-	-
OO	0.48	OA	0.34	OO	0.5	0.0819	0.5	0.5	-	-	-	-
OO	0.48	OB	0.08	OO	0.5	0.0203	0.5	-	0.5	-	-	-
OO	0.48	AA	0.06	OO	-	-	-	1	-	-	-	-
OO	0.48	AB	0.03	OO	-	-	-	0.5	0.5	-	-	-
OO	0.48	BB	<0.01	OO	-	-	-	-	1	-	-	-
OO	0.48	OO	0.48	OA	-	-	1	-	-	-	-	-
OO	0.48	OA	0.34	OA	0.5	0.0819	0.5	0.5	-	-	-	-
OO	0.48	OB	0.08	OA	-	-	0.5	-	0.5	-	-	-
OO	0.48	AA	0.06	OA	1	0.0291	-	1	-	-	-	-
OO	0.48	AB	0.03	OA	0.5	0.0072	-	0.5	0.5	-	-	-
OO	0.48	BB	<0.01	OA	-	-	-	-	1	-	-	-
OO	0.48	OO	0.48	OB	-	-	1	-	-	-	-	-
OO	0.48	OA	0.34	OB	-	-	0.5	0.5	-	-	-	-
OO	0.48	OB	0.08	OB	0.5	0.0203	0.5	-	0.5	-	-	-
OO	0.48	AA	0.06	OB	-	-	-	1	-	-	-	-
OO	0.48	AB	0.03	OB	0.5	0.0072	-	0.5	0.5	-	-	-
OO	0.48	BB	<0.01	OB	1	0.0018	-	-	1	-	-	-
OO	0.48	OO	0.48	AA	-	-	1	-	-	-	-	-
OO	0.48	OA	0.34	AA	-	-	0.5	0.5	-	-	-	-
OO	0.48	OB	0.08	AA	-	-	0.5	-	0.5	-	-	-
OO	0.48	AA	0.06	AA	-	-	-	1	-	-	-	-
OO	0.48	AB	0.03	AA	-	-	-	0.5	0.5	-	-	-
OO	0.48	BB	<0.01	AA	-	-	-	-	1	-	-	-
OO	0.48	OO	0.48	AB	-	-	1	-	-	-	-	-
OO	0.48	OA	0.34	AB	-	-	0.5	0.5	-	-	-	-
OO	0.48	OB	0.08	AB	-	-	0.5	-	0.5	-	-	-
OO	0.48	AA	0.06	AB	-	-	-	1	-	-	-	-
OO	0.48	AB	0.03	AB	-	-	-	0.5	0.5	-	-	-
OO	0.48	BB	<0.01	AB	-	-	-	-	1	-	-	-
OO	0.48	OO	0.48	BB	-	-	1	-	-	-	-	-
OO	0.48	OA	0.34	BB	-	-	0.5	0.5	-	-	-	-
OO	0.48	OB	0.08	BB	-	-	0.5	-	0.5	-	-	-
OO	0.48	AA	0.06	BB	-	-	-	1	-	-	-	-
OO	0.48	AB	0.03	BB	-	-	-	0.5	0.5	-	-	-
OO	0.48	BB	<0.01	BB	-	-	-	-	1	-	-	-
OA	0.34	OO	0.48	OO	0.50	0.0819	0.5	0.5	-	-	-	-
OA	0.34	OA	0.34	OO	0.25	0.0291	0.25	0.5	-	0.25	-	-
OA	0.34	OB	0.08	OO	0.25	0.0072	0.25	0.25	0.25	-	0.25	-
OA	0.34	AA	0.06	OO	-	-	-	0.5	-	0.5	-	-
OA	0.34	AB	0.03	OO	-	-	-	0.25	0.25	0.25	0.25	-

Maternal genotype		Paternal genotype		Genotype of second child		Combination of genotypes	Probability of genotype first child based on genotype mother, father and second child					
Genotype	Genotype probability[149]	Genotype	Genotype probability[149]	Genotype	Genotype probability	Probability of combination	OO	OA	OB	AA	AB	BB
OA	0.34	BB	<0.01	OO	-	-	-	-	0.5	-	0.5	-
OA	0.34	OO	0.48	OA	0.50	0.0819	0.5	0.5	-	-	-	-
OA	0.34	OA	0.34	OA	0.50	0.0582	0.25	0.5	-	0.25	-	-
OA	0.34	OB	0.08	OA	0.25	0.0072	0.25	0.25	0.25	-	0.25	-
OA	0.34	AA	0.06	OA	0.50	0.0103	-	0.5	-	0.5	-	-
OA	0.34	AB	0.03	OA	0.25	0.0026	-	0.25	0.25	0.25	0.25	-
OA	0.34	BB	<0.01	OA	-	-	-	-	0.5	-	0.5	-
OA	0.34	OO	0.48	OB	-	-	0.5	0.5	-	-	-	-
OA	0.34	OA	0.34	OB	-	-	0.25	0.5	-	0.25	-	-
OA	0.34	OB	0.08	OB	0.25	0.0072	0.25	0.25	0.25	-	0.25	-
OA	0.34	AA	0.06	OB	-	-	-	0.5	-	0.5	-	-
OA	0.34	AB	0.03	OB	0.25	0.0026	-	0.25	0.25	0.25	0.25	-
OA	0.34	BB	<0.01	OB	0.50	0.0006	-	-	0.5	-	0.5	-
OA	0.34	OO	0.48	AA	-	-	0.5	0.5	-	-	-	-
OA	0.34	OA	0.34	AA	0.25	0.0291	0.25	0.5	-	0.25	-	-
OA	0.34	OB	0.08	AA	-	-	0.25	0.25	0.25	-	0.25	-
OA	0.34	AA	0.06	AA	0.50	0.0103	-	0.5	-	0.5	-	-
OA	0.34	AB	0.03	AA	0.25	0.0026	-	0.25	0.25	0.25	0.25	-
OA	0.34	BB	<0.01	AA	-	-	-	-	0.5	-	0.5	-
OA	0.34	OO	0.48	AB	-	-	0.5	0.5	-	-	-	-
OA	0.34	OA	0.34	AB	-	-	0.25	0.5	-	0.25	-	-
OA	0.34	OB	0.08	AB	0.25	0.0072	0.25	0.25	0.25	-	0.25	-
OA	0.34	AA	0.06	AB	-	-	-	0.5	-	0.5	-	-
OA	0.34	AB	0.03	AB	0.25	0.0026	-	0.25	0.25	0.25	0.25	-
OA	0.34	BB	<0.01	AB	0.50	0.0006	-	-	0.5	-	0.5	-
OA	0.34	OO	0.48	BB	-	-	0.5	0.5	-	-	-	-
OA	0.34	OA	0.34	BB	-	-	0.25	0.5	-	0.25	-	-
OA	0.34	OB	0.08	BB	-	-	0.25	0.25	0.25	-	0.25	-
OA	0.34	AA	0.06	BB	-	-	-	0.5	-	0.5	-	-
OA	0.34	AB	0.03	BB	-	-	-	0.25	0.25	0.25	0.25	-
OA	0.34	BB	<0.01	BB	-	-	-	-	0.5	-	0.5	-
OB	0.08	OO	0.48	OO	0.50	0.0203	0.5	-	0.5	-	-	-
OB	0.08	OA	0.34	OO	0.25	0.0072	0.25	0.25	0.25	-	0.25	-
OB	0.08	OB	0.08	OO	0.25	0.0018	0.25	-	0.5	-	-	0.25
OB	0.08	AA	0.06	OO	-	-	-	0.5	-	-	0.5	-
OB	0.08	AB	0.03	OO	-	-	-	0.25	0.25	-	0.25	0.25
OB	0.08	BB	<0.01	OO	-	-	-	-	0.5	-	-	0.5
OB	0.08	OO	0.48	OA	-	-	0.5	-	0.5	-	-	-
OB	0.08	OA	0.34	OA	0.25	0.0072	0.25	0.25	0.25	-	0.25	-
OB	0.08	OB	0.08	OA	-	-	0.25	-	0.5	-	-	0.25
OB	0.08	AA	0.06	OA	0.50	0.0026	-	0.5	-	-	0.5	-
OB	0.08	AB	0.03	OA	0.25	0.0006	-	0.25	0.25	-	0.25	0.25
OB	0.08	BB	<0.01	OA	-	-	-	-	0.5	-	-	0.5
OB	0.08	OO	0.48	OB	0.50	0.0203	0.5	-	0.5	-	-	-

Maternal genotype		Paternal genotype		Genotype of second child		Combination of genotypes	Probability of genotype first child based on genotype mother, father and second child					
Genotype	Genotype probability[149]	Genotype	Genotype probability[149]	Genotype	Genotype probability	Probability of combination	OO	OA	OB	AA	AB	BB
OB	0.08	OA	0.34	OB	0.25	0.0072	0.25	0.25	0.25	-	0.25	-
OB	0.08	OB	0.08	OB	0.50	0.0036	0.25	-	0.5	-	-	0.25
OB	0.08	AA	0.06	OB	-	-	-	0.5	-	-	0.5	-
OB	0.08	AB	0.03	OB	0.25	0.0006	-	0.25	0.25	-	0.25	0.25
OB	0.08	BB	<0.01	OB	0.50	0.0002	-	-	0.5	-	-	0.5
OB	0.08	OO	0.48	AA	-	-	0.5	-	0.5	-	-	-
OB	0.08	OA	0.34	AA	-	-	0.25	0.25	0.25	-	0.25	-
OB	0.08	OB	0.08	AA	-	-	0.25	-	0.5	-	-	0.25
OB	0.08	AA	0.06	AA	-	-	-	0.5	-	-	0.5	-
OB	0.08	AB	0.03	AA	-	-	-	0.25	0.25	-	0.25	0.25
OB	0.08	BB	<0.01	AA	-	-	-	-	0.5	-	-	0.5
OB	0.08	OO	0.48	AB	-	-	0.5	-	0.5	-	-	-
OB	0.08	OA	0.34	AB	0.25	0.0072	0.25	0.25	0.25	-	0.25	-
OB	0.08	OB	0.08	AB	-	-	0.25	-	0.5	-	-	0.25
OB	0.08	AA	0.06	AB	0.50	0.0026	-	0.5	-	-	0.5	-
OB	0.08	AB	0.03	AB	0.25	0.0006	-	0.25	0.25	-	0.25	0.25
OB	0.08	BB	<0.01	AB	-	-	-	-	0.5	-	-	0.5
OB	0.08	OO	0.48	BB	-	-	0.5	-	0.5	-	-	-
OB	0.08	OA	0.34	BB	-	-	0.25	0.25	0.25	-	0.25	-
OB	0.08	OB	0.08	BB	0.25	0.0018	0.25	-	0.5	-	-	0.25
OB	0.08	AA	0.06	BB	-	-	-	0.5	-	-	0.5	-
OB	0.08	AB	0.03	BB	0.25	0.0006	-	0.25	0.25	-	0.25	0.25
OB	0.08	BB	<0.01	BB	0.50	0.0002	-	-	0.5	-	-	0.5
AA	0.06	OO	0.48	OO	-	-	-	1	-	-	-	-
AA	0.06	OA	0.34	OO	-	-	-	0.5	-	0.5	-	-
AA	0.06	OB	0.08	OO	-	-	-	0.5	-	-	0.5	-
AA	0.06	AA	0.06	OO	-	-	-	-	-	1	-	-
AA	0.06	AB	0.03	OO	-	-	-	-	-	0.5	0.5	-
AA	0.06	BB	<0.01	OO	-	-	-	-	-	-	1	-
AA	0.06	OO	0.48	OA	1	0.0291	-	1	-	-	-	-
AA	0.06	OA	0.34	OA	0.50	0.0103	-	0.5	-	0.5	-	-
AA	0.06	OB	0.08	OA	0.50	0.0026	-	0.5	-	-	0.5	-
AA	0.06	AA	0.06	OA	-	-	-	-	-	1	-	-
AA	0.06	AB	0.03	OA	-	-	-	-	-	0.5	0.5	-
AA	0.06	BB	<0.01	OA	-	-	-	-	-	-	1	-
AA	0.06	OO	0.48	OB	-	-	-	1	-	-	-	-
AA	0.06	OA	0.34	OB	-	-	-	0.5	-	0.5	-	-
AA	0.06	OB	0.08	OB	-	-	-	0.5	-	-	0.5	-
AA	0.06	AA	0.06	OB	-	-	-	-	-	1	-	-
AA	0.06	AB	0.03	OB	-	-	-	-	-	0.5	0.5	-
AA	0.06	BB	<0.01	OB	-	-	-	-	-	-	1	-
AA	0.06	OO	0.48	AA	-	-	-	1	-	-	-	-
AA	0.06	OA	0.34	AA	0.50	0.0103	-	0.5	-	0.5	-	-
AA	0.06	OB	0.08	AA	-	-	-	0.5	-	-	0.5	-

Maternal genotype		Paternal genotype		Genotype of second child		Combination of genotypes	Probability of genotype first child based on genotype mother, father and second child					
Genotype	Genotype probability[149]	Genotype	Genotype probability[149]	Genotype	Genotype probability	Probability of combination	OO	OA	OB	AA	AB	BB
AA	0.06	AA	0.06	AA	1	0.0037	-	-	-	1	-	-
AA	0.06	AB	0.03	AA	0.50	0.0009	-	-	-	0.5	0.5	-
AA	0.06	BB	<0.01	AA	-	-	-	-	-	-	1	-
AA	0.06	OO	0.48	AB	-	-	-	1	-	-	-	-
AA	0.06	OA	0.34	AB	-	-	-	0.5	-	0.5	-	-
AA	0.06	OB	0.08	AB	0.50	0.0026	-	0.5	-	-	0.5	-
AA	0.06	AA	0.06	AB	-	-	-	-	-	1	-	-
AA	0.06	AB	0.03	AB	0.50	0.0009	-	-	-	0.5	0.5	-
AA	0.06	BB	<0.01	AB	1	0.0002	-	-	-	-	1	-
AA	0.06	OO	0.48	BB	-	-	-	1	-	-	-	-
AA	0.06	OA	0.34	BB	-	-	-	0.5	-	0.5	-	-
AA	0.06	OB	0.08	BB	-	-	-	0.5	-	-	0.5	-
AA	0.06	AA	0.06	BB	-	-	-	-	-	1	-	-
AA	0.06	AB	0.03	BB	-	-	-	-	-	0.5	0.5	-
AA	0.06	BB	<0.01	BB	-	-	-	-	-	-	1	-
AB	0.03	OO	0.48	OO	-	-	-	-	0.5	-	-	-
AB	0.03	OA	0.34	OO	-	-	-	-	0.25	0.25	0.25	-
AB	0.03	OB	0.08	OO	-	-	-	-	0.25	-	0.25	0.25
AB	0.03	AA	0.06	OO	-	-	-	0.5	-	0.5	0.5	-
AB	0.03	AB	0.03	OO	-	-	-	0.25	-	0.25	0.5	0.25
AB	0.03	BB	<0.01	OO	-	-	-	0.25	-	-	0.5	0.5
AB	0.03	OO	0.48	OA	0.50	0.0072	-	-	0.5	-	-	-
AB	0.03	OA	0.34	OA	0.25	0.0026	-	-	0.25	0.25	0.25	-
AB	0.03	OB	0.08	OA	0.25	0.0006	-	-	0.25	-	0.25	0.25
AB	0.03	AA	0.06	OA	-	-	-	-	-	0.5	0.5	-
AB	0.03	AB	0.03	OA	-	-	-	-	-	0.25	0.5	0.25
AB	0.03	BB	<0.01	OA	-	-	-	-	-	-	0.5	0.5
AB	0.03	OO	0.48	OB	0.50	0.0072	-	0.5	0.5	-	-	-
AB	0.03	OA	0.34	OB	0.25	0.0026	-	0.25	0.25	0.25	0.25	-
AB	0.03	OB	0.08	OB	0.25	0.0006	-	0.25	0.25	-	0.25	0.25
AB	0.03	AA	0.06	OB	-	-	-	-	-	0.5	0.5	-
AB	0.03	AB	0.03	OB	-	-	-	-	-	0.25	0.5	0.25
AB	0.03	BB	<0.01	OB	-	-	-	-	-	-	0.5	0.5
AB	0.03	OO	0.48	AA	-	-	-	0.5	0.5	-	-	-
AB	0.03	OA	0.34	AA	0.25	0.0026	-	0.25	0.25	0.25	0.25	-
AB	0.03	OB	0.08	AA	-	-	-	0.25	0.25	-	0.25	0.25
AB	0.03	AA	0.06	AA	0.50	0.0009	-	-	-	0.5	0.5	-
AB	0.03	AB	0.03	AA	0.25	0.0002	-	-	-	0.25	0.5	0.25
AB	0.03	BB	<0.01	AA	-	-	-	-	-	-	0.5	0.5
AB	0.03	OO	0.48	AB	-	-	-	0.5	0.5	-	-	-
AB	0.03	OA	0.34	AB	0.25	0.0026	-	0.25	0.25	0.25	0.25	-
AB	0.03	OB	0.08	AB	0.25	0.0006	-	0.25	0.25	-	0.25	0.25
AB	0.03	AA	0.06	AB	0.50	0.0009	-	-	-	0.5	0.5	-
AB	0.03	AB	0.03	AB	0.50	0.0005	-	-	-	0.25	0.5	0.25

Maternal genotype		Paternal genotype		Genotype of second child		Combination of genotypes	Probability of genotype first child based on genotype mother, father and second child					
Genotype	Genotype probability[149]	Genotype	Genotype probability[149]	Genotype	Genotype probability	Probability of combination	OO	OA	OB	AA	AB	BB
AB	0.03	BB	<0.01	AB	0.50	0.0001	-	-	-	-	0.5	0.5
AB	0.03	OO	0.48	BB	-	-	-	0.5	0.5	-	-	-
AB	0.03	OA	0.34	BB	-	-	-	0.25	0.25	0.25	0.25	-
AB	0.03	OB	0.08	BB	0.25	0.0006	-	0.25	0.25	-	0.25	0.25
AB	0.03	AA	0.06	BB	-	-	-	-	-	0.5	0.5	-
AB	0.03	AB	0.03	BB	0.25	0.0002	-	-	-	0.25	0.5	0.25
AB	0.03	BB	<0.01	BB	0.50	0.0001	-	-	-	-	0.5	0.5
BB	<0.01	OO	0.48	OO	-	-	-	-	1	-	-	-
BB	<0.01	OA	0.34	OO	-	-	-	-	0.5	-	0.5	-
BB	<0.01	OB	0.08	OO	-	-	-	-	0.5	-	-	0.5
BB	<0.01	AA	0.06	OO	-	-	-	-	-	-	1	-
BB	<0.01	AB	0.03	OO	-	-	-	-	-	-	0.5	0.5
BB	<0.01	BB	<0.01	OO	-	-	-	-	-	-	-	1
BB	<0.01	OO	0.48	OA	-	-	-	-	1	-	-	-
BB	<0.01	OA	0.34	OA	-	-	-	-	0.5	-	0.5	-
BB	<0.01	OB	0.08	OA	-	-	-	-	0.5	-	-	0.5
BB	<0.01	AA	0.06	OA	-	-	-	-	-	-	1	-
BB	<0.01	AB	0.03	OA	-	-	-	-	-	-	0.5	0.5
BB	<0.01	BB	<0.01	OA	-	-	-	-	-	-	-	1
BB	<0.01	OO	0.48	OB	1	0.0018	-	-	1	-	-	-
BB	<0.01	OA	0.34	OB	0.50	0.0006	-	-	0.5	-	0.5	-
BB	<0.01	OB	0.08	OB	0.50	0.0002	-	-	0.5	-	-	0.5
BB	<0.01	AA	0.06	OB	-	-	-	-	-	-	1	-
BB	<0.01	AB	0.03	OB	-	-	-	-	-	-	0.5	0.5
BB	<0.01	BB	<0.01	OB	-	-	-	-	-	-	-	1
BB	<0.01	OO	0.48	AA	-	-	-	-	1	-	-	-
BB	<0.01	OA	0.34	AA	-	-	-	-	0.5	-	0.5	-
BB	<0.01	OB	0.08	AA	-	-	-	-	0.5	-	-	0.5
BB	<0.01	AA	0.06	AA	-	-	-	-	-	-	1	-
BB	<0.01	AB	0.03	AA	-	-	-	-	-	-	0.5	0.5
BB	<0.01	BB	<0.01	AA	-	-	-	-	-	-	-	1
BB	<0.01	OO	0.48	AB	-	-	-	-	1	-	-	-
BB	<0.01	OA	0.34	AB	0.50	0.0006	-	-	0.5	-	0.5	-
BB	<0.01	OB	0.08	AB	-	-	-	-	0.5	-	-	0.5
BB	<0.01	AA	0.06	AB	1	0.0002	-	-	-	-	1	-
BB	<0.01	AB	0.03	AB	0.50	0.0001	-	-	-	-	0.5	0.5
BB	<0.01	BB	<0.01	AB	-	-	-	-	-	-	-	1
BB	<0.01	OO	0.48	BB	-	-	-	-	1	-	-	-
BB	<0.01	OA	0.34	BB	-	-	-	-	0.5	-	0.5	-
BB	<0.01	OB	0.08	BB	0.50	0.0002	-	-	0.5	-	-	0.5
BB	<0.01	AA	0.06	BB	-	-	-	-	-	-	1	-
BB	<0.01	AB	0.03	BB	0.50	0.0001	-	-	-	-	0.5	0.5
BB	<0.01	BB	<0.01	BB	1	<0.0001	-	-	-	-	-	1

Table S4. Probability of incompatible first pregnancy based on the ABO blood group of mother, father and second child in general population

Maternal phenotype	Paternal phenotype	Phenotype of second child	Probability of phenotype first child based on phenotype mother and father[a]				Probability of incompatible first pregnancy
			O	A	B	AB	
O	O	O	1	-	-	-	-
O	A	O	0.5	0.5	-	-	0.5
O	B	O	0.5	-	0.5	-	0.5
O	AB	O	-	-	-	-	-
O	O	A	-	-	-	-	-
O	A	A	0.37	0.63	-	-	0.63
O	B	A	-	-	-	-	-
O	AB	A	-	0.5	0.5	-	1
O	O	B	-	-	-	-	-
O	A	B	-	-	-	-	-
O	B	B	0.46	-	0.54	-	0.54
O	AB	B	-	0.5	0.5	-	1
O	O	AB	-	-	-	-	-
O	A	AB	-	-	-	-	-
O	B	AB	-	-	-	-	-
O	AB	AB	-	-	-	-	-
A	O	O	0.5	0.5	-	-	-
A	A	O	0.25	0.75	-	-	-
A	B	O	0.25	0.25	0.25	0.25	0.5
A	AB	O	-	-	-	-	-
A	O	A	0.37	0.63	-	-	-
A	A	A	0.16	0.84	-	-	-
A	B	A	0.18	0.32	0.18	0.32	0.5
A	AB	A	-	0.5	0.21	0.29	0.5
A	O	B	-	-	-	-	-
A	A	B	-	-	-	-	-
A	B	B	0.23	0.23	0.27	0.27	0.54
A	AB	B	-	0.5	0.25	0.25	0.5
A	O	AB	-	-	-	-	-
A	A	AB	-	-	-	-	-
A	B	AB	0.17	0.29	0.20	0.34	0.54
A	AB	AB	-	0.5	0.18	0.32	0.5
B	O	O	0.5	-	0.5	-	-
B	A	O	0.25	0.25	0.25	0.25	0.5
B	B	O	0.25	-	0.75	-	-
B	AB	O	-	-	-	-	-
B	O	A	-	-	-	-	-
B	A	A	0.18	0.32	0.18	0.32	0.63
B	B	A	-	-	-	-	-
B	AB	A	-	0.25	0.5	0.25	0.5
B	O	B	0.46	-	0.54	-	-
B	A	B	0.23	0.23	0.27	0.27	0.5

Maternal phenotype	Paternal phenotype	Phenotype of second child	Probability of phenotype first child based on phenotype mother and father[a]				Probability of incompatible first pregnancy
			O	A	B	AB	
B	B	B	0.22	-	0.78	-	-
B	AB	B	-	0.24	0.5	0.26	0.5
B	O	AB	-	-	-	-	-
B	A	AB	0.17	0.29	0.20	0.34	0.63
B	B	AB	-	-	-	-	-
B	AB	AB	-	0.23	0.5	0.27	0.5
AB	O	O	-	-	-	-	-
AB	A	O	-	-	-	-	-
AB	B	O	-	-	-	-	-
AB	AB	O	-	-	-	-	-
AB	O	A	-	0.5	0.5	-	-
AB	A	A	-	0.5	0.21	0.29	-
AB	B	A	-	0.25	0.5	0.25	-
AB	AB	A	-	0.25	0.25	0.5	-
AB	O	B	-	0.5	0.5	-	-
AB	A	B	-	0.5	0.25	0.25	-
AB	B	B	-	0.24	0.5	0.26	-
AB	AB	B	-	0.25	0.25	0.5	-
AB	O	AB	-	-	-	-	-
AB	A	AB	-	0.5	0.18	0.32	-
AB	B	AB	-	0.23	0.5	0.27	-
AB	AB	AB	-	0.25	0.25	0.5	-

Grey cells reflect possible incompatible combinations.
[a]Calculated from the probabilities per ABO genotype shown in supplemental Table 1.

Table S5. Calculation of ABO (in)compatibility in cases and general population based on mothers and fathers

ABO combination mother and father	Probability incompatible pregnancy[a]	Occurrence of					
		ABO combinations		Determined[b] compatible first pregnancy		Possible[c] incompatible first pregnancy	
		Cases, n (%)	Population[d], %	Cases, %	Population[d], %	Cases, %	Population[d], %
O x O	-	45 (22.4)	23.0	22.4	23.0	-	-
O x A	0.58	21 (10.5)	19.3	-	-	6.0	11.1
O x B	0.52	8 (4.0)	4.2	-	-	2.1	2.2
O x AB	1.00	1 (0.5)	1.4	-	-	0.5	1.4
A x O	-	43 (21.4)	19.3	21.4	19.3	-	-
A x A	-	34 (16.9)	16.2	16.9	16.2	-	-
A x B	0.52	6 (3.0)	3.5	-	-	1.6	1.8
A x AB	0.50	7 (3.5)	1.2	-	-	1.7	0.6
B x O	-	15 (7.5)	4.2	7.5	4.2	-	-
B x A	0.58	6 (3.0)	3.5	-	-	1.7	2.0
B x B	-	2 (1.0)	0.8	1.0	0.8	-	-
B x AB	0.50	2 (1.0)	0.3	-	-	0.5	0.1
AB x O	-	2 (1.0)	1.4	1.0	1.4	-	-
AB x A	-	4 (2.0)	1.2	2.0	1.2	-	-
AB x B	-	1 (0.5)	0.3	0.5	0.3	-	-
AB x AB	-	4 (2.0)	0.1	2.0	0.1	-	-
Total		**201 (100)**	**100**	**74.6**	**66.5**	**14.1**	**19.4**

[a]See supplemental tables 1 and 2 for calculations.
[b]The probability of an incompatible first pregnancy was 0.
[c]The probability of an incompatible first pregnancy was >0.
[d]Calculated from the probabilities per ABO genotype shown in supplemental Table 1.

Table S6. Calculation of ABO (in)compatibility in first pregnancy of cases and general population based on mothers, fathers and second children

ABO combination mother, father and second child	Probability incompatible pregnancy[a]	Occurrence of ABO combinations		Occurrence of Determined[b] compatible first pregnancy		Occurrence of Possible[c] incompatible first pregnancy	
		Cases, n (%)	Population[d], %	Cases, %	Population[d], %	Cases, %	Population[d], %
O x O x O	-	30 (24.4)	23.0	24.4	23.0	-	-
O x A x O	0.5	6 (4.9)	8.2	-	-	2.4	4.1
O x A x A	0.63	8 (6.5)	11.1	-	-	4.1	7.0
O x B x O	0.5	2 (1.6)	2.0	-	-	0.8	1.0
O x B x B	0.54	3 (2.4)	2.2	-	-	1.3	1.2
O x AB x A	1	-	0.7	-	-	0	0.7
O x AB x B	1	1 (0.8)	0.7	-	-	0.8	0.7
A x O x O	-	6 (4.8)	8.2	4.1	8.2	-	-
A x O x A	-	18 (14.6)	11.1	14.6	11.1	-	-
A x A x O	-	5 (4.1)	2.9	4.1	2.9	-	-
A x A x A	-	21 (17.1)	13.2	17.1	13.2	-	-
A x B x O	0.5	-	0.7	-	-	-	0.4
A x B x A	0.5	1 (0.8)	1.0	-	-	0.4	0.5
A x B x B	0.54	-	0.8	-	-	-	0.4
A x B x AB	0.54	-	1.1	-	-	-	0.6
A x AB x A	0.5	3 (2.4)	0.6	-	-	1.2	0.3
A x AB x B	0.5	-	0.3	-	-	-	0.1
A x AB x AB	0.5	-	0.4	-	-	-	0.2
B x O x O	-	5 (4.1)	2.0	4.1	2.0	-	-
B x O x B	-	4 (3.3)	2.2	3.3	2.2	-	-
B x A x O	0.5	-	0.7	-	-	-	0.4
B x A x A	0.63	1 (0.8)	1.0	-	-	0.5	0.6
B x A x B	0.5	1 (0.8)	0.8	-	-	0.4	0.4
B x A x AB	0.63	-	1.1	-	-	-	0.7
B x B x O	-	1 (0.8)	0.2	0.8	0.2	-	-
B x B x B	-	-	0.6	-	0.6	-	-
B x AB x A	0.5	-	0.1	-	-	-	-
B x AB x B	0.5	-	0.1	-	-	-	0.1
B x AB x AB	0.5	-	0.1	-	-	-	-
AB x O x A	-	-	0.7	-	0.7	-	-
AB x O x B	-	2 (1.6)	0.7	1.6	0.7	-	-
AB x A x A	-	1 (0.8)	0.6	0.8	0.6	-	-
AB x A x B	-	-	0.3	-	0.3	-	-
AB x A x AB	-	1 (0.8)	0.4	0.8	0.4	-	-
AB x B x A	-	-	0.1	-	0.1	-	-
AB x B x B	-	-	0.1	-	0.1	-	-
AB x B x AB	-	1 (0.8)	0.1	0.8	0.1	-	-
AB x AB x A	-	-	-	-	-	-	-
AB x AB x B	-	2 (1.6)	-	1.6	-	-	-
AB x AB x AB	-	1 (0.8)	0.1	0.8	0.1	-	-
Total	**-**	**124 (100)**	**100**	**79.0**	**66.5**	**11.9**	**19.4**

[a]See supplemental tables 1 and 2 for calculations. [b]The probability of an incompatible first pregnancy was 0. [c]The probability of an incompatible first pregnancy was >0. [d]Calculated from the probabilities per ABO genotype shown in supplemental Table 1.

CHAPTER 3

DOES DISEASE SEVERITY ALWAYS INCREASE IN SUBSEQUENT PREGNANCIES WITH D IMMUNIZATION?

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ABSTRACT

Objective

To evaluate whether the severity of HDFN increases in every subsequent pregnancy with D immunization and a D-positive child.

Methods

This study was part of the Dutch nationwide OPZI 2.0 study, including all pregnant women with D antibodies from July 1, 2014 to March 31, 2015 and from August 1, 2015 to February 28, 2017. Women with two subsequent D immunized pregnancies with D-positive children were included in the present analysis. Data on all previous pregnancies of the women were collected. The severity of HDFN was compared between the first and subsequent pregnancy at risk using a Wilcoxon Signed Rank Test. Predictive factors for severe disease in the subsequent pregnancy were assessed by multivariate analysis.

Results

The cohort comprised of 62 D immunized women with a total of 150 D-positive children after immunization occurred. In general, the severity of HDFN increased significantly in the subsequent pregnancy, compared to the first immunized pregnancy (*P*<.001). The severity however remained equal or even decreased in 44% of the cohort. Especially if antibodies were already detected at first trimester screening in the first immunized pregnancy, no significant increase in severity of HDFN was noted (*P*=.197). If no therapy or only non-intensive phototherapy was indicated during the first pregnancy in this group, also no or only mild HDFN was observed in the next pregnancy. Contrarily, women with antibodies detected late (>= 27th week) during the first immunized pregnancy, most often even before they had ever given birth to a D-positive child, were most prone for increasing severity (*P*<.001) and for severe disease in a subsequent pregnancy. The highest ADCC test result in the first immunized pregnancy appeared a reliable predictor for severe disease in the next pregnancy: if the ADCC test did not exceed 10% in the first pregnancy, 74-99% of subsequent D-positive children will not be treated with intrauterine transfusion(s).

Conclusion

Whereas severity of D-mediated HDFN in general increases in every subsequent pregnancy with a D-positive child, this does not apply to 44% of cases. Based on the time of antibody detection, severity in the first immunized pregnancy and the ADCC result, the risk of severe disease in a subsequent pregnancy can be assessed.

INTRODUCTION

Hemolytic disease of the fetus and newborn (HDFN) is a serious, and nowadays rare, condition, caused by maternal alloantibodies against fetal red blood cells (RBCs). The destruction of RBCs may result in neonatal anemia and hyperbilirubinemia, evoking the need for phototherapy, red cell transfusions or exchange transfusions. In severe cases, anemia occurs prenatally and intervention with intrauterine transfusion(s) (IUT) is needed. A recent national cohort study on all IUTs performed in the Netherlands reported that between 2011 and 2016 83% of these procedures are performed for D immunization.[150] D immunization is thus not only the most frequent type of immunization,[17] it also causes the great majority of severe HDFN cases.

Maternal alloimmunization may occur as a result of an incompatible pregnancy or blood transfusion. As blood transfusions are always ABO- and D-matched, D alloimmunization is now mostly the result of maternal exposure to foreign fetal red cell antigens, inherited from the father.[11] The risk of alloimmunization depends on the duration and amount of fetomaternal hemorrhage, characteristics of the maternal immune system and of the red blood cell antigens.[17]

To prevent D immunization induced by pregnancy, D-negative mothers carrying D-positive fetuses receive both antenatal and postnatal anti-D prophylaxis (RhIg) in most developed countries. As a result, the immunization rate of D-negative women after giving birth to a D-positive child has been reduced impressively from 5% in the early 1960s to 0.3% nowadays.[5,7,13]

A generally accepted idea is that the severity of HDFN increases in every subsequent pregnancy complicated by D immunization, although the course of disease varies and can also be surprisingly mild.[23] As HDFN becomes more rare, the exposure of obstetricians to alloimmunized mothers in clinical practice decreases. As a result, fewer clinicians are familiar with the natural course of the disease. In order to properly counsel and manage these women after a first D immunized pregnancy, accurate data on the severity of HDFN in subsequent pregnancies are needed. This current study therefore aims to assess if, and so, how the severity of HDFN increases in consecutive pregnancies with D immunization and D-positive fetuses.

METHODS

Setting

In the Netherlands, all pregnant women are screened for the presence of allo-antibodies in the first trimester of pregnancy. Furthermore, D-negative and c-negative women are additionally screened in week 27. The coverage of this screening program is almost 100%.[14] All maternal blood samples with a positive screening result, identified at routine screening or at any other moment in pregnancy, are sent to one of the two national referral laboratories (Sanquin Diagnostic services and Special Institute for Blood group Investigations (BIBO)). Here, the clinical relevance of the antibody is evaluated by assessing the antibody specificity, IgG or IgM type, and by assessing whether the fetus is antigen-positive. Although this assessment is nowadays often made directly by non-invasive genotyping of the fetus, serological typing of the father was still the first step in most of the pregnancies in this study. If the fetus is D-positive, the risk on fetal hemolysis is assessed by serially determining the antibody titer and antibody-dependent cell-mediated cytotoxicity (performed only at Sanquin Diagnostic Services), a monocyte based assessment of the destructive capacity of the antibodies.[24,151]

Study design and population

This study was part of the OPZI 2.0 study, a nationwide cohort study on D immunization in pregnancy. All pregnant women with a positive screening for D antibodies, identified at Sanquin Diagnostic Services during our study period, were eligible for inclusion. Positive screenings as a result of a RhIg administration were not included. Women were identified from two time periods (for practical reasons): from July 1, 2014 to March 31, 2015 and from August 1, 2015 to February 28, 2017.

The local care provider of eligible pregnant women was contacted in order to obtain patient's informed consent. If written consent was obtained, clinical data were collected from the care provider by means of a detailed questionnaire. In case outcome data were incomplete, the researchers made at least three attempts to contact care providers or study participants directly in order to obtain complete data. Furthermore, if it was unclear whether women received RhIg in a previous pregnancy, this information was obtained from the Department for Vaccine Supply and Prevention Programs (RIVM-DVP). Women were excluded if the mother additionally had another antibody with a titer higher than that of D (and an antigen-positive child).

To test the hypothesis that HDFN is more severe in the subsequent pregnancy with D immunization than in the first immunized pregnancy, we selected all women with more than one pregnancy with D antibodies and D-positive fetuses from the OPZI 2.0 cohort. In order to assess the risk of selection and non-response bias, characteristics of included and non-included cases were compared (supplemental text).

Sample size calculation

Based on the literature[5,152] and an interim analysis, we expected approximately 20% of cases to be treated with IUT, exchange transfusion or ending in fetal or neonatal death in the first immunized pregnancy, and 45% in the second pregnancy. With a significance of 0.05 and a power of 0.8, a total of 56 women with two immunized pregnancies of D-positive fetuses would be required.

Data collection and outcome definitions

Relevant clinical data from all previous non-immunized and immunized pregnancies were collected in the OPZI 2.0 database: data on maternal characteristics (age, ethnicity, moment of antibody detection) and pregnancy and birth details (possible sensitizing or boosting events, all RhIg administrations, mode of delivery of both child and placenta, perinatal bleeding, etc.). Furthermore, we obtained treatment details to assess the severity of HDFN of all pregnancies **with D antibodies and D-positive fetuses** (intrauterine transfusion details, hospital stay, neonatal bilirubin and hemoglobin levels, duration and intensity of phototherapy, red blood cell or exchange transfusions). From Sanquin Diagnostic Services, laboratory data was retrieved (including antibody titers, ADCC results and the presence of additional antibodies).

In the current study, '**first immunized pregnancy**' is defined as the first pregnancy with D antibodies and a D-positive child. '**Subsequent pregnancy'** is defined as the second pregnancy with D antibodies and a D-positive child.

Our main outcome was disease severity, which was based on HDFN treatment and categorized as follows:

1. No HDFN: no antenatal or postnatal treatment
2. Mild HDFN: non-intensive phototherapy (≤2 lamps), or only one day intensive phototherapy (>2 lamps), with or without a red blood cell transfusion during the first month after birth

3. Moderate HDFN: intensive phototherapy (>2 lamps) for more than one day or neonatal exchange transfusion (in the Netherlands neonatal exchange transfusion has been gradually replaced by intensive phototherapy)
4. Severe HDFN: intrauterine transfusion or HDFN-related death.

In case of missing data on disease severity, patients were assigned to a disease category based on the other, non-missing disease parameters (laboratory results, phototherapy duration and intensity, etc.).

Ethical considerations

The medical ethics committee of the Leiden University Medical Center approved the protocol (P15.101/NV/nv). Written informed consent was obtained from all mothers included in this study.

Statistical analysis

All outcomes were analyzed according to a predefined analysis strategy that was conducted in collaboration with our clinical epidemiologist (JGB).

For our main outcome, sensitivity and subgroup analyses on the difference in severity of HDFN between two subsequent pregnancies, a Wilcoxon Signed-Rank test was used. Differences in severity of HDFN between two non-paired groups were analyzed with a multinomial logistic regression. In other, non-paired analyses, the Pearson's Chi-square test or logistic regression (or Fisher's exact test if appropriate) was used for the comparison of proportions. Comparisons of non-parametric outcomes were analyzed with the Mann-Whitney U test. A sensitivity analysis was performed among patients in whom all the information on disease outcome was available. As the risk of HDFN might be different if D-antibodies are found at first trimester screening or around 27 weeks in the first immunized pregnancy, a subgroup analysis was performed in these 'early' and 'late' groups.

In order to identify factors possibly predicting severe HDFN (IUT or death) in a subsequent pregnancy for counseling purposes, a prediction model was constructed including all variables known or thought to be associated with increasing HDFN severity from the literature, the potential predictors. All potential predictors with a *P*-value<.25 in univariate analysis were included in a multivariate logistic regression model. The prediction model was further improved by applying manual backward selection, excluding the variable with

the highest *P*-value at every step. Eventually, all variables with a *P*-value<.1 remained in the final prediction model (supplemental Table 3).

RESULTS

Selection and characteristics of study population

During the study period, 311 pregnant women with D immunization were found eligible for inclusion in the OPZI 2.0 study. Figure 1 shows how the study population for the present analysis on HDFN severity in subsequent pregnancies was selected (N=62 women). Of the 62 women included, 19 experienced three pregnancies with D antibodies and a D-positive child, three women had four pregnancies, and one woman delivered six D-positive children after her D antibodies were detected, including a total of 150 D-positive children. Table 1 shows the characteristics of included women and their children.

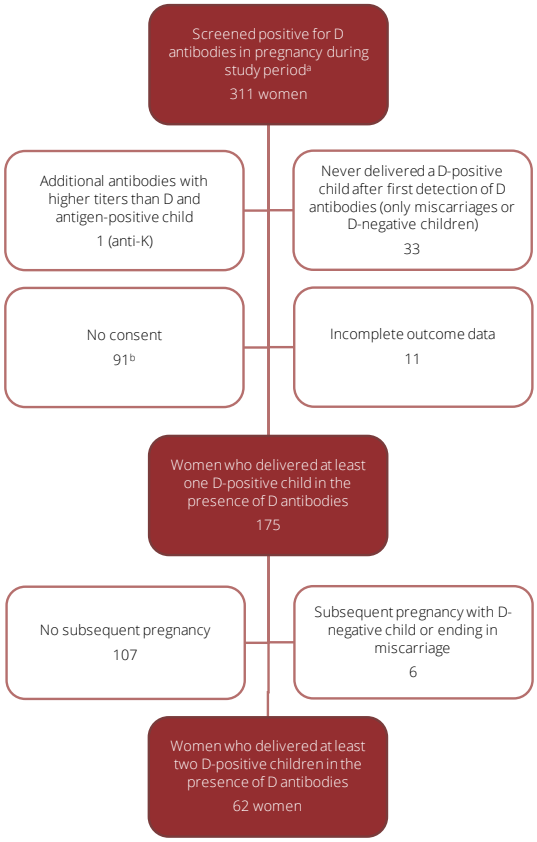


Figure 1. Composition of the study population. [a]Newly immunized women or a new pregnancy after previous immunization. [b]In 21 of these women the antibody was first detected at 27th week screening and no subsequent pregnancy occurred during the study period.

Table 1. Baseline characteristics of 62 women with two or more pregnancies with D antibodies and a D-positive child

First immunized pregnancy		
Caucasian origin		44 (73)
Maternal age at first positive screening		27 [19-35]
Number of previous births (D-positive and D-negative)		1 [0-3]
Gestational age at first positive screening		
Early first trimester screening		21 (34)
Delayed 'first trimester' screening, ≥20 weeks		2 (3)
27th week screening		35 (57)
Between 27th week screening and birth		4 (7)
Between first and subsequent pregnancy with a D-positive child		
Time, years[a]		2 [1-15]
Number of (spontaneous) abortions		0 [0-5]
Number of births of D-negative fetuses 0 1 2		0 [0-2] 58 (94) 3 (5) 1 (2)
	First immunized pregnancy (N=64 children, 2 twins)	**Subsequent pregnancy (N=62 children)**
Pregnancy year	2010 [1999-2015]	2015 [2001-2017]
Gender of child Male Female	 41 (64) 23 (36)	 32 (53) 29 (48)

Data presented in N (%) or median [range].
The first immunized pregnancy is the first pregnancy with D antibodies and a D-positive child, the subsequent pregnancy is the second pregnancy with D antibodies and a D-positive child.
[a]Years between due dates of first and subsequent immunized pregnancy.

Severity of HDFN in the first immunized and the subsequent pregnancy

In this cohort of 150 D-positive children out of pregnancies complicated by D antibodies, no children died as a result of HDFN. One fetal death occurred to a cause other than HDFN. In the third pregnancy of this mother, antibodies were first detected at 27th week screening and upon referral to a gynecologist, perinatal death was noted at 34 weeks and 3 days. The highest antibody titer was 1:4 and the highest ADCC result 10%, both indicating a low risk of fetal hemolysis. A severe growth restriction was noted (between the third and fifth percentile), the baby was non-hydropic and pathological examination of the placenta showed approximately 20% infarction. The fetal death was thus considered the result of placental dysfunction. Symptoms and severity of HDFN of this and the next D-positive child of this mother are not reported in tables and figures and was not analyzed.

Table 2. Severity of HDFN in the first immunized and the subsequent pregnancy with a D-positive child

HDFN severity	First immunized pregnancy N=63 children[a]	Subsequent pregnancy N=61 children	*P*-value
No HDFN	19 (30)	11 (18)	<.001
Mild HDFN	22 (35)[b]	14 (23)[c]	
Moderate HDFN	20 (32)	17 (28)	
Severe HDFN	2 (3)	19 (31)	

Data shown in n (%). Wilcoxon Signed Rank test performed to compare HDFN severity in first and subsequent immunized pregnancy.
[a]61 women, two twins (all mild disease).
[b]Intensity of phototherapy missing in one child (2 days of phototherapy), interpreted as mild.
[c]Intensity of phototherapy missing in one child ('short phototherapy'), interpreted as mild.

Table 2 demonstrates that overall the severity of HDFN was significantly higher in the subsequent pregnancy, compared to the first immunized pregnancy (*P*<.001). HDFN was more severe in the subsequent pregnancy in 34/61 women (56%, maximum of three categories more), equally severe in 19/61 (31%) and less severe in 8 women compared to the first immunized pregnancy (13%, maximum of one HDFN category less). For two patients, the intensity of phototherapy was missing in one of two pregnancies and HDFN severity was therefore imputed, the sensitivity analysis without these patients with imputed HDFN severity showed a similar result (*P*<.001). Figure 2 demonstrates the severity of HDFN in subsequent pregnancies in relation to the severity in the first immunized pregnancy.

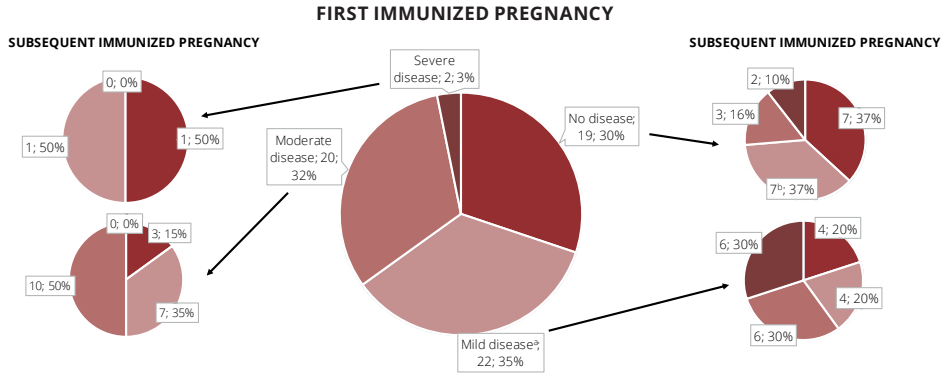


Figure 2. Severity of HDFN in the first and subsequent immunized pregnancy in 61 women (63 vs. 61 D-positive children). Outcome of woman with fetal death to a cause other than HDFN not shown. [a]Intensity of phototherapy missing in one child (2 days of phototherapy), interpreted as mild. [b]Intensity of phototherapy missing in one child ('short phototherapy'), interpreted as mild.

Characteristics of D immunized women and their sequential D-positive children

Table 3 presents the raw data on indicators of HDFN and treatment details in first immunized pregnancies and in subsequent pregnancy with a D-positive child. Most of these factors were more severe in the second immunized compared to the first immunized pregnancy.

Severity of HDFN according to the time of antibody detection

Supplemental figures 1a and 1b demonstrate severity of HDFN in subsequent pregnancies in relation to the severity in the first immunized pregnancy for the subgroups with D antibodies detected either at first trimester screening ('early') or around the 27th week screening ('late') in the first immunized pregnancy. The change in HDFN severity between the first and subsequent pregnancy at risk was significantly different between patients with antibodies detected early (median 0 HDFN categories change, range -1 to +1) and late (median +1, range -1 to +3, *P*=.015). In the early detected subgroup, HDFN became less severe in the subsequent pregnancy of 5 women (22%), was equally severe in 8 (35%) women and more severe in 10 women (43%). This difference in severity was not significant (*P*=.197). All 13 women in the group with early detected antibodies having no or only mild HDFN in the first immunized pregnancy also had a subsequent child with no or mild disease. From the 11 women with moderate to severe HDFN, 9 continued to have moderate or severe HDFN in the subsequent pregnancy.

In contrast, if antibodies were detected late in the first immunized pregnancy, the severity of HDFN increased significantly in the subsequent compared to the first immunized pregnancy (*P*<.001), as HDFN was less severe in 3 out of 37 women (8%), equal in 11 (30%) and more severe in 24 women (65%). This was most pronounced when the antibody was detected during the first pregnancy of a D-positive child, before RhIg could even have been administered (supplemental Table S2).

Predicting severe disease in the second pregnancy with D antibodies

Factors from the first immunized pregnancy possibly predicting severe disease (intrauterine treatment) in the subsequent pregnancy with a D-positive fetus were assessed in a multivariate prediction model (supplemental Table S3). After univariate preselection and manual backward selection, the highest ADCC result in the first immunized pregnancy remained as the only significant predicting factor for receiving intrauterine transfusion(s) in the subsequent pregnancy.

Table 3. HDFN Characteristics of 62 D immunized women and their sequential D-positive children

	First immunized pregnancy N=63 children[a]	Subsequent pregnancy N=62 children
Additional antibodies present	14 (23)	24 (39)
Rh (C, E, G)	12	22
Non-Rh (Fy(a), Jk(a), Wr(a)	2	2
Highest ADCC result	30 [10-80]	50 [10-80]
Highest antibody titer	64 [1-4000]	128 [2-2000]
Perinatal death as a result of HDFN	0	0
Fetuses treated with intrauterine transfusion(s)	2 (3)	19 (31)
Gestational age at first IUT	20+2 and 35+4 (N=2)	30+4 [20-34] (N=19)
Gestational age at birth, weeks+days	37+5 [33-43]	37+2 [35-41]
Births induced before 37 weeks' gestation for HDFN	2 (4)	15 (25)
Arterial umbilical cord pH	7.24 ± 0.08	7.25 ± 0.08
First neonatal hemoglobin, g/dL	15.4 ± 3.1	13.2 ± 3.4
First neonatal hematocrit, %	46% ± 9.2	40% ± 10.4
First neonatal bilirubin, mg/dL	4.5 ± 3.9	4.9 ± 2.5
Lowest hemoglobin in first week of life, g/dL	14.1 ± 3.2	11.9 ± 3.2
Lowest hematocrit in first week of life, %	42% ± 8.1	34% ± 8.9
Highest bilirubin in first week of life, mg/dL	12.3 ± 5.5	12.8 ± 4.6
Neonates treated with phototherapy	43 (68)	50 (82)
Neonates treated with exchange transfusion	10 (16)	6 (10)
Neonates treated with red cell transfusion in first month after birth	14 (23)	20 (33)
Number of red cell transfusions in first month after birth	0.35 ± 0.8 0 [0-4]	0.49 ± 0.8 0 [0-3]

Data presented in N(%), median [range] or mean ± standard deviation.
The first immunized pregnancy is the first pregnancy with D antibodies and a D-positive child, the subsequent pregnancy is the second pregnancy with D antibodies and a D-positive child.
[a]61 women, two twins (all mild disease), one fetal death with a cause other than HDFN not shown.

The predictive value of this test is summarized in Table 4. The negative predictive value of an ADCC test result >10% (thus ADCC result ≤10%) appeared most useful: if the ADCC test did not exceed 10% in the first pregnancy, 74-99% of subsequent D-positive children will not be treated with intrauterine transfusion(s).

Disease severity in third and later pregnancies at risk for HDFN

Compared to the second pregnancy with D antibodies and D-positive children, HDFN severity in the third pregnancy at risk (N=23) was less severe in 23%, equal in 46% and more severe in 32% (no significant increase, *P*=.741). This resulted in 5/23 (22%)

children without treatment, 6 that were mildly affected (26%), 6 moderate (26%) and 6 severe (26%) in third pregnancies at risk. Supplemental Table S4 provides a more detailed description of the outcome of the four women with more than three pregnancies at risk.

Table 4. The predictive value of the highest ADCC result in the first pregnancy on severe disease in the second affected pregnancy.

ADCC	Sensitivity (95% CI)	Specificity (95% CI)	Negative predictive value (95% CI)	Positive predictive value (95% CI)	Area under the curve*
>10%	94 (73-99)	46 (31-62)	94 (74-99)	44 (30-60)	Area .767
>30%	65 (41-83)	65 (49-79)	80 (63-91)	46 (28-65)	95% CI (.636-.898)
>50%	65 (41-83)	78 (63-89)	83 (67-92)	58 (36-77)	P=.002[a]
80% or higher	35 (17-59)	89 (75-96)	75 (61-85)	60 (31-83)	

*Area under the curve of ADCC as a continuous variable.

DISCUSSION

In this unselected national cohort of 150 D-positive children of 62 women with D antibodies, HDFN severity in the first pregnancy with anti-D antibodies with a D-positive child and subsequent pregnancies at risk was evaluated. Although we found that in general the severity of HDFN was more profound in the second immunized ('subsequent') pregnancy compared to the first, the severity remained equal or even decreased in 44% of the cohort. Especially in the group with antibodies detected early in the first immunized pregnancy, no significant increase in severity of HDFN was noted. If no therapy or only non-intensive phototherapy was indicated during their first pregnancy, also no or only mild HDFN was observed in the next pregnancy. Contrarily, women with antibodies detected late (>= 27th week) during the first immunized pregnancy, most often even before they had ever given birth to a D-positive child, were most prone for increasing severity and for severe disease in a subsequent pregnancy.

The major strength of our study is the unselected study population: as coverage of the national screening program is near 100% in the Netherlands[14] and serological assessment (titers and ADCC tests) for the risk on HDFN is performed at Sanquin Diagnostic services only, all women with D antibodies in the Netherlands that were pregnant during our study period were identified. Single center or regional studies inevitably deal with the referral status of the clinic(s), causing a selection bias.

Another strong point of this study was our satisfactory response rate of 73% (see supplemental text), indicating that also the risk of non-response bias is low. To assess this risk, we evaluated whether the moment of antibody detection, an important factor influencing our main outcome, was equally distributed among women with or without consent or complete data. Reassuringly, this was indeed the case (supplemental text). Furthermore, no selection bias seems to be induced by selecting women with two or more subsequent pregnancies only, as having a subsequent pregnancy or not was not associated with HDFN severity in the first pregnancy (supplemental text and Table S1).

A limitation of this study is however that the clinical rationale of treatment decisions is unclear in retrospect and might vary over time. This means that cut-offs for the disease categories used to assess the course of subsequent pregnancies with HDFN inevitably remain subject to a certain amount of arbitrariness. Our main finding that disease severity in general increases in subsequent pregnancies at risk is however supported by the increase in almost all raw disease characteristics in Table 3.

In this study, severe HDFN occurred more often in subsequent (31%) compared to first immunized (3%) pregnancies, in line with findings of others. For example, Tiblad et al. found 1.7% (5/288) severe HDFN in first immunized pregnancies, according to our definitions, and 19% in the second pregnancy at risk.[152] Similar to our findings, second children at risk of mothers that were already immunized during their first pregnancy received more treatment for HDFN, although not significantly. Other authors observed 0% severe disease in first immunized pregnancies and 19% in 'reactivation' of D immunization.[153] Our study is however the first study directly comparing the first and subsequent immunized pregnancy of the same D immunized woman, which demonstrated that the severity of HDFN did not increase in 44% of the cohort. This challenges the general accepted concept that every next child at risk for HDFN will be more severely affected.

We found that the severity of HDFN increased only significantly in women with antibodies first detected at 27th week screening of their first immunized pregnancy, after a negative early screening. This increase has a logical explanation: the relatively short exposure to maternal antibodies in the first immunized pregnancy may not induce substantial hemolysis, and the pathogenic response of the antibodies thus appears in the subsequent pregnancy. However, 13 fetuses of the 37 (35%) women with late detected antibodies already showed severe HDFN in the subsequent pregnancy (6/23 (26%) in the early

detected group). Almost all of these 13 mothers had never given birth to a D-positive child before immunization occurred and were thus immunized before RhIg could even have been administered (Table S2). Based on these findings, immunization during the first pregnancy of a D-positive fetus, detected around the 27th week of pregnancy, seems to be a sign of a strong maternal respondership, possibly related to a combination of genetic risk factors such as carrying HLA-DRB1*1501 and FCRIIC-ORF alleles.[29,154,155] If in the future 'high responders' could be identified early, additional anti-D prophylaxis before the conventional antenatal administration might prevent immunization during the first pregnancy at risk. Severe disease hardly ever occurred in the women with late detected antibodies as a result of giving birth to a D-positive child, despite receiving full prophylaxis. This suggests that in these women the immune response presumably is not prevented but is merely suppressed, which has earlier been suggested by others.[5,6,152,156]

Our findings suggest that the time of first antibody detection and the severity of HDFN in the first immunized pregnancy is determining subsequent HDFN severity. If women get D immunized around the 27th week of their first pregnancy of a D-positive child, after a negative first trimester antibody screening, there is a substantial risk of severe HDFN in a next pregnancy. If women become immunized during delivery of a prior D-positive child, the D antibodies are detected early in the next ('first immunized') pregnancy and HDFN still remains mild, the risk of severe disease in a next pregnancy is virtually absent. In early detected cases with moderate and severe HDFN in their first pregnancy, there is a high risk of severe disease in the next pregnancy. The risk of HDFN has been found to correlate with the IgG-Fc-glycosylation profile of anti-D antibodies.[27] Interestingly, we have previously shown that there exists immunological memory for this Fc-glycosylation profile, meaning that this profile is sustained in subsequent pregnancies[28]. Our observation that the ADCC result, which is greatly influenced by the Fc-glycosylation profile,[28] is the best predicting factor for HDFN in the next pregnancy supports the hypothesis that the pathogenicity of the antibodies as revealed in the first pregnancy, remains stable and therefore predicts the severity of disease in the next pregnancy. We plan to test this hypothesis by analysing the glycosylation patterns in this cohort, comparing women with and without increasing disease severity. Lastly, an additional factor influencing the relation between severity in the first and subsequent immunized pregnancies might be the inherited fetal Fc-receptor profiles, as we have previously shown that this profile influences the risk of severe HDFN.[29]

An important final note is that even if severe disease occurs, fetal death as a result of HDFN is nowadays very rare. Furthermore, the outcome of fetuses treated with IUT has improved significantly over time, due to strict and reliable monitoring, early referral (less fetal hydrops) and declining complication rates.[57,150] In case of a very fulminant course of disease, indicated by the need for IUT, the presence of hydrops or even fetal death before 20 weeks' gestation, non-invasive treatment with intravenous immunoglobulins or other (new) immunomodulatory agents should be considered.[157]

CONCLUSION

The severity of anti-D mediated HDFN in general, but not always, increases in subsequent pregnancies with D-positive fetuses. This is mainly observed in mothers with antibodies occurring during the first immunized pregnancy, detected at 27th week screening, often before RhIg can even be administered. Based on the moment of antibody detection, antibody characteristics as reflected by ADCC test results and the severity of HDFN in the first immunized pregnancy, the risk of severe HDFN in a subsequent pregnancy can now be more accurately assessed.

SUPPLEMENTAL MATERIAL

Risk of bias

Table S1. Characteristics of first immunized pregnancies of women with and without a subsequent pregnancy

General characteristics	With subsequent pregnancy (68 women, 70 children)	Without subsequent pregnancy (107 women, 110 children)	Univariate analysis	Multivariate analysis[a]
Number of previous births	1 [0-3]	1 [0-8]	.001[a]	.030
Maternal age at first positive screening	27 [18-35]	30 [18-42]	<.001[a]	<.001
Gestational age at first positive screening			.018[b]	.797
<20 weeks	26 (38)	61 (57)		
≥20 weeks	42 (62)	46 (43)		
HDFN severity				
No HDFN	20 (29)	40 (36)	Ref[a]	Ref
Mild HDFN	23 (33)	21 (19)	.054	.367
Moderate HDFN	23 (33)	37 (34)	.568	.345
Severe HDFN	3 (43)	12 (11)	.323	.667

[a]Logistic regression
[b]Chi-Square

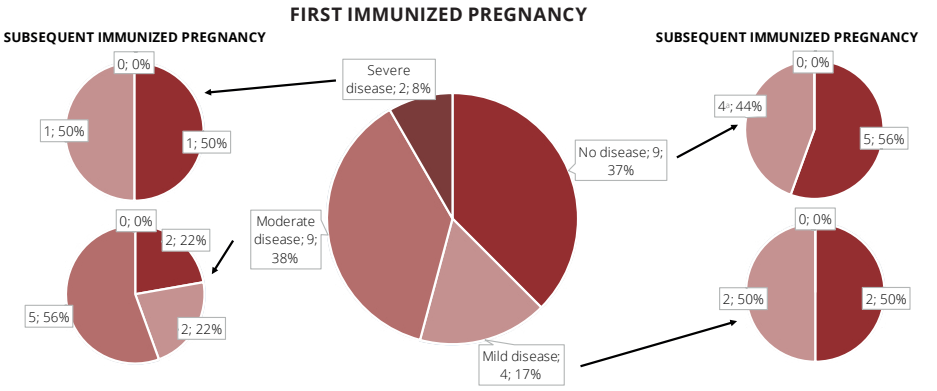
During our study period, 311 women were found eligible for inclusion, as they were pregnant and screened positive for D-antibodies. 55 women were excluded because they had no subsequent pregnancy, only D-negative children or miscarriages after developing D antibodies or because they had other antibodies with higher titers than D. Of the 256 remaining women, 186 gave informed consent and complete data was obtained, resulting in a response rate of 73%.

To assess the risk of non-response bias, the occasion of antibody detection was compared between women with and without consent or complete data. Of the women with consent and complete data, 75% were detected at first trimester screening and 25% at 27th week screening. This was 77% and 23%, and thus similar, in the group with no consent or incomplete data.

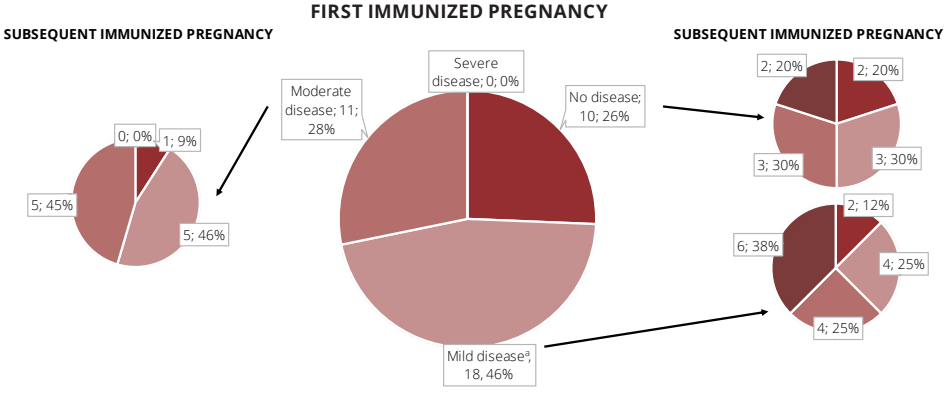
Subsequently, the risk of selection bias was assessed, possibly introduced by selecting women with a subsequent pregnancy only. Data was collected on all pregnancies of women with a positive D antibody screening in pregnancy at the time of our study

period and thus not only if the pregnancy itself occurred during the study period. We were therefore able to determine that 68 of the 175 women with D immunization and a D-positive child became pregnant again after the first immunized pregnancy. To evaluate whether selecting women with two subsequent pregnancies induced selection bias, characteristics of these 68 women were compared to the characteristics of the 107 women without a subsequent pregnancy (supplemental Table 1). The group with a subsequent pregnancy was associated with lower maternal age (P<.001) and less previous births (P=.030) and seemed not associated with HDFN severity and the occasion of first detection of D antibodies in the first pregnancy.

Severity of HDFN in consecutive pregnancies according to the moment of antibody detection



Supplemental Figure 1a. Disease severity in first and second pregnancies at risk for HDFN in 24 women (23 vs. 23 children), subgroup with antibodies detected early in the first pregnancy at risk. [a]1 child with at least mild disease ('short phototherapy'), interpreted as mild.



Supplemental Figure 1b. Disease severity in first and second pregnancies at risk for HDFN in 37 women (40 vs. 38 children), subgroup with antibodies detected late in the first pregnancy at risk. [a]1 child with at least mild disease in first immunized pregnancy (2 days of phototherapy), interpreted as mild.

Table S2. Severity of HDFN in cases with antibodies detected at 27th week screening, with and without a previous D-positive child

	First immunized pregnancy N=40 children[a]		Subsequent pregnancy N=38 children[a]	
	Birth of a D-positive child before immunization		Birth of a D-positive child before immunization	
Disease severity	**No N=26**	**Yes N=14**	**No N=25**	**Yes N=13**
No HDFN	6 (23)	4 (29)	3 (12)	1 (8)
Mild HDFN	12 (46)	7 (50)	4 (16)	4 (31)
Moderate HDFN	8 (31)	3 (21)	6 (24)	7 (54)
Severe HDFN	0	0	12 (48)	1 (8)

[a]38 women, two twins in first pregnancy (all mild disease), one fetal death with a cause other than HDFN and subsequent pregnancy of same women not shown.

Table S3. Univariate and multivariate logistic regression to predict severe disease in the second immunized pregnancy with a D-positive child, based on characteristics of the first immunized pregnancy

		Multivariate (*P* out=.1)																		
	Univariate (*P* out= .25)	Step 1			Step 2			Step 3			Step 4			Step 5			Step 6		
Characteristic		B	95% CI	*P*	B	95% CI	*P*	B	95% CI	*P*	B	95% CI	*P*	B	95% CI	*P*	B	95% CI	*P*
Maternal age	.857																		
Occasion of first positive screening	.550																		
Highest ADCC result (per 10%)	.003	0.194	0.61-2.41	.581	0.196	0.63-2.34	.558	0.183	0.64-2.07	.572	0.312	0.76-2.45	.296	0.406	1.05-2.14	.024	0.333	1.12-1.74	.003
Highest D titer[a]	.019	0.154	0.62-2.19	.631	0.153	0.64-2.12	.617	0.144	0.64-2.07	.629	0.099	0.62-1.95	.735						
Premature induction for HDFN	.343																		
Gender of child	.587																		
First neonatal hemoglobin, g/dL	.052	-0.166	0.49-1.48	.559	-0.168	0.51-1.42	.524	-0.175	0.51-1.39	.497	-0.092	0.57-1.46	.703	-0.078	0.58-1.49	.746			
First neonatal bilirubin, mg/dL	.146	0.004	0.97-1.04	.823	0.004	0.97-1.04	.823	0.003	0.98-1.03	.861									
Phototherapy needed	.010	0.265	0.05-32.26	.871	.257	0.06-28.42	.870												
Need for simple transfusion in first month of life	.108	-0.021	0.10-9.90	.986															
Possible antibody-enhancing moment at first delivery	.564																		
Years between pregnancies	.719																		

Grey boxes reflect the variables excluded at each step.

[a]Log2 transformation to achieve normal distribution.

Disease severity in third and later pregnancies at risk for HDFN

Table S4. Characteristics of cases with more than three subsequent pregnancies with D antibodies and D-positive children

		Pregnancy number[a]					
Patient	**Characteristic**	**First**	**Second**	**Third**	**Fourth**	**Fifth**	**Sixt**
1	Disease severity	No	No	No	No		
	Highest ADCC	10	10	10	10		
	GA at birth	41	40+6	41	41+2		
2	Disease severity	Moderate (intensive PT)	Mild	Mild	Mild		
	Highest ADCC	unknown	30	60	30		
	GA at birth	37+2	37+6	36+3	38		
3	Disease severity	Moderate (ET)	Severe (IUT)	Severe (IUT and ET)	Severe (IUT)		
	Highest ADCC	60	50	80	80		
	GA at birth	38	37+1	31+2	37+1		
4	Disease severity	Moderate (ET)	Moderate (intensive PT)	Moderate (ET)	Moderate (ET)	Severe (IUT and ET)	Severe (IUT)
	Highest ADCC	10	25	80	80	80	80
	GA at birth	39+5	37	37+1	31+5	36	37+1

PT:phototherapy; ET:exchange transfusion; IUT:intrauterine transfusion.
[a]Pregnancies with D immunization and D-positive children only.

CHAPTER 4

THE NEAR DISAPPEARANCE OF FETAL HYDROPS IN RELATION TO CURRENT STATE-OF-THE-ART MANAGEMENT OF RED CELL ALLOIMMUNIZATION

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ABSTRACT

Objective

In this study, we aim to evaluate trends in the condition of fetuses and neonates with hemolytic disease at the time of first intrauterine transfusion (IUT) and at birth, in relation to routine first-trimester antibody screening, referral guidelines, and centralization of fetal therapy.

Method

We conducted a 30-year cohort study including all women and fetuses treated with IUT for red-cell alloimmunization at the Dutch national referral center for fetal therapy.

Results

Six hundred forty-five fetuses received 1852 transfusions between 1 January 1987 and 31 December 2016. After the introduction of routine first-trimester antibody screening, the hydrops rate declined from 39% to 15% (OR 0.284, 95% CI, 0.19-0.42, $P < 0.001$). In the last time cohort, only one fetus presented with severe hydrops (OR 0.482, 95% CI, 0.38-0.62, $P < 0.001$). Infants are born less often <32 weeks (OR 0.572, 95% CI, 0.39-0.83, $P = 0.004$) and with higher neonatal hemoglobin ($P < 0.001$). Neonatal hemoglobin was positively independently associated with gestational age at birth, fetal hemoglobin, and additional intraperitoneal transfusion at last IUT.

Conclusion

Severe alloimmune hydrops, a formerly often lethal condition, has practically disappeared, most likely as a result of the introduction of routine early alloantibody screening, use of national guidelines, and pooling of expertise in national reference laboratories and a referral center for fetal therapy.

INTRODUCTION

Hemolytic disease of the fetus and newborn (HDFN) is a serious complication in pregnancy that has long been a major cause of perinatal mortality. The disease is caused by maternal alloimmunization to fetal red blood cell antigens, mostly concerning D, followed by K, c, and E.[17] The maternal antibodies may cross the placenta and cause fetal hemolysis and anemia, which, if untreated, may lead to fetal heart failure, hydrops, and death.

In the 1980s, important progress in the treatment of fetuses with severe HDFN was made by the introduction of intravascular intrauterine transfusion (IUT), now the cornerstone in prenatal management.[33] However, survival of especially severely hydropic fetuses remained significantly lower than that of anemic fetuses without hydrops.[31] Severe hydrops is furthermore associated with neurodevelopmental impairment on the long term.[32] Preventing hydrops is therefore of utmost importance to improve both survival and long-term outcome.

In the first 3 years after the introduction of intravascular IUT in the Netherlands (1987-1989), the majority of cases (55%) presented with hydrops.[53] A recent study from our group showed that the incidence of fetal alloimmune hydrops has declined to approximately 13%.[57]

In this study, we aimed to evaluate trends in the condition of fetuses and newborns with hemolytic disease at the time of first IUT and at birth and discuss possible contributing factors.

METHODS

Study design, setting and study population

We conducted a retrospective cohort study including all patients with red cell alloimmunization requiring IUT in the Leiden University Medical Center (LUMC) from January 1987 until January 2017. The LUMC serves as the Dutch national referral center for fetal therapy since 1965. In the Netherlands, the annual live born number was 187 000 in 1987 and 173 000 in 2016 (with a maximum of 206 000 in 2000).[158] The prevalence of a

clinically relevant alloantibody (father positive for the antigen) at first-trimester screening was 0.14% to -0.33% in 2016.[14]

Patient data, IUT details, and information on pregnancy outcome of these patients were collected from our electronic prospectively filled Rhesus database. Cases were excluded if fetal or neonatal death occurred from causes other than red cell immunization. Diagnostic fetal blood samplings not followed by IUT, owing to adequate pre-transfusion fetal hemoglobin, were excluded. If the diagnostic fetal blood sampling confirmed fetal anemia, but the following transfusion failed due to technical difficulties or complications, the procedure was not excluded.

Suspected severe fetal anemia requiring IUT was defined as (1) a peak systolic velocity in the fetal middle cerebral artery (MCA) of 1.5 multiples of the median for gestational age (MoM), detected by Doppler measurement, and/or (2) the presence of other signs of anemia at ultrasound examination (cardiomegaly, ascites, hydrops), or (3) amniotic fluid delta optical density measurements reaching the upper part of the Liley's zone II or zone III (only in the early years of this study).

Prevention, screening, diagnostics, and treatment

As a primary preventive measure, anti-D immunoglobulin (RhIg) is administered to all D-negative women, carrying a D-positive child, at 30 weeks' gestation (since 1998) and after birth (since 1969).[17] RhIg prophylaxis has nowadays reached a coverage of approximately 100% in the Netherlands.[139]

To prevent non-D immunizations, blood transfusions administered to women of reproductive age (<45 y) are K- (since 2004), c-, and E-matched in the Netherlands (since 2011).[11] The order of implementation of these and other preventive measures for red cell immunization in the Netherlands is summarized in Table S1.

Despite these preventive measures, alloimmunization may still occur. Since the late 1960s, **D-negative** women are routinely screened for the presence of alloantibodies at week 32 of pregnancy. This screening was brought forward to 30 weeks in 1998 and again forward to 27 weeks in 2011. From 1998 onwards, **all** pregnant women (D-negative and D-positive) are routinely screened for antibodies in the first-trimester. Since 2011, the 27 weeks screening was furthermore broadened to include **c-negative** women.[15]

If screened positive, the antigen status of the fetus is first assessed by testing the paternal zygosity or by cell-free DNA testing in maternal plasma (available for D, c/C, E, and K). If the fetus is assumed or determined to be antigen-positive, antibody titers and antibody-dependent cell-mediated cytotoxicity (ADCC) are measured every 2 weeks, to assess the risk of fetal hemolysis. All laboratory tests are performed at Sanquin Blood Supply in Amsterdam or the Special Institute for Blood Group Investigation in Groningen, the two national referral laboratories.[15,20]

In case of a high risk of severe hemolysis, indicated by serological testing or abnormal ultrasound findings, national obstetric guidelines advise the patient to be referred to the LUMC. The current screening and prevention protocol in the Netherlands is summarized in a recent review of de Haas et al and in a national obstetric guideline.[17,20] Patients with D antibodies are to be referred to the LUMC when titers rise grossly above 1:16 or when the ADCC is higher than 50% (>1:16 and >30% for other Rh antibodies and 1:2 and 30% for anti-K). The management of pregnancies complicated by HDFN in our center consists of weekly monitoring with MCA Doppler until anemia is suspected and intervention is needed, and information is described in more detail previously.[55,57]

Data collection and outcome definitions

Data on maternal characteristics, primary antibody type, treatment details, the severity of fetal hemolytic disease (fetal Hb and presence of hydrops at first IUT), and perinatal outcome were collected.

In order to identify changes over time, all pregnancies were divided into time groups of 6 years each, based on the year of first IUT (1987-1992, 1993-1998, 1999-2004, 2005-2010, and 2011-2016).

Hydrops was classified as mild, if a distinct rim of ascites in the absence or presence of pericardial effusion was seen, whereas abundant ascites, in the absence or presence of pericardial and pleural effusion and skin edema, was considered as severe hydrops.[31]

Primary outcome was the presence of hydrops (total, mild and severe) at the time of first IUT. Furthermore, we assessed the fetal (Z)Hb concentration at first IUT, which is the number of standard deviations of fetal Hb from the mean for gestational age (1 SD = 1 g/dL deviation).[59] Other outcome measures were as follows: perinatal survival, defined as surviving the first month of life or surviving hospitalization (when hospitalized >1 mo

after birth), neonatal Hb, and the proportion of neonates requiring postnatal exchange transfusion(s).

Perinatal survival in consecutive time cohorts was assessed for all fetuses and for those with or without alloimmune hydrops.

Ethical considerations

Only the caregivers knew the identity of their patients, and study data were analyzed anonymously. Therefore, the medical ethics committee of the LUMC approved this research (C15.094) and decided, according to the Medical Research Involving Human Subjects Act (WMO), that written informed consent was not needed.

Statistical analysis

As outcome of multiple pregnancies in the same woman might be interrelated, outcomes were compared using generalized estimating equations (GEE). Within the GEE, a linear, binary logistic or ordinal logistic model was used for comparison of estimated means or odds, respectively. "Time cohort" was used as a continuous predictor to assess a linear trend in variables over time, rather than an overall difference in the variable between all time groups.

Before the use of MCA Doppler became the gold standard to set the indication for IUT around 2000, amniotic fluid delta optical density measurements were used. These are now known to be less accurate in predicting moderate to severe anemia[47] and therefore, a sensitivity analysis was performed for our main outcome analysis, with only patients treated from 2000 onwards.

Numerical outcomes that were not normally distributed were transformed to normality (log2 transformation for titers, gestational age at birth raised to the power of 10).

If important outcomes showed a significant trend over time, we assessed possible factors associated with this outcome by multivariate regression using backward selection (Table S2). The choice for inclusion of variables was made on theoretical grounds, and a *P* value of > 0.1 was used for exclusion of variables.

P values < 0.05 were considered statistically significant.

RESULTS

Characteristics of the study population

During the 30-year study period, IUTs were started in 653 fetuses in 645 pregnancies of 551 women. There were eight twin pregnancies in which both twins were treated with IUT and one more in which only one fetus required IUTs; the co-twin was D-negative. Six singleton pregnancies were excluded because fetal death occurred from causes other than red -cell alloimmunization, described in more detail previously.[91] Two more cases were excluded, as fetal blood sampling did not reveal fetal anemia, and therefore, no transfusion was performed.

Table 1. Trends in characteristics of 645 fetuses and 637 pregnancies treated with intrauterine transfusion over time

Group	Total N=645	1987-1992 N=92	1993-1998 N=127	1999-2004 N=159	2005-2010 N=152	2011-2016 N=115	*P*
Number of IUTs per fetus[a]	2.9 ± 1.43 [1-8]	3.4 ± 1.54 [1-8]	2.9 ± 1.33 [1-6]	3.0 ± 1.33 [1-6]	2.8 ±1.43 [1-6]	2.7 ± 1.33 [1-6]	0.003
Maternal age at first IUT	32 ± 4.5	31 ± 4.2	32 ±4.4	32 ± 4.3	32 ±5.0	32 ± 4.4	0.125
Antibody against D	524 (81)	80 (87)	110 (87)	126 (80)	113 (74)	95 (83)	0.074[b]
K	83 (13)	7 (8)	11 (9)	22 (14)	29 (19)	14 (12)	
c	23 (4)	4 (4)	4 (3)	8 (5)	6 (4)	1 (1)	
Other[c]	15 (2)	1 (1)	2 (2)	3 (2)	4 (3)	5 (4)	
GA at first IUT, total	27+3 [16-36]	26+4.5 [18-34]	28+1 [17-35]	27 [17-36]	27+4.5 [16-35]	28+2 [16-35]	0.408[d]
D immunizations	27+5 [16-36]	26+4.5 [18-34]	28+1.5 [17-35]	27+0.5 [17-36]	28+6 [16-35]	28+3 [17-35]	
K immunizations	24+2 [16-35]	21+1 [18-30]	24 [20-31]	23+4 [19-31]	25+4 [18-35]	26+5 [16-33]	

Data in n (%), mean ± standard deviation or median [range] if not normally distributed.
[a]If born alive.
[b]Ordinal logistic generalized estimating equations.
[c]Other include: E, e, Fya, Jka, and very infrequent antibodies.
[d]Overall GA over time.

A total of 645 fetuses remained for analysis in 637 pregnancies of 543 women, receiving a total of 1852 transfusions. In the first time cohort, fetuses received a median of 4 (range 1-8) transfusions per pregnancy, declining to 3 (range 1-6) in the last time cohort (P = 0.003); 55% of fetuses treated with IUT were male, and 45% female. Table 1 shows overall baseline characteristics of these pregnancies and trends in characteristics over time.

Table 2. Outcome of 645 fetuses over time

Group	Total N=645	1987-1992 N=92	1993-1998 N=127	1999-2004 N=159	2005-2010 N=152	2011-2016 N=115	OR per time cohort (95% CI)	P
Hydrops	150 (23)	38 (41)	47 (37)	40 (25)	18 (12)	7 (6)	0.556 (0.48-0.65)	< .001
Mild	93 (14)	18 (20)	28 (22)	28 (18)	13 (9)	6 (5)	0.690 (0.59-0.81)	< .001
Severe	57 (9)	20 (22)	19 (15)	12 (8)	5 (3)	1 (1)	0.482 (0.38-0.62)	< .001
Overall survival[a]	598/641 (93)	73/92 (79)	115/127 (91)	157/159 (99)	146/152 (96)	107/111 (96)	1.898 (1.37-2.37)	< .004
Without hydrops	473/492 (96)	49/54 (91)	75/80 (94)	118/119 (99)	130/134 (97)	101/105 (96)	1.343 (0.89-2.02)	0.158
With mild hydrops	87/92 (95)	16/18 (89)	27/28 (96)	27/28 (96)	12/13 (92)	5/5 (100)	1.379 (0.52-3.67)	0.518
With severe hydrops	38/57 (67)	8/20 (40)	13/19 (68)	12/12 (100)	4/5 (80)	1/1 (100)	3.343 (1.28-8.71)	0.013
Fetal Hb at first IUT, g/dL	5.9 ± 2.4	5.0 ± 2.2	5.2 ± 2.5	5.6 ± 2.3	6.4 ± 2.2	6.8 ± 2.6	-	<0.001
ZHb at first IUT[b]	-7.2 ± 2.2	-7.9 ± 2.2	-8.0± 2.2	-7.4 ± 1.9	-6.7 ± 1.9	-6.2 ± 2.2	-	<0.001
GA at last transfusion	33+5 [16-37]	32+6 [18-37]	34 [18-37]	33+6 [26-36]	34 [16-36]	32+3 [21-36]	-	0.114
Time between last transfusion and birth, d	19 [0-99]	13 [0-29]	14 [0-99]	19 [0-57]	20 [0-56]	21 [0-91]	-	<0.001
GA at birth[c]	36+3 [28-39]	35+6 [29-39]	36+2 [30-39]	36+2 [28-39]	36+5 [31-38]	36+4 [29-39]	-	<0.001[d]
Births before 32-wk gestation	23 (4)	8 (10)	5 (4)	7 (4)	1 (1)	2 (2)	0.572 (0.39-0.83)	0.004
Neonates requiring exchange transfusion(s)[c]	298 (51)	68 (91)	76 (64)	94 (64)	42 (29)	18 (17)	0.420 (0.36-0.49)	<0.001
Hb at birth, g/dL	11.3 ± 2.7	9.2 ± 2.5	11.0 ± 2.5	11.2 ± 2.3	11.9 ± 2.7	12.6 ± 2.6	-	<0.001[e]

Data in n (%) or mean ± SD.
Abbreviation: GA, gestational age; IUT, intrauterine transfusion.
[a]Survivors/total number of fetuses with a specific hydrops category (no/mild/severe).
[b]Number of standard deviations from the mean Hb for gestational age (1 SD=1g/dL).
[c]If born alive.
[d]Raised to power 10 to achieve normal distribution.
[e]Linear generalized estimated equation adjusted for gestational age at birth.

Trends in fetal condition at first transfusion over time

All primary and secondary outcomes are shown in Table 2. In the total 30-year cohort, at the time of first transfusion, 23% of fetuses showed signs of hydrops. Hydrops was classified as mild in 14.4% and as severe in 8.8%. The incidence of hydrops declined significantly over time, from 41% in the first time cohort (1987-1992) to 6% in the final cohort, 2011-2016 (OR for the presence of hydrops in a later time cohort 0.556, 95% CI, 0.48-0.65, $P < 0.001$). In Figure 1, the proportion of fetuses with hydrops (mild and severe) at first transfusion is shown over time. Trends in hydrops are shown separately for D and K immunizations.

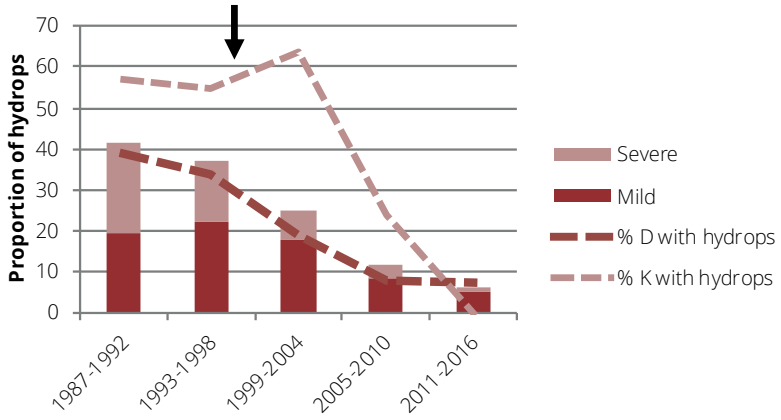

Figure 1. Proportion of fetuses with hydrops at time of first intrauterine transfusion. The proportion of fetuses with alloimmune hydrops at first intrauterine transfusion, out of all fetuses treated with intrauterine transfusion, is presented in columns per time cohort. The introduction of routine first-trimester antibody screening is marked by an arrow.

The great majority of cases with hydrops were associated with D, K, or c immunization (97%). Furthermore, we noted one hydrops case with anti-e, one with anti-Fy^a, and three cases in pregnancies with antibodies against low frequent red blood cell antigens: one anti-Cw and two pregnancies of a woman with "Verdegaal" alloimmunization.

After the introduction of routine first-trimester antibody screening in 1998, the hydrops rate declined significantly, from 39% (up to 1998) to 15% (after 1998, OR 0.284, 95% CI, 0.19-0.42, $P < 0.001$); and the incidence of severe hydrops dropped from 18% to 4% (OR 0.204, 95% CI, 0.11-0.37, $P < 0.001$). From 1999 onwards, 21 out of 65 fetuses (32%) with K immunization developed hydrops despite routine screening, compared with 40/334 (12%) of fetuses affected by anti-D (OR 3.508, 95% CI, 1.82-6.78, $P < 0.001$). The median

gestational age at first transfusion in K affected pregnancies was approximately 3 weeks earlier than in D immunizations (24 wk and 6 d, range 16-35, vs. 27 wk and 6 d [16-36], $P < 0.001$).

Sensitivity analysis

A sensitivity analysis was performed with only cases included treated from the year 2000 onwards, thus excluding cases in which the decision for IUT was based on the Liley curve. For this analysis, the time cohort 1999-2004 was thus adjusted to 2000-2004, in which 32/138 (23%) of cases presented with hydrops at first IUT, significantly decreasing to 7/115 (6%) (OR for the presence of hydrops in a later time cohort 0.459, 95% CI, 0.30-0.70, $P < 0.001$).

Substandard factors in the management of cases with severe hydrops

To identify possible substandard care factors that might have contributed to the development of hydrops, charts of the 18 pregnancies that presented with severe hydrops after the implementation of first-trimester screening were reviewed.

Eleven of these 18 patients (61%) were treated with IUT within a day after presentation with severe hydrops at our fetal therapy center, indicating a possible late referral. Detailed information was not available for one of these cases, and in two cases, hydrops resulted from patient delay (first prenatal visit at 20 and 25 wk, despite two previous affected pregnancies). In a fourth patient, first-trimester screening was negative, but severe hydrops was noted at a routine ultrasound around week 29 and appeared to result from immunization to a not previously detected private antigen (Verdegaal). The other seven patients were subject to protocol violations: delays in respectively diagnostics (N=1, blood sent to reference laboratory a month after positive screening in regional laboratory, father not typed for antigen against which antibodies were formed) and referral from midwife to gynecologist despite positive screening (N=3) and from gynecologist to our tertiary center although serological risk assessment prescribed referral (N=3). Most of these cases (5/7) occurred in the first 3 years after screening implementation.

Seven out of the 18 cases were timely referred to our center but still developed severe hydrops. Management factors that may be associated with the development of severe hydrops in these cases are as follows: alternating weekly monitoring with the referring hospital, not yet optimally skilled in MCA Doppler measurements (four cases prior to 2000), postponing IUT to optimize birth timing despite amniotic fluid delta optical density

measurement in "Liley 2c" zone (1 case) and lack of monitoring in the time span awaiting paternal genotyping in a case with high risk of anemia (ADCC>80%). No substandard care factors were found in the last patient.

Trends in survival and neonatal condition over time

Trends in survival of hydropic and nonhydropic fetuses are shown in Figure 2 and in Table 2. Survival of fetuses without hydrops did not increase significantly (from 49/54 [91%] in the first 6 years to 101/105 [96%] in the last 6 years of the study (OR for survival in a later time cohort 1.343, 95% CI, 0.89-2.02, *P* = 0.158), while survival of fetuses with hydrops increased from 24/38 (63%) to 6/6 (100%, OR 2.635, 95% CI, 1.37-5.07, *P* = 0.004).

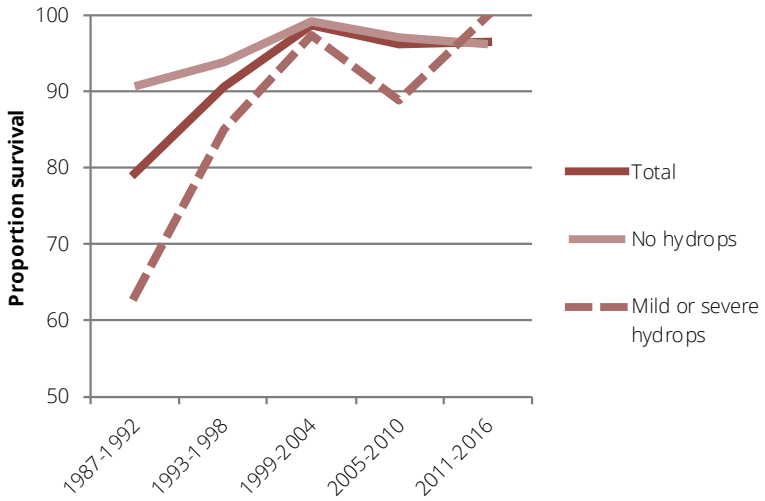

Figure 2. Survival rates of fetuses with and without hydrops. Lines reflect the proportion of fetuses treated with intrauterine transfusion for red cell alloimmunization that survived up to 28 d after birth (or until hospital discharge if admitted more than 28 d).

The rate of children born very prematurely (before 32-wk gestation) declined significantly over time, from 10% of all live born children in the early years to 2% in the last time cohort (OR for very premature birth in a later time cohort 0.572, 95% CI, 0.39-0.83, *P* = 0.004). Hemoglobin at birth increased significantly over time, from 9.2 to 12.6 g/dL in the last cohort (*P* < 0.001). Multivariate regression was performed to assess factors contributing to this rise; factors included in this analysis were as follows: number of IUTs performed, type of antibody (D or K), highest antibody titer, gestational age at birth, time interval between the last IUT and birth, transfusion volume (last IUT), additional intraperitoneal transfusion (last IUT) and pretransfusion fetal hemoglobin at last IUT (Table S2). With

the use of backward selection with a *P*-out of 0.1, gestational age at birth and additional intraperitoneal transfusion and pretransfusion fetal hemoglobin at last IUT remained and were positively independently associated with a higher hemoglobin at birth (Hb 0.187 g/dL higher with every week gestational age, $P = 0.010$, Hb 1.3 g/dL higher after intraperitoneal transfusion, $P < 0.001$, and Hb 0.62 g/dL higher/point less fetal Hb deficit, and $P < 0.001$). In the first years of the study, only 1.1% of patients received an additional intraperitoneal transfusion, increasing to 37.4% in the last time cohort.

Currently, only 17% of neonates treated with IUTs receive one or more exchange transfusions after birth, significantly declining from 91% in the first cohort (OR for receiving exchange transfusion(s) in a later time cohort 0.420, 95% CI, 0.36-.49, $P < 0.001$).

DISCUSSION

This 30-year cohort study describes factors that contribute to the gradual and near disappearance of severe alloimmune hydrops, a serious condition that has negatively influenced fetal survival and long-term outcome for decades. The hydrops rate at first IUT keeps declining since the introduction of routine first-trimester antibody screening in 1998. In the last years of this study, less than 1% (1/115) of fetuses suffered from severe hydrops. Survival of fetuses with and without hydrops is nowadays almost equal, rising up to or above 95%. Furthermore, fetuses are nowadays born less anemic, which was associated with additional intraperitoneal transfusion at the last IUT.

Since the introduction of early screening of all pregnant women in the Netherlands in 1998, the overall hydrops rate in fetuses requiring IUT declined from 39% to 15%, and the incidence of severe hydrops reduced more than fourfold, from 18% to 4%. This impressive currently low incidence of fetal hydrops indicates that fetuses with HDFN are nowadays identified and treated more timely. We hypothesize that this is mainly due to the implementation of national guidelines on management of alloimmunization in pregnancy, covering screening, laboratory, and clinical monitoring and protocols for timely referral to the (single) fetal therapy center. The hydrops rates we found are comparable with those reported in other Western countries with similar screening programs, which vary between 8% and 16%.[58,62,64,66,67,93,159] A study from Belgium, also reported severe hydrops in 5% of red cell immunized pregnancies.[58]

Although the hydrops rate in pregnancies complicated by K antibodies has declined impressively since the introduction of routine first-trimester antibody screening, the odds of developing hydrops are still 3.5 times higher than in D immunization and transfusions are performed approximately 3 weeks earlier in anti-K cases. To ensure timely referral to a tertiary center and prevent hydrops, fast determination of the fetal phenotype in K immunizations is therefore of uttermost importance.

In developing countries, where routine early antibody screening is less well organized or lacking,[160] the incidence of hydrops at time of first IUT is remarkably higher. A group from Brazil reported a 34% hydrops rate in their cohort with mainly D immunized pregnancies, which they contributed to late referral.[60] In the largest tertiary referral center in India, the hydrops rate was 22% in patients with D immunization requiring IUT between 2011 and 2014. Equal to our findings, survival was approximately the same for hydropic and non-hydropic fetuses in this cohort (94% vs. 90%, respectively).[65]

An additional explanation for the declining incidence of hydrops is the optimization of diagnostic accuracy to predict severe fetal anemia over time. The previously used amniotic fluid delta optical density measurements[161] showed limited accuracy in the second pregnancy trimester and in K immunized pregnancies[47,162] and were gradually replaced by measuring the peak flow velocity in the MCA by Doppler ultrasound, which is standard diagnostic care since around 2000.[30] To account for this change in diagnostic management, we performed a sensitivity analysis with only cases included from 2000 onwards. This analysis showed a similar result to the main analysis: OR for the presence of hydrops in a later time cohort 0.459, $P < 0.001$.

An important finding is that survival of fetuses with hydrops increased significantly, leading to a similar survival rate in hydropic and nonhydropic fetuses in recent years. This confirms findings by others[65,67] and is likely partly due to early detection and timely referral for treatment of fetal anemia. Thus, hydrops, if it occurs at all, is nowadays usually only mild, which is associated with favorable outcome. Furthermore, the increase in survival is most profound in cases with severe hydrops (OR 3.3). A more advanced gestational age at birth in later years of the study and/or advancements in prenatal and especially neonatal management in the past decades may have contributed, as well as a decrease in IUT complications.[57] Centralization of fetal therapy and neonatal care in large volume centers is increasingly important for quality of care.[95]

Infants born alive after treatment with IUT(s) seem to be in better neonatal condition nowadays, being less anemic at birth (despite longer intervals between the last transfusion and birth) and needing less exchange transfusions. We found that both additional intraperitoneal transfusion, in adjunct to intravascular transfusion, and more advanced gestational age at birth were independently associated with higher neonatal hemoglobin. As we previously demonstrated, transfusions through the intrahepatic route gained popularity over the years and are associated with less procedure-related complications.[57] An additional advantage of this transfusion technique is the possibility to deposit some extra blood intraperitoneally with the aim to prolong the interval between transfusions or between the last transfusion and birth.[89]

We hypothesize that the found reduction in need for exchange transfusions is mainly caused by the gradual replacement of this invasive procedure by high quality intensive phototherapy and the corresponding more restrictive exchange transfusion protocol in 2004.[163]

This study is conducted in a well-organized health care system for alloimmunized pregnant women. The centralization of screening and treatment gives us great insight and overview in the prevalence of alloimmunization and the details of intrauterine therapy. A limitation might be that we were not able to include fetuses with hydrops, but without IUT. It is therefore possible that some women lost their fetuses to severe hydrops before referral to our national referral center or that labor was induced before performing an IUT. We hypothesize that the subsequent underestimation of hydrops deaths will be greatest in the early years of the study, as this scenario is highly unlikely in the present screening setting.

CONCLUSION

In summary, the incidence of alloimmune fetal hydrops has decreased importantly over the past decades, most likely as a result of the introduction of early screening for alloantibodies in all pregnancies, use of national guidelines, and the availability of both national reference laboratories and the pooling of knowledge and expertise in a referral center for fetal therapy. In particular, severe alloimmune hydrops, a formerly often lethal condition, has practically disappeared, not only leading to improved survival of HDFN but also diminishing risk of long term impairment.

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SUPPLEMENTAL MATERIAL

Table S1. Evolvement of national screening and prevention program in the Netherlands

Year	Preventive measure
1960s	3rd trimester screening for D antibodies in D-negative women D-matching of RBC transfusions
1969	Postnatal RhIg prophylaxis for ABO compatible pregnancies in D-negative women without a living child
1970	Postnatal RhIg for D-negative women with one living child regardless of ABO blood group
1971	Postnatal RhIg for all D-negative pregnant women
1998	Antenatal 30 weeks RhIg prophylaxis for D-negative women without a living child
	1st trimester screening for all pregnant women
2004	Additional K-matching of RBC transfusions in women <45 years of age
2008	Antenatal RhIg prophylaxis for all D-negative women
2011	3rd trimester antibody screening for c-negative women Additional c- and E-matching of RBC transfusions in women <45 years of age Fetal D-typing in maternal plasma introduced, allowing targeted antenatal prophylaxis for D-negative women carrying a D-positive child only

Table S2. Multivariate regression to identify factors associated with Hb at birth in fetuses with D or K immunization and complete data for all possible risk factors

Characteristic	Multivariate analysis (*P* out=.1)							
	Step 1	Step 2	Step 3	Step 4	Step 5	Step 6		
	P					B	SE	*P*
Total number of IUTs performed	.597	.714						
Type of antibody								
D (reference)								
K	.243	.276	.223	.169	.205			
Highest antibody titer[a]	.561	.576	.501					
Gestational age at birth, weeks+days	.256	.391	.362	.003	.011	.187	.07	.010
Days between last IUT and birth	.341	.444	.430	.208				
Fetal ZHb at last IUT[b]	<.001	<.001	<.001	<.001	<.001	.619	.07	<.001
Transfusion volume at last IUT[c]	.743							
Complementary intraperitoneal transfusion performed at last IUT	<.001	<.001	<.001	<.001	<.001	1.302	.37	<.001

Grey boxes reflect the variables excluded at each step. Multivariate analysis perfomed by generalized estimating equation. Model fit tested with regular linear regression: adjusted R square .231, *P* F-test <.001.
[a]Log2 transformation performed, 1 step rise in titer reflects rise from 2 to 4 or 32 to 64, etc.
[b]Number of standard deviations from the mean Hb for gestational age (1 SD=1g/dL).
[c]Intravenous volume + intraperitoneal volume.

CHAPTER 5

COMPLICATIONS OF INTRAUTERINE INTRAVASCULAR BLOOD TRANSFUSION: LESSONS LEARNED AFTER 1678 PROCEDURES

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ABSTRACT

Background

Maternal alloimmunization to fetal red blood cell antigens is a major cause of fetal anemia, which can lead to hydrops and perinatal death if untreated. The cornerstone of management during pregnancy is intrauterine intravascular blood transfusion (IUT). Although this procedure is considered relatively safe, complications continue to occur. The aim of this study was to evaluate rates of procedure-related complications and perinatal loss following IUT, and their change over time, in order to identify factors leading to improved outcome.

Methods

This was a retrospective analysis of IUTs for red-cell alloimmunization performed at the national referral center for fetal therapy in the Netherlands, from 1988 to 2015. Differences in complication rates and their associations with alterations in transfusion technique before and after 2001 were assessed.

Results

Between 1988 and 2015, 1678 IUTs were performed in 589 fetuses. For IUTs performed in 2001 and onwards, there was significant improvement in survival (88.6% vs 97.0%, $P<0.001$) and a decline in procedure-related complication rates per fetus (9.8% vs. 3.3%, $P=0.001$) and per procedure (3.4% vs. 1.2%, $P=0.003$) compared with those before 2001. Procedure-related perinatal loss declined from 4.7% to 1.8% per fetus ($P=0.053$). Beneficial changes in transfusion technique were routine use of fetal paralysis increased use of intrahepatic transfusion and avoidance of arterial puncture.

Conclusions

IUT has become an increasingly safe procedure in recent years when performed by experienced hands. The chosen technique should be fine-tuned according to the patient's individual situation. The declining complication rates are most likely related to center volume: this rare procedure is best performed in experienced fetal therapy centers.

INTRODUCTION

Since its introduction in 1981, intrauterine intravascular blood transfusion (IUT) has become the cornerstone of treatment for fetal anemia in pregnancies complicated by red-cell alloimmunization.[50] In experienced hands, it is nowadays considered a safe procedure, significantly improving perinatal outcome in fetuses suffering with severe anemia.[53,55]

One way to improve fetal outcome further is to minimize the occurrence of procedure-related (PR) complications associated with IUT. A PR fetal-loss rate of approximately 2% per procedure was reported in a review by Schumacher and Moise.[69] However, the small studies included in their review used a variety of definitions for complications and treatment techniques. In 2005, we reported PR complications and fetal-loss rates of 3.1% and 1.6% per procedure, respectively, in a single-center series of 740 IUTs.[91] In our study, transamniotic 'free loop' needling, inadvertent arterial puncture and refraining from the use of fetal paralysis were identified as risk factors for adverse outcome.[91] Apart from the technical aspects, operator and team experience are known to be of utmost importance for performing successful and safe IUTs.[95]

In recent years, a few smaller studies have been performed on this subject, revealing no relevant new insights or tools to improve perinatal outcome further after IUT.[58,66]

Our study aimed to evaluate PR complications and perinatal loss rates after IUT, including assessment of their change over time, over a period of nearly three decades in a national single-center cohort, in order to identify factors leading to improved outcome.

METHODS

Patients

We included all patients treated with IUT between January 1988 and January 2015 at the national referral center for fetal therapy in Leiden, The Netherlands, for fetal anemia caused by red-blood-cell alloimmunization. The findings of IUTs performed between 1988 and 2001 have been analyzed and published previously.[91]

Patient data, technical aspects of IUT and complications were collected from our custom-built electronic Rhesus database. As early IUT is known to be associated with higher perinatal loss rates,[96-99] we compared outcomes performed after IUTs before and after 20 weeks' gestation, to determine whether expected improvements in survival also apply to very young anemic fetuses. To identify changes in procedural techniques and perinatal outcome, pregnancies were divided into two cohorts, according to year of procedure. The first cohort included patients with a first IUT before January 2001, described previously,[91] and the second cohort included patients with a first IUT performed from 1 January 2001 onwards. This policy was chosen as it was assumed that the findings of our first study in 2005[91] may have resulted in gradual changes in transfusion techniques and therefore also in perinatal survival.

Complications after IUT were classified independently into (PR) or non-procedure related (NPR)[91] by two operators (I.L.v.K, D.O.). In summary, fetal condition before IUT was assessed by ultrasound (presence of hydrops, biophysical profile), fetal heart rate tracing in fetuses > 26 weeks' gestation and blood gas analysis of the fetal blood sample obtained before transfusion (a blood gas result of ≤ 7.25 was considered a sign of compromise).[91] If a complication occurred during or after a complicated procedure in a fetus with a reasonable condition prior to IUT based on the abovementioned findings, the complication was considered to be related to the procedure i.e. PR. In fetuses with an unfavorable condition prior to an uncomplicated procedure, the complication was classified as not related to the procedure i.e. NPR. If the experts could not agree on classification, the complication remained unclassified.

The following complications were taken into account: rupture of membranes or preterm delivery within 7 days after IUT, if occurring before 34 weeks of gestation; and intrauterine infection and fetal distress resulting in either emergency Cesarean section (CS) within 24 hours after IUT or fetal or neonatal death.

Primary outcomes assessed were perinatal survival and PR complications. Furthermore, we assessed primary antibody type, gestational age at first IUT, fetal hemoglobin concentration and presence of hydrops at first IUT, procedure access site and other technical details of IUT, number of transfusions per fetus and gestational age at delivery.

Diagnostics

Patients with red-cell alloimmunization were referred, according to national guidelines, to our center in Leiden, The Netherlands, which has served as the national referral center for fetal therapy since 1965. Indication for referral is based on type and titer of antibody, level of antibody-dependent cell-mediated cytotoxicity assay,[24] and obstetric history.

In the first decade of the study period, amniotic fluid delta optical density measurements at 450 nm were used to assess the likelihood of fetal anemia. In later years, the peak systolic velocity in the fetal middle cerebral artery (MCA-PSV) was used to determine the optimal timing of first and subsequent IUTs.[30,46,63] Cordocentesis was performed if MCA-PSV exceeded 1.5 multiples of the median and/or if signs of hydrops were detected on ultrasound, and was followed by transfusion if the fetal blood sample showed fetal anemia.

Intrauterine intravascular blood transfusion technique

Our IUT technique has been described comprehensively in a previous publication.[55] No routine antibiotic prophylaxis or corticosteroid was administered prior to IUT. In nearly all recent cases, atracurium was given intravenously (or intramuscularly) to the fetus prior to transfusion to cause fetal paralysis.

IUT was carried out under aseptic conditions, using a 20- or 22-G needle. In this study, all IUT attempts were made intravascularly. As a result of our prior study,[91] fetal paralysis was applied more often and known risk factors for PR complications, such as transamniotic needling (also known as 'free loop' needling) and arterial puncture, were avoided more consciously in the second half of the study. A tailored mode of transfusion was chosen depending on the fetal anatomy, preferably transfusing into the placental cord insertion in the case of an anterior placenta and into the fetal intrahepatic umbilical vein in the case of a posterior placenta, often in combination with additional intraperitoneal transfusion. For both time cohorts, a maximum of four experienced operators were involved, all capable of applying different transfusion techniques. IUT was considered to be successful if more blood than was acquired for fetal blood sampling was transfused to the fetus, as verified by ultrasound.

Fetal condition was monitored before, during and after transfusion. If the condition of the mother and fetus was satisfactory, patients were discharged within 6 h after IUT.

Statistical analysis

To compare proportions, Fisher's exact test (or Pearson's chi-square test, when appropriate), binary logistic regression (Wald test) or Mann – Whitney U-test was used. The independent t-test was used for comparison of means. All variables with P-value of ≤ 0.15 in univariate analysis were included in a multiple logistic regression model to identify possible independent risk factors for severe PR complications (emergency CS or death). A P-value of < 0.05 was considered statistically significant.

RESULTS

During the 27-year study period, 595 fetuses in 587 pregnancies of 497 women received a total of 1685 IUTs, of which 740 were described previously.[91] In eight twin pregnancies, both twins had anemia as a result of red-cell immunization and received IUTs. In one additional twin pregnancy, one fetus was affected and was included in this study; the co-twin was Rhesus D (RhD) negative.

Six singleton pregnancies, in which fetal death occurred from causes unrelated to red-cell alloimmunization, were excluded, as described in detail previously.[91] Therefore, 589 fetuses were treated with a total of 1678 IUTs in 581 pregnancies of 491 women. Of these, 741 procedures were performed in 255 fetuses before 2001 and 937 procedures were performed in 334 fetuses from 2001 onwards.

Overall perinatal survival was 93.4%, significantly increasing from 88.6% in the first time-cohort to 97.0% in the second time-cohort (odds ratio (OR), 4.2 (95% CI, 2.0 – 8.7), $P < 0.001$). Characteristics of the study population in both time-cohorts are summarized in Table1.

Table 1. Characteristics of 1678 intrauterine intravascular blood transfusions (IUTs) in 589 fetuses with anemia caused by red-cell alloimmunization, according to study period in which procedure was performed

Characteristic	1988-2000 (n=255 fetuses/741 IUTs)	2001-2014 (n=334 fetuses/937 IUTs)	P
Primary immunization against			
Rhesus D	217 (85.1)	255 (76.3)	0.009
Kell	25 (9.8)	53 (15.9)	0.037
Other[a]	13 (5.1)	26 (7.8)	0.242
GA at first IUT (weeks)	27 (17 to 36)	27 (16 to 35)	0.787
Hydrops at first IUT	97 (38.0)	43 (12.9)	<0.001
Hemoglobin at first IUT (g/dL)	4.8 (1.1 to 13.2)	6.3 (1.5 to 12.9)	<0.001
Z-Hemoglobin at first IUT[b]	-8.3 (-12.2 to -0.24)	-6.9 (-11.7 to -0.5)	<0.001
Δ Hemoglobin (after IUT – before IUT) (g/dL)	4.5 (-0.5 to 11.2)	4.4 (0.5 to 9.2)	0.039
Number of IUTs per fetus	3 (1 to 7)	3 (1 to 6)	0.337
GA at delivery of liveborn (weeks)	37 (30 to 39)	36 (28 to 39)	<0.001

Data are given as *n* (%) or median (range). GA: gestational age.
[a]Rhesus c, E or e, Duffy (Fya), Kidd (Jka), rare or low-frequency antigens
[b]Number of SDs from median concentration for gestational age.

The main type of immunization was for RhD, although this significantly decreased from 85.1% before 2001 to 76.3% from 2001 onwards ($P = 0.009$), whereas the proportion of Kell immunization increased (9.8% to 15.9%, $P = 0.037$, Table 1). In the second time-cohort, four patients were treated with intravenous immunoglobulin prior to the first transfusion.

Complications

In a total of 1678 IUTs, 69 complications occurred. Forty-four of these were considered as being directly related to the procedure (PR). In eight patients, one PR complication led to another (five emergency CSs followed by perinatal death, two intrauterine infections followed by perinatal death and one intrauterine infection followed by emergency CS). After correction for this, the actual PR complication rates for the total cohort over 27 years were 6.1% per fetus and 2.1% per procedure. Survival and PR complications are listed in Table 2.

In the first time-cohort, there were 51 complications (seven women each had two complications; actual complication rate = 5.9% per procedure) and in the second time-cohort there were 18 (one women had two complications; actual complication rate = 1.7% per procedure) ($P < 0.001$). Compared with the first cohort, the incidence of PR

complications significantly declined in the second cohort, from 9.8% to 3.3% per fetus (OR, 0.3 (95% CI, 0.2–0.7), *P* = 0.001) and from 3.4% to 1.2% per procedure (OR, 0.3 (95% CI, 0.2 – 0.7), *P* = 0.003). The risk of PR perinatal loss decreased over time from 4.7% to 1.8% per fetus and from 1.6% to 0.6% per procedure (Table 2).

Preterm prelabor rupture of membranes and preterm delivery

In three cases, preterm prelabor rupture of membranes (PPROM) occurred within 7 days after transfusion, leading to preterm delivery before 34weeks. One PR and one NPR classified PPROM are described in more detail in our previously published cohort study.[91] In the second time-cohort, PPROM occurred in one case, the day after the first IUT at 30 weeks' gestation. The baby was liveborn 8 days later. As it took three attempts to complete the transfusion successfully, this complication was classified as PR. The PR-PPROM rate was thus low in both cohorts and did not differ significantly (Table 2).

Table 2. Outcome and procedure-related complications after 1678 intrauterine intravascular blood transfusions (IUTs) in 589 fetuses with anemia caused by red-cell alloimmunization, according to study period in which procedure was performed

Outcome	1988-2000 (n=255 fetuses/741 IUTs)	2001-2014 (n=334 fetuses/937 IUTs)	OR (95% CI)	P
Survival (*n* (%))[a]	226 (88.6)	324 (97.0)	4.16 (2.0-8.7)	<0.001
Procedure-related complication (*n*)	32	12		
Per fetus (*n* (%))[b]	25 (9.8)	11 (3.3)	0.31 (0.2-0.7)	0.001
Per procedure (*n* (%))[b]	25 (3.4)	11 (1.2)	0.34 (0.2-0.7)	0.003
Procedure-related PPROM (*n*)	1	1		
Per fetus (%)	0.4	0.3	0.76 (0.0-12.3)	1.000
Per procedure (%)	0.1	0.1	0.79 (0.0-12.7)	1.000
Procedure-related infection (*n*)	2	1		
Per fetus (%)	0.8	0.3	0.38 (0.0-4.2)	0.581
Per procedure (%)	0.3	0.1	0.40 (0.0-4.4)	0.587
Procedure-related emergency CS (*n*)	17	4		
Per fetus (%)	6.7	1.2	0.17 (0.1-0.5)	<0.001
Per procedure (%)	2.3	0.4	0.18 (0.1-0.5)	<0.001
Procedure-related loss (*n*)	12	6		
Per fetus (%)	4.7	1.8	0.37 (0.1-1.0)	0.053
Per procedure (%)	1.6	0.6	0.39 (0.1-1.0)	0.059

CS: Cesarean section; OR: odds ratio; PPROM: preterm prelabor rupture of membranes.
[a]Alive at discharge from tertiary center.
[b]Actual number and rate (eight patients had two interrelated complications).

Infection

Three cases of culture-proven intrauterine infection with *Escherichia coli* were observed, and all three were classified as PR. Two cases were part of our previously published cohort with IUTs before 2001.[91] A third case occurred in the second cohort after IUT at 18 weeks. The infection led to fetal loss and was considered to be PR. The decrease in PR infections over time, from 0.8% to 0.3% per fetus and from 0.3% to 0.1% per procedure, was not significant (Table 2).

Emergency Cesarean section

Within 24h after IUT, 24 fetuses were delivered by emergency CS for fetal distress, which was considered PR in 21 cases. Five children died subsequently; all deaths were PR and have been described previously.[91] As fetal condition in two of the six cases in the second cohort was unfavorable prior to an uncomplicated IUT, these complications were considered NPR. One case of PR intrauterine infection resulted in emergency CS.[91]

In one case, emergency CS was performed at 34 weeks' gestation for persistent tachycardia after transfusion, probably triggered by volume overload, and was considered as PR. The three remaining cases of CS at 32, 34 and 35 weeks were all classified as PR. All neonates in the second cohort survived after emergency CS. In summary, the occurrence of PR emergency CS decreased from 6.7% in the first. In one case, emergency CS was performed at 34 weeks' gestation for persisting tachycardia after transfusion, probably triggered by volume overload, and this complication was considered PR. The three remaining CSs at 32, 34 and 35 weeks were all classified as procedure-related. All neonates in the new cohort survived after emergency CS. In summary, the occurrence of procedure-related emergency CS declined from 6.7% of fetuses in the first cohort to 1.2% in the second cohort ($P < 0.001$) and from 2.3% to 0.4% per procedure ($P < 0.001$).

Fetal or neonatal death

During the study period, a total of 39 cases of fetal or neonatal death occurred after IUT, of which 10 occurred from 2001 onwards. In this second cohort, one patient with RhD immunization was treated initially with (a possibly incomplete) interstitial laser for twin reversed arterial perfusion syndrome and fetal death was detected the day after the third uncomplicated IUT. Because of the complexity of this case, this complication could not be clearly classified as PR or NPR. Three of the nine remaining cases of loss were considered to be NPR. One NPR loss occurred after the decision to stop intrauterine treatment, as a result of refractory severe hydrops that was diagnosed relatively late and

was caused by antibodies against a low-frequency antigen. In the two other cases, fetal death was detected 19 and 22 days after an uncomplicated IUT and, because of the time lapse between the procedure and the death, these deaths were considered as being NPR.

Therefore, a total of six PR losses occurred in the second cohort, including one with *E.coli* infection that was described earlier. In three patients, multiple attempts at intravenous access led to bradycardia during the procedure, leading to fetal loss directly, and at 1 and 8 days later. Two other PR fetal losses occurred within 1week after IUTs at 16 and 26weeks in fetuses with a reasonable condition prior to IUT. We thus saw a decrease in PR death rates from 4.7% to 1.8% per fetus (P = 0.053) and from 1.6% to 0.6% per procedure (P = 0.059).

Fetal death occurred after eight (17.0%) of 47 IUTs performed before 20 weeks. Four of these were classified as NPR and four as PR, one of the latter was preceded by infection. Fetal demise in the total cohort occurred more often before 20 weeks than after 20 weeks, accounting for both NPR (8.5% *vs* 1.0% per procedure; P=0.002) and PR (8.5% *vs* 0.9%; P = 0.001) fetal death. No significant difference was found in PR fetal-loss rate before 20weeks between the first and the second time-cohort (P = 0.083).

Technical details

In the second time-cohort, significantly more procedures were completed successfully than in the first time-cohort (96.8% *vs* 99.0%, P = 0.001). The number of attempts per procedure decreased (median, 1 (range, 1 – 7) vs 1 (range, 1 – 5), P < 0.001). From 2001 onward, the fetal liver was the most frequently chosen procedure access site (48.0% in the second cohort *vs* 13.8% in the first cohort, P < 0.001). Trends in procedure access sites over time are presented in Figure 1.

Fetal paralysis was applied significantly more frequently (97.8% *vs* 81.3%, P < 0.001) and transamniotic needling in a free loop of cord was performed less frequently (3.7% *vs* 33.1%, P < 0.001) from 2001 onwards compared with before 2001. No arterial punctures were performed after 2001 (0.0% *vs* 3.2%, P < 0.001).

In Table 3, procedures that were followed by severe PR complications (fetal distress resulting in emergency CS or death) are compared with the remaining procedures by univariate analysis. Hydrops was not associated with severe PR complications (P = 0.315). Z-hemoglobin (number of SDs from median concentration for gestational age),

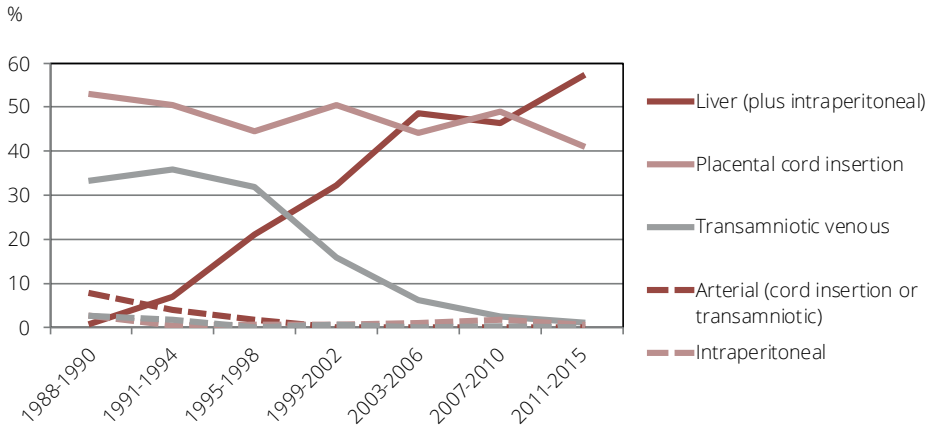

Figure 1. Trends in procedure access sites for intrauterine intravascular blood transfusion between January 1988 and January 2015.

fetal paralysis, procedure access site and unsuccessful IUT were included in a multiple regression model. A positive association with severe PR complications was found for transamniotic (P = 0.030) and arterial (P < 0.001) transfusion sites, compared with transfusion into the fetal liver. There was a negative association of PR complications with fetal paralysis (P = 0.034). Intrahepatic and placental cord insertion sites were equally safe (P = 0.597).

DISCUSSION

In this cohort of 589 fetuses treated with 1678 IUTs for fetal anemia caused by red-cell immunization, we found an improvement in perinatal survival over time, from 88.6% in 1988–2000 to 97.0% from 2001 onwards. The incidence of PR complications decreased significantly in the last decade. PR loss rates declined from 4.7% to 1.8% per fetus and from 1.6% to 0.6% per procedure, a decrease that approached statistical significance. In spite of our policy not to apply routine antibiotics prior to transfusion, PR infection rate was extremely low (0.1% per procedure). These results suggest that intrauterine treatment for red-cell immunization has become significantly safer in the past decade.

Recently, three other European fetal therapy centers reported on PR complications and loss rates.[58,66,67] Pasman et al. found a comparable PR complication rate (1.5%) in 135 procedures performed between 2000 and 2014, although no perinatal death occurred in

the 56 fetuses in their study.[58] We found lower overall PR perinatal loss rates compared with another recent European study by Tiblad *et al.*, in which four of 85 fetuses died as a direct result of the procedure (4.7% per fetus, 1.4% per procedure) performed between 1990 and 2010,[66] and with the third study by Sainio *et al.*, which had a 3.8% PR fetal-loss rate.[67] None of these studies compared trends in results over time.

The increase in survival in our study may be explained, in part, by reduced severity of the disease at referral in the second cohort, reflected by a lower hydrops rate and higher hemoglobin concentrations at first IUT.[55,164] This improvement reflects the optimization of the Dutch program for detection and prevention of red-cell antibodies.[16,17]

Table 3. Characteristics of intrauterine transfusions followed by severe procedure-related complications, compared to remaining procedures

Characteristic	IUT with PR complication[a] (n=34)	Remaining IUTs n=1644	OR (95% CI)	P
Hydrops at IUT	7 (20.6)	231 (14.1)	1.6 (0.7 - 3.7)	0.315
GA at IUT (weeks)	31.1 (16.0 to 35.1)	29.9 (16.4 to 37.0)	-	0.411
Z-hemoglobin at IUT[b]	-7.4 (-12.2 to -3.6)	-6.8 (-11.7 to 1.4)	0.8 (0.7 - 1.0)	0.016
Fetal paralysis	23 (67.6)	1440 (87.6)	0.2 (0.1 - 0.5)	0.001
Procedure access site				
Liver	6 (17.6)	546 (33.2)	0.4 (0.2 - 1.0)	0.065
Placental cord insertion	11 (32.4)	787 (47.9)	0.5 (0.3 - 1.1)	0.083
Transamniotic 'free loop'	10 (29.4)	270 (16.4)	2.1 (1.0 - 4.5)	0.060
Artery	4 (11.8)	20 (1.2)	10.8 (3.5 - 33.6)	0.001
Intraperitoneal	0 (0)	13 (0.8)	-	1.000
Other[c]	3 (8.8)	8 (0.5)	19.8 (5.0–78.2)	0.001
Unsuccessful IUT	3 (8.8)	30 (1.8)	5.2 (1.5 - 18.0)	0.027

Data are given as *n* (%) or median (range). OR: odds ratio.
[a]Procedures followed by fetal distress resulting in emergency Cesarean section within 24 h or fetal death.
[b]Number of SDs from median concentration for gestational age (GA).
[c]Unknown vessel, heart, chorionic vein.

We showed previously that fetal hydrops was associated with adverse outcome, both short- and long-term,[31,164] but not with occurrence of PR complications, [91] as confirmed in the present study. The decline in number of RhD immunizations during the study period is probably best explained by the introduction of routine prophylactic administration of anti-D in the 30th week of gestation in 1998,[5] in addition to postnatal anti-D.

It is likely that the most important factor associated with our low complication rates is the large number of IUTs performed annually at our center (mean, 62 per year *vs* 10, 14 and 38 per year in Tiblad *et al.*,[7] Pasman *et al.*[8] and Sainio *et al.*,[13] respectively), which enhances operator and team experience and thus also diminishes PR complication rates.[95]

We hypothesize that the extensive decline in PR complications is the result of avoiding possibly hazardous techniques in the more recent procedures. Risk factors for adverse outcome were identified previously[91] and were confirmed in the current study as being arterial puncture, transamniotic 'free loop' needling and refraining from fetal paralysis. These technical aspects occurred significantly less frequently in procedures performed from 2001 onwards in our study, compared with procedures carried out before this timepoint. Furthermore, the fetal liver gained impressive popularity as a procedure access site, as this is associated with very low complication and loss rates and is considered to be a safe route of access.[91,92,165] In the previously mentioned studies with higher reported complication rates, 15.5%[7] and 63.8%[13] of transfusions were transamniotic.

Our current study shows that early IUT is still a hazardous procedure, as both NPR and PR complications occur more often before 20 weeks. Early IUT is technically more challenging, resulting in a higher complication risk. Unfortunately, evidence-based studies on the benefit of intravenous immunoglobulin treatment to postpone the first IUT are still lacking. We are currently evaluating the effect of intravenous immunoglobulin in an international multicenter cohort study.

One of the strengths of our study is that all complications were independently and thoroughly classified as PR or NPR. Furthermore, the size of our cohort is considerably larger than those in other published studies. The retrospective design of this study carries some limitations. For example, the rationale for decisions on technical details of procedures by individual operators is difficult to determine retrospectively. However, because of the available evidence supporting transfusion techniques, randomization into different strategies to identify risk factors prospectively could be considered as unethical.[58,91] Another limitation of this study could be that we did not address neonatal outcomes other than death. This was a deliberate choice, as our focus was on severe PR complications. Furthermore, short- and long-term outcomes of IUTs have recently been thoroughly addressed by our group.[164]

Our study demonstrates that IUTs should be a tailored treatment, with the chosen technique fine-tuned to the patient's situation. We advocate that every operator should master all transfusion techniques and maintain experience by performing a sufficient number of transfusions per year in an experienced team. A minimum number of 10 transfusions annually for experienced operators has been suggested.[95] In order to achieve this target, centralization of fetal therapy is necessary.

In summary, we found that IUT for red-cell immunization has become a safer treatment option for fetal anemia. We believe that our current PR fetal-loss rates (1.8% per fetus and 0.6% per procedure) can be considered 'as good as it gets' in experienced hands. For the future, we are focusing our research on non-invasive treatment, such as immunomodulation with intravenous immunoglobulin.

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CHAPTER 6

POSTPONING EARLY INTRAUTERINE TRANSFUSION WITH INTRAVENOUS IMMUNOGLOBULIN TREATMENT; THE PETIT STUDY ON SEVERE HEMOLYTIC DISEASE OF THE FETUS AND NEWBORN

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ABSTRACT

Background

Intrauterine transfusion for severe alloimmunization in pregnancy performed before 20 weeks' gestation is associated with a higher fetal death rate. Intravenous immunoglobulins may prevent hemolysis and could therefore be a non-invasive alternative for early transfusions.

Objective(s)

We evaluated whether maternal treatment with intravenous immunoglobulins defers the development of severe fetal anemia and its consequences in a retrospective cohort to which 12 fetal therapy centers contributed.

Study design

We included consecutive pregnancies of alloimmunized women with a history of severe hemolytic disease and by propensity analysis compared index pregnancies treated with intravenous immunoglobulins (n=24) with pregnancies managed without intravenous immunoglobulins (n=28).

Results

In index pregnancies with intravenous immunoglobulin treatment, fetal anemia developed on average 15 days later compared to previous pregnancies (8% less often before 20 weeks' gestation). In pregnancies without intravenous immunoglobulin treatment anemia developed 9 days earlier compared to previous pregnancies (10% more before 20 weeks), an adjusted 4-day between-group difference in favor of the immunoglobulin group (95%CI -10 to 18, *P*=.564). In the subcohort in which immunoglobulin treatment was started before 13 weeks, anemia developed 25 days later and 31% less before 20 weeks' gestation (54% compared to 23%) than in the previous pregnancy. Fetal hydrops occurred in 4% of immunoglobulin-treated pregnancies and in 24% of those without intravenous immunoglobulin treatment (OR 0.03, 95%CI 0 to 0.5, *P*=.011). Exchange transfusions were given to 9% of neonates born from pregnancies with and in 37% without immunoglobulin treatment (OR 0.1, 95%CI 0 to 0.5, *P*=.009).

Conclusion(s)

Intravenous immunoglobulin treatment in mothers pregnant with a fetus at risk for hemolytic disease seems to have a potential clinically relevant, beneficial effect on the course and severity of the disease. Confirmation in a multicenter randomized trial is needed.

INTRODUCTION

Hemolytic disease of the fetus and newborn (HDFN) is caused by maternal alloimmunization against fetal red blood cells. The maternal antibodies can destruct fetal red blood cells and consequently cause fetal anemia, hydrops and perinatal death.[17,166] Intrauterine blood transfusion (IUT) is currently the only treatment option to prevent fetal death and reduce neurological impairment of these fetuses.[55]

Although relatively safe in experienced hands, IUT remains an invasive procedure, and complications may occur.[57] Early transfusions are technically challenging, especially when performed before 22 weeks' gestation, and carry a significantly higher risk of fetal loss compared with procedures performed later in gestation.[96,97,99] In the largest single-center cohort series from the Leiden University Medical Center (LUMC) (the Netherlands), procedure-related fetal death rates after intravascular intrauterine transfusions performed either before or after 20 weeks' gestation were 8.5% and 0.9% per procedure, respectively.[57] Alternatively, some suggested the use of technically easier intraperitoneal transfusions.[100,167] To date, no other treatment option for fetuses suffering from severe anemia early in pregnancy has been proven to be effective.

The use of intravenous immunoglobulin(s) (IVIg) may postpone or even replace invasive intrauterine treatment in fetuses of mothers with severe alloimmunization in previous pregnancies.[112,167,168] IVIg may theoretically dilute circulating maternal antibodies and induce competition at the placenta, reducing transplacental transfer of maternal antibodies. Furthermore, it might increase antibody turnover and thus lower maternal alloantibody levels and, after transfer to the fetus, block fetal macrophage function.[169,170] As a result, IVIg might prevent hemolysis, but cannot treat existing fetal anemia.[118]

Administration of IVIg in pregnancy is considered safe, although side effects may include urticaria, myalgia, chills, headache, nausea or fever.[171] Another disadvantage is that IVIg treatment is relatively expensive (approximately $6,000/week).[128]

A few single-center case series have reported on the possible effects of intravenous immunoglobulins on morbidity and mortality in hemolytic disease of the fetus and newborn.[126,167,168] In the largest study, from the 1990s, IVIg appeared to lead to a major reduction in fetal mortality from 51% to 20%.[112]

Although several fetal therapy centers occasionally use IVIg treatment in pregnancies at risk for recurrence of severe HDFN, there is still much uncertainty about the indications and true effects of IVIg. In this study, we gathered the international experience of treatment with IVIg to evaluate whether (early) administration in high-risk alloimmunized pregnancies is successful in delaying the onset of severe fetal anemia and thus diminishing its clinical consequences.

MATERIAL AND METHODS

Study design, setting and study population

We conducted a retrospective multicenter cohort study. The cohort consisted of pregnancies of women with an earlier pregnancy with severe HDFN ('previous pregnancy'), managed in the first trimester of a new pregnancy between January 2010 and June 2016 ('index pregnancy'). A list of participating centers is provided as supplemental data. Patients from the Leiden University Medical Center (LUMC) were included from 2001 onward, as the antenatal management of HDFN has not changed since the early 2000's in our center.[57]

In all included current ('index') pregnancies women were either treated with IVIg ('IVIg group') or were managed without IVIg ('non-IVIg group'). Severe HDFN was defined as either a previous fetal and neonatal death as a result of HDFN, or the need for IUT prior to 24 weeks' gestation in the previous pregnancy**.**

All eligible pregnancies of all mothers were included. We excluded pregnancies in which a previous fetal or neonatal death was the result of a lack of diagnostic or therapeutic care, rather than caused by severe HDFN.

In the participating centers, 10 to140 women with red cell immunization are seen annually, receiving 5-60 IUTs that are performed by 1-4 operators. A 20 or 22 Gauge needle was used for intrauterine intravascular transfusion in all participating centers. The preferred transfusion access sites were the placental cord insertion and the intrahepatic part of the umbilical vein.

Treatment with IVIg was preferably started before 13 completed weeks' gestation. Most cases were treated with Nanogam® or Privigen® IVIg. Alternatively, Gammagard®,

Intragam®, Vigam®, Flebogamma® or a combination was used. Most centers dosed IVIg at 1g/kg maternal weight and administered it in weekly doses.

We documented patient characteristics, laboratory results, Doppler measurement results, data on additional treatments, IUT details, delivery details and data on neonatal outcome (up to three months of age) from all pregnancies. Furthermore, details on IVIg treatment were collected of all index pregnancies.

Outcome definitions

We chose the difference in gestational age at onset of severe fetal anemia, requiring IUT, between the index and previous pregnancy ('delta gestational age') as our primary outcome, because the expert opinion is that fetal anemia tends to occur earlier in gestation in subsequent pregnancies of the same alloimmunized mother.[72] As anemia may be present for days before it is diagnosed, the exact onset of severe anemia is impossible to determine. Therefore, we use 'onset of severe anemia' when we mean 'diagnosis of severe anemia' throughout this manuscript. The (diagnosis of) onset of severe anemia was defined as either the day of IUT, the day fetal death was diagnosed, or the day the Doppler peak systolic velocity in the middle cerebral artery (MCA-PSV)[30] was measured above 1.5 MoM, in case fetal death followed at an unknown time point.

We elected the need for IUT before 20 weeks as a secondary outcome, because of the clinical relevance of this endpoint due to the associated increased risk for procedure-related complications.[57]

Furthermore, we assessed perinatal survival, fetal hemoglobin (Hb) and Z hemoglobin (ZHb) and the presence of hydrops at time of the first IUT, the occurrence of complications (premature rupture of membranes, emergency cesarean section and fetal or neonatal death), the number of IUTs per pregnancy and the proportion of neonates needing exchange transfusions. ZHb is the deviation of fetal Hb from the mean for gestational age (1 standard deviation corresponds to 1 g/dL deviation).[59]

Ethical considerations

Depending on the local regulations of the participating centers, this research was approved by the relevant institutional review boards or ethics committees and accordingly, written informed consent was obtained if prescribed. All study data were analyzed anonymously and only the local caregivers knew the identity of their patients.

Therefore, the medical ethics committee of the LUMC approved this research (P15.327/SH/sh) and decided, according to the Medical Research Involving Human Subjects Act (WMO), that written informed consent was not needed from Dutch cases.

Statistics

All primary and secondary outcomes were analyzed in collaboration with our statistician and clinical epidemiologist.

As several women were included with more than one index pregnancy and because pregnancies of the same woman are interrelated, outcomes were compared using generalized estimating equations (GEE). Within the GEE, a binary logistic or linear model was used for comparison of estimated odds or means, respectively.

To adjust for possible confounding by indication, propensity scores were calculated that represent the probability that women would be selected for IVIg treatment by their caregivers in the index pregnancy.[172] Factors included were gestational age at onset of anemia in the previous pregnancy, pregnancy interval and the number of previous births, type of antibody (D or Kell), maternal BMI and number of IUTs performed in the previous pregnancy. More information on how these factors were included in the propensity score is available as supplemental data. We used inverse probability of treatment weighing (IPTW) based on the generated propensity score in all GEE analyses.

Seven sensitivity analyses were performed for the primary outcome and one for the secondary outcome hydrops. Details on in- and excluded cases and the results of these analyses are available as supplemental data.

RESULTS

Characteristics of the mothers and their pregnancies

A total of 50 pregnancies of women with a severe HDFN were included. Five of these women had more than one pregnancy eligible for inclusion; four women were included with two and one woman with three index pregnancies. One woman was included as a non-IVIg case with her 9th pregnancy. In her 10th pregnancy she received IVIg.

After exclusion of four pregnancies because death in the previous pregnancy did not result from the severity of HDFN, but was most likely caused by lack of timely diagnostics or treatment options, a total of 52 pregnancies remained; 28 in the non-IVIg group and 24 in the IVIg group. One mother started IVIg treatment at the time of her first IUT and this pregnancy was therefore analysed in the non-IVIg group for outcomes at the time of first transfusion only (gestational age, Hb, hydrops) and excluded for other outcomes. Table 1 shows the baseline characteristics of all index pregnancies/fetuses.

In previous pregnancies of the IVIg group, more women had experienced a fetal or neonatal death (63% vs. 44%), anemia occurred 3 weeks earlier (20 vs. 23 weeks) and more IUTs were performed per pregnancy (5 vs. 4), compared to the group that was not treated with IVIg in their current (index) pregnancies. These and other patient characteristics were used to generate propensity scores.

In 51 of 52 index pregnancies an IUT was needed. There was only one index pregnancy without IUT, of a woman with a previous neonatal death at 36 weeks and 5 days. IVIg was started from 13 weeks onwards and no signs of fetal anemia were detected. In the first week of life, the Hb was 9.7 g/dL.

Gestational age at onset of severe fetal anemia

Primary and secondary outcomes are shown as unadjusted and adjusted data in Table 2. The gestational age at onset of severe fetal anemia in the pregnancies with IVIg treatment was on average 15 days later (95% confidence interval (CI) 0 to +31 days) than in the mother's previous pregnancy, whereas this was 9 days earlier (CI -20 to +2) in the non-IVIg group. The adjusted estimated mean difference in this 'delta gestational age' between treatment groups was 4 days (CI -10-18, *P*=.564). If IVIg was started before 13 weeks' gestation, anemia occurred 25 days later than in the previous pregnancy (unadjusted data).

The gestational age at onset of severe anemia in the individual cases is provided by Figure 1. This figure also illustrates that in both groups (those with and without IVIg treatment), the course of disease was very heterogeneous. Although anemia on average developed 9 days earlier in subsequent pregnancies without IVIg treatment, 11 out of 28 fetuses in this group had a later onset of fetal anemia than in the previous pregnancy.

Table 1. Patient demographics and baseline characteristics

	Unadjusted data			Weighted data[a]		
Characteristic	**IVIg group N=24**	**Non-IVIg group N=28**	**SMD**	**IVIg group[b] N=24**	**Non-IVIg group[b] N=25**	**SMD**
Previous pregnancy						
Fetal or neonatal death	15 (63)	12 (44)	37	8 (32)	9 (36)	-7
Gestational age at onset of anemia, weeks+days	20+0 [16-36]	23+0 [17-31]	-66	24+0 [16-36]	21+0 [17-31]	4
IUTs before 20 weeks, n(%)	11 (46)	3 (11)	85	5 (19)	4 (14)	15
Number of IUTs[c]	5 [0-7]	4 [3-7]	-10	4 [0-7]	5 [4-7]	3
Index pregnancy						
Years between pregnancies	2 [0-6]	3 [0-9]	-35	3 [0-6]	2 [0-9]	-4
Gender of child Boy Girl	 12 (50) 12 (50)	 15 (68) 7 (32)	 38	 6 (24) 18 (76)	 16 (74) 6 (26)	 117
Antibody against D Kell	 19 (79) 5 (21)	 19 (68) 9 (32)	 -26	 20 (87) 3 (14)	 19 (74) 6 (26)	 -29
Number of previous births	3 [1-9]	3 [1-8]	-7	3 [1-9]	2 [1-8]	1
Maternal age at first IUT, years	33 [24-41]	32 [24-43]	10	37 [24-41]	29 [24-43]	129
Maternal body mass index	24 [20-41]	28 [22-38]	-72	24 [20-41]	26 [22-38]	-11
Laboratory predictor of disease Highest titer in pregnancy Highest Quantitation in pregnancy (IU/mL)	 1024 [256-16000] 47 [13-305]	 512 [32-8000] 70 [22-381]	 32 -19	 256 [256-16000] 39 [13-305]	 1000 [32-8000] 75 [23-381]	 17 49
IVIg dose, g/kg maternal weight[d]	0.63 [0.32-0.73]	-	-	-	-	-
Number of days between IVIg treatments	7 [7-28]	-	-	-	-	-
Treated with plasmapheresis	8 (33.3)	0	82	4 (15)	0	46

Data presented in median [range] or N (%). Abbreviation: *N,* number of index pregnancies; *SMD,* Standardized Mean Difference; *IUT*, Intrauterine transfusion; *IVIg,* intravenous immunoglobulins.

[a]Weighted by the propensity score.

[b]For variables expressed in N (%), the weighted N was calculated from the weighted proportions and rounded.

[c]If no death occurred.

[d]Amount of IgG1 and IgG3 administrated, calculated as IVIg dose in g/kg maternal weight * percentage IgG in substrate * percentage IgG1 and IgG3.

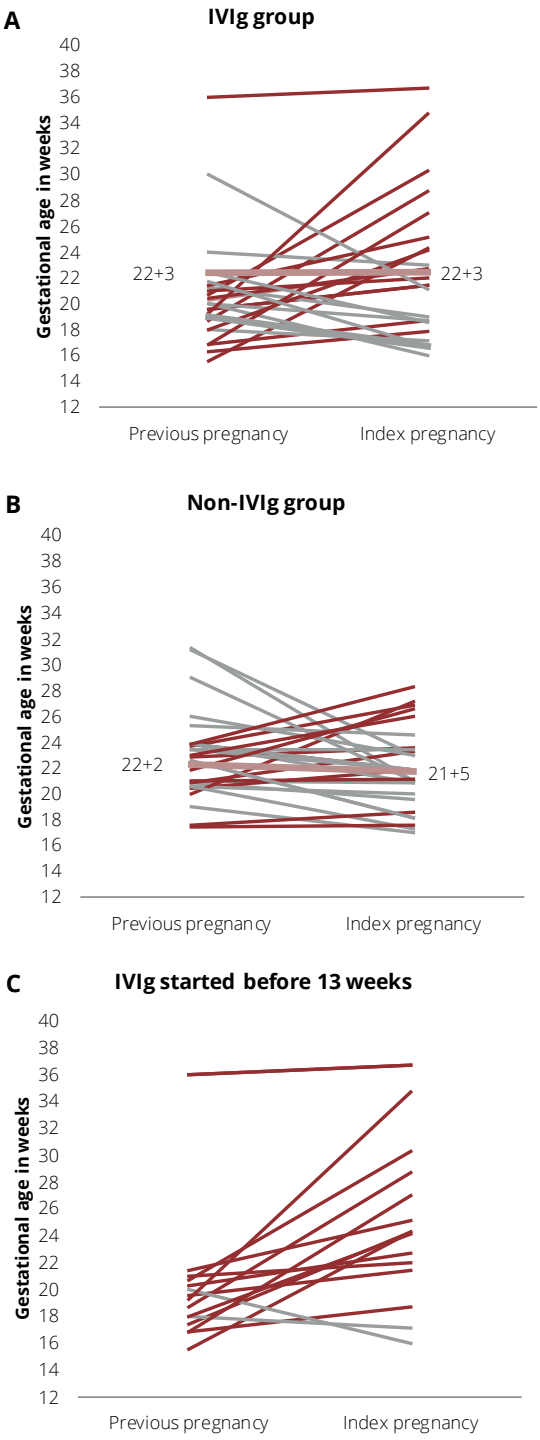

Figure 1. Gestational age at onset of severe anemia in previous and index pregnancy. Grey lines reflect earlier onset of severe anemia in the index pregnancy compared to previous pregnancies, red lines reflect later anemia. The pink, bold lines reflect the weighted estimated means. (A) Group treated with intravenous immunoglobulins. (B) Reference patients without intravenous immunoglobulin treatment in index pregnancy. (C) Subgroup treated with intravenous immunoglobulins started before 13 weeks' gestation.

The development of fetal anemia before and after 20 weeks' gestation, in patients treated or not treated with IVIg and in the subcohort of patients treated with early IVIg, is displayed in Figure 2. In the subcohort in which immunoglobulin treatment was started before 13 weeks, anemia developed 31% less before 20 weeks' gestation (54% compared to 23%) than in the previous pregnancy. In pregnancies with early onset of anemia (before 20 weeks, N=9) IVIg was started at a median of 14 weeks, this was 12 weeks and 4 days in pregnancies in which anemia developed later (N=15). The dose of IgG administered was 0.67 g/kg (range 0.32-0.69) and 0.54 g/kg (range 0.32-0.73) maternal weight respectively, in pregnancies with early and later anemia.

In five pregnancies, IVIg was continued after the first IUT and the second IUT was performed a median of 18 days later (range 13-26 days).

Other clinical outcomes

Overall survival was 45/51 (88%) and did not differ between treatment groups (adjusted OR 1.2 (0.1-11.7), *P*=.894; Table 2). In the index pregnancies of the IVIg group, one fetal and one neonatal death occurred, respectively due to a CMV infection at 18 weeks' gestation and to necrotizing enterocolitis. In the non-IVIg group, four index pregnancies ended in fetal death, following the first IUT performed at gestational ages of 18, 21, 22 and 27 weeks. The cause of fetal death in these cases was either procedure-related or due to the compromised fetal condition.

The decisive MCA Doppler measurement, used to set the indication IUT, was 1.69 MoM in the IVIg group and 1.84 in the non-IVIg group (adjusted estimated mean difference -0.3 MoM, CI -0.6 to 0 MoM, *P*=.043). In both treatment groups, patients received 0.8 MCA Doppler measurements per week, or one MCA Doppler measurement every 8 to 9 days (adjusted estimated mean difference 0.1 measurement/week, CI -0.2-0.3, *P*=.537).

In both treatment groups, three first transfusions were performed intraperitoneally without intravascular access. For 8 non-IVIg patients the degree of anemia was measured in hematocrit only. Consequently, a fetal Hb measurement was available for 20/23 IVIg (and IUT) treated and 14/28 non-IVIg pregnancies. At the time of first IUT, fetuses in both groups had similar Hb levels: 6.4 SD's below the mean for gestational age in the IVIg group and 7.6 SD's in the non-IVIg group (adjusted estimated mean difference 0.9 SDs less deviation from the mean in the IVIg group, CI -0.4 to 2.3 SDs, *P*=.171).[59] Fetuses in the non-IVIg group had signs of hydrops in 24% at the time of first IUT, compared to

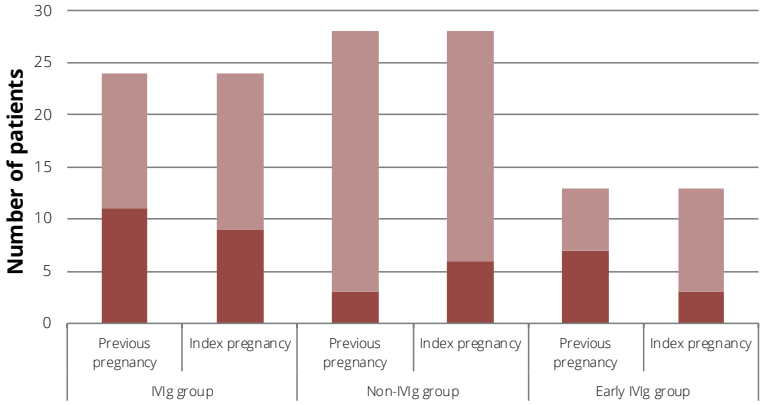

Figure 2. Onset of fetal anemia before and after 20 weeks' gestation in previous and index pregnancies. Red bars reflect onset of severe anemia before 20 weeks' gestations, pink bars reflect anemia after 20 weeks' gestation.

4% in IVIg-treated subjects (adjusted OR 0.03, CI 0-0.5, *P*=.011). After birth, 2/22 (9%) IVIg treated neonates with available neonatal data required an exchange transfusion, which was significantly less than the 7/19 (37%) newborns in the non-IVIg group (adjusted OR 0.1, 95% CI 0-0.5, *P*=.009).

Sensitivity analyses

The results of the sensitivity analyses are shown in supplemental Table 1. The estimated mean differences of the difference ('delta') in gestational age at onset of anemia in the index and previous pregnancy between treatment groups were all in favor of IVIg treatment, although not statistically significant. The largest raw and adjusted effect sizes were seen in patients with D antibodies only, where fetal anemia occurred on average 18 days later in IVIg treated pregnancies, compared to previous pregnancies, and 14 days earlier in pregnancies from women not treated with IVIg, compared to previous pregnancies (adjusted estimated mean difference between groups 10 days, 95% CI -6 to + 26, *P*=.202).

In eight patients IVIg treatment was combined with plasmapheresis. Additionally, one of these patients received corticosteroids. None of the patients in the non-IVIg group received plasmapheresis or other additional treatments. The sensitivity analysis without pregnancies in which plasmapheresis was performed had a similar result to the original analysis (supplemental Table 1).

In patients that were monitored with MCA Doppler in the participating center at least twice before IUT was performed, hydrops was noted in none of the 21 IVIg-treated fetuses and in 4/18 fetuses of the non-IVIg group (weighted *P*<.001).

Table 2. Primary and secondary outcomes

Outcome	Unadjusted				Propensity analysis			
	IVIg group N=24	Non-IVIg group N=28	Effect size[a]	*P*	IVIg group[b] N=24	Non-IVIg group[b] N=26	Effect size[a]	*P*
Delta gestational age, days[c]	15 (0-31)	-9 (-19-1)	24 (6-43)	.011	0 (-11-11)	-4 (-13-5)	4 (-10-18)	.564
Gestational age at onset of anemia, weeks+days	22+6 (21-25)	22+0 (21-23)	5 (-12-22)	.560	22+3 (21-24)	21+5 (20-23)	5 (-9-19)	.475
IUTs before 20 weeks, n(%)	9 (38)	6 (21)	0.5 (0.1-1.5)	.207	6 (24)	6 (22)	1.1 (0.1-8.9)	.908
Survival, n (%)	22 (92)	23 (85)	1.9 (0.3-11.4)	.477	22 (92)	23 (89)	1.2 (0.1-11.7)	.894
Fetal hemoglobin at first IUT, g/dL	5.8 (4.7-7.0)	4.6 (3.3-5.9)	1.2 (-0.5-3.0)	.151	5.4 (4.6-6.2)	4.4 (3.0-5.7)	1.1 (-0.5-2.6)	.184
Hydrops at time of first IUT, n (%)	1 (4)	6 (24)	0.1 (0-1.4)	.092	1 (3)	12 (46)	0.03 (0-0.5)	.011
Number of IUTs[d]	5 (4-6)	5 (4-5)	0.1 (-1.3-1.6)	.859	4 (4-5)	5 (4-6)	-0.4 (-1.5-0.8)	.505
Complication after first IUT, n (%)			0.3 (0-2.6)	.261			0.2 (0-2.1)	.159
PROM or preterm delivery	0	1 (3.7)[e]			0	0		
Intrauterine infection	0	0			0	0		
Emergency cesarean section	1 (5)	0			0 (1.5)	0		
Fetal death	0	4 (15)[e]			0	3 (12)		
Need for exchange transfusion, n (%)	2/22 (9)	7/19 (37)	0.2 (0-0.96)	.045	1 (6)	9 (47)	0.1 (0-0.5)	.009

Data presented in estimated mean (95% CI) or N (%). Abbreviation: *N*, number of index pregnancies; *PROM*, premature rupture of membranes.

[a]Expressed in estimated mean difference (95% CI) for numerical outcomes or OR (95% CI) for proportions.

[b]For variables expressed in N (%), the weighted N was calculated from the weighted proportions and rounded.

[c]Difference in gestational age at onset of severe anemia between index and previous pregnancy.

[d]If no death occurred.

[e]PROM led to fetal death.

COMMENT

In this study, 12 fetal therapy centers from Europe, North-America, Australia and New Zealand collaborated to evaluate the effect of IVIg on the onset of fetal anemia in pregnant women with severe fetal anemia in a previous pregnancy. We found that the onset of severe fetal anemia was later in IVIg treated pregnancies, compared to previous pregnancies. A larger (unadjusted) effect size was seen in the subgroup where IVIg was started ≤13 weeks. In the pregnancies not treated with IVIg, severe fetal anemia occurred earlier than in previous pregnancies. The adjusted difference between groups in this 'delta gestational age' was a non-statistically significant 4 days in favor of the IVIg group. The largest effect was observed in pregnancies with HDFN resulting from D immunization. IVIg started at or before 13 weeks appeared to positively influence the number of transfusions before 20 weeks' gestation.

Additionally, we observed that only one fetus of the 24 IVIg-treated pregnancies developed hydrops, whereas 6/28 of the non-IVIg fetuses did. Furthermore, the neonatal exchange transfusion rate was impressively and significantly lower for neonates from the IVIg group compared to those of the non-IVIg group.

Our international collaborative study is the first study in the past two decades on the effect of IVIg in severe HDFN, comparing fetuses from IVIg treated pregnancies with non-IVIg treated pregnancies. The only comparable study was published in 1997, when monitoring of fetal anemia was entirely different, and mainly based on serial amniocentesis for bilirubin levels.[112] Nevertheless, in an IVIg-treated group of 30 severely alloimmunized pregnancies, they found less hydrops, less fetal death and a mean delay in the need for intrauterine treatment of 1,5 weeks, compared to 39 controls. Their conclusion that 'IVIg treated patients seem to have better fetal outcome' is similar to ours.

The positive effect of IVIg treatment on the time of onset of severe fetal anemia was not statistically significant. However, the point estimates of all outcomes point in the same positive direction, supporting a potential clinically relevant and beneficial effect of IVIg on the course of HDFN. For example, IVIg was associated with less fetal hydrops and a lesser need for neonatal exchange transfusions compared to treatment without IVIg. Furthermore, early IVIg treatment appeared to delay the onset of severe anemia with 3.5 weeks compared to previous pregnancies (unadjusted data, supplemental Table 1).

We hypothesize that the effect of IVIg on time of onset of fetal anemia may have been underestimated. Although fetuses in both groups were monitored with equal intervals, those in the IVIg group might have been monitored more thoroughly, as decisive Doppler measurements before IUT were lower than compared to pregnancies without IVIg. This may reflect an earlier suspicion of severe fetal anemia and intervention with IUT in IVIg treated pregnancies compared to non-IVIg treated pregnancies. A more expectant management in the IVIg group could have resulted in longer recorded time to onset of anemia.

Mothers in the IVIg group seemed to have a history of more aggressive course of disease (based on the onset of anemia and the number of previous deaths), however we found strikingly less hydrops in currently IVIg-treated pregnancies. This is in accordance with the study of Voto et al.[112] Although in our study, there may be an effect of relatively early IUT timing in the IVIg group, a real preventive effect of IVIg on the development of fetal hydrops cannot be ruled out and needs further investigation. Preventing fetal hydrops is known to be very beneficial for perinatal survival and long-term outcome.[32]

A new finding was the reduced need for neonatal exchange transfusions after prenatal IVIg treatment. The rate of exchange transfusions in the IVIg group appeared to be significantly lower than in the non-IVIg group and comparable to the expected 15% observed in other (D-only) studies.[173,174] Apart from the limited sample sizes, a restriction in this finding is the relatively high number of missing data in the non-IVIg group, as chances on missing data are higher in cases were no exchange transfusion is performed. In the scenario that indeed none of these patients with missing data would have received an exchange transfusion, the difference in exchange transfusion rate would still be 6 vs 23% in favor of the IVIg group. If this could be confirmed in a prospective study, it would be highly relevant for the clinical setting. These complex procedures become increasingly rare, with only a few centers able to maintain the necessary skills, and the risks and complications are often underestimated.[175]

Lastly, this study is the first to provide valuable insight in the course of disease in subsequent pregnancies with a high risk of HDFN. A surprising finding was the relatively high number of women in the non-IVIg group (11/28) in which the disease did not worsen in the subsequent pregnancy. Although caution is needed in challenging the accepted concept that the disease is more severe in every following pregnancy, we feel that this deserves further study.

Strengths of this study were the relatively large dataset, obtained through international multicentre collaboration, and the performance of weighted analyses based on the propensity score, to correct for potential confounders. All centers used practically identical strategies for diagnosis and treatment of fetal anemia. As it is possible that centers that do not offer IVIg treatment may serve a less severely affected patient population, the propensity score analysis may be influenced by this unmeasured 'case mix'[176] differences in population between centers. We addressed this by performing a sensitivity analysis including only centers that offer IVIg, which resulted in a similar outcome.

Furthermore, we addressed other potential differences in disease or management by performing additional sensitivity analyses. The effect sizes of these analyses all appeared to be similar to the original analysis (supplemental Table 1).

The most important limitation of our study was the heterogeneity of the groups, due to the rarity of severe HDFN, the heterogeneous course of disease and the retrospective nature of the study. Only a prospective, preferably randomized and ideally blinded study could overcome this issue. Together with the still limited sample size, and the rarity of adverse outcomes, absence of statistical significance of observed differences was not unexpected. However, all observed differences point towards a potential clinically relevant benefit of IVIg in this group of alloimmunized pregnancies with very high risk on severe disease.

Another limitation is that the definition of 'onset of severe fetal anemia' is relatively broad. Patients are not monitored daily and anemia may be present a few days before their scheduled appointment. Although this can never be fully circumvented, we did perform a sensitivity analysis without patients in which this uncertainty was larger than a few days (for example, fetal death noticed at a scheduled appointment). The result of this analysis was similar to the original analysis.

Finally, despite the propensity analysis, a possible residual confounding effect of the type of antibody remained. This is reflected by the standardized mean difference of this variable being >10% (Table 1), which is the proposed threshold below which imbalance seems to be negligible.[177]

Despite the limitations, we feel that gathering the international experience on the use of IVIg in red cell alloimmunization in a conjoint cohort, is an important first step for future research on this subject.

Conclusion

In women pregnant with a fetus at high risk for early hemolytic disease, treatment with weekly IVIg seems to positively influence the course and severity of disease. To truly assess the beneficial effects of IVIg, a prospective and preferably randomized controlled trial would be required, and is planned. Due to the rarity of the disease, conducting such a study will be challenging and requires further intensive international collaboration.

ACKNOWLEDGEMENTS

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SUPPLEMENTAL MATERIAL

Glossary of terms

GEE – generalized estimating equations. This type of analysis does not require that all observations are independent. This is important in the present study, as pregnancies of the same woman are by definition interrelated.

HDFN – hemolytic disease of the fetus and newborn

IUT – (intravascular) intrauterine transfusion

IVIg – intravenous immunoglobulins

Propensity score –in retrospective studies, there is usually a reason that treated subjects received the treatment and untreated subjects didn't. The propensity score calculates the baseline probability the studied treatment was assigned to each study subject, based on the characteristics of these patients. The aim of the propensity score is to balance the treatment groups in order to distribute the values of covariates equally between treated and untreated subjects, like in randomized trials.[172]

SMD - Standardized mean difference. This value represents the balance of a covariate between groups and is presented in the % difference in the value of this covariate. For example, if the means of a specific variable differ 50% between groups, the sample is less balanced than if only 10% difference exists. If the SMD is below 10%, the imbalance is thought to be neligible.[177]

List of participating centers

- the Leiden University Medical Center, the Netherlands;
- Liverpool Hospital, Australia;
- University Hospitals KU Leuven, Belgium;
- The Queen Elizabeth Hospital, Scotland;
- Karolinska University Hospital, Sweden;
- Children's Memorial Hermann Hospital, USA;
- Pränatal Medizin München, Germany;
- University of Birmingham, United Kingdom;
- St Michael's Hospital Bristol, United Kingdom;

- Copenhagen University Hospital Rigshospitalet, Denmark;
- Evergreen Hospital, USA;
- Auckland District Health Board, New Zealand.

Propensity analysis

Included factors were gestational age at onset of anemia in the previous pregnancy, years between previous and index pregnancy and the number of previous births (continuous variables), type of antibody (D or Kell), maternal BMI and number of IUTs performed in the previous pregnancy. Body mass index (BMI) was categorized based on the overall cohort median (27 kg/m^2) to create 'low', 'high' and 'missing' categories. For the number of IUTs, a categorical variable was made based on the overall cohort median (5 transfusions) to create 'low', 'high' and 'unable to determine' categories. This last category consisted of pregnancies ending in fetal or neonatal death or missing values for the number of IUTs. Two index pregnancies were excluded in the adjusted analyses, as the propensity score could not be generated due to missing values in any of the factors.

For analyses with <90% of all pregnancies included, new propensity scores were calculated based on the same confounders. 'GA at birth' could be corrected for all abovementioned potential confounders except for maternal BMI due to smaller numbers in this analysis.

Sensitivity analyses

Seven sensitivity analyses were performed for the primary outcome:

- By center: to examine whether the results were influenced by 'case-mix'[1] differences between centers that do and centers that do not offer IVIg, we repeated the analyses with only pregnancies managed in hospitals that did offer IVIg;
- Certain GA only: to examine whether the results were influenced by using the (possibly imprecise) gestational age at determination of fetal death as a cut-off for 'onset of anemia', we selected pregnancies with a more precisely known moment of onset of anemia;
- By early IVIg treatment: exclusion of the pregnancies in which IVIg was started after 13 completed weeks.
- D antibodies only: all Kell pregnancies were excluded;
- Every woman included once: to evaluate whether the inclusion of multiple pregnancies for some women influenced the results, we repeated the analysis with

every woman included just once, with the pregnancy that was most comparable to the other treatment group;
- No plasmapheresis performed: all patients that received both plasmapheresis and IVIg were excluded;
- By early previous anemia: as alloimmunization in patients with a previous fetal or neonatal death at later gestation might be different from patients requiring IUT before 24 weeks, the analysis was repeated with only patients in which anemia occurred before 24 weeks' gestation in the previous pregnancy.

As hydrops could also occur as a result of late referral, we performed an additional sensitivity analysis with only patients that were monitored with MCA Doppler in the participating center at least twice before IUT was performed.

Table S1. Sensitivity analyses

	Unadjusted				Propensity analysis			
	IVIg group	**Non-IVIg group**	**EMD (95% CI)**	***P***	**IVIg group**	**Non-IVIg group**	**EMD (95% CI)**	***P***
By center	N=24	N=16			N=24	N=16		
Delta GA[a]	15 (0-31)	-11(-23-2)	26 (6-47)	.011	1 (-11-13)	-9 (-21-4)	10 (-8-27)	.274
Certain GA only	N=20	N=20			N=20	N=18		
Delta GA[a]	25 (10-40)	-6 (-16-4)	31 (13-49)	.001	-2 (-11-8)	-4 (-14-5)	3 (-11-16)	.700
By early IVIg treatment	N=13	N=28			N=13	N=25		
Delta GA[a]	25 (10-41)	-9 (-19-1)	34 (15-52)	<.001	-1 (-29-26)	-6 (-17-5)	5 (-25-34)	.746
D antibodies only	N=19	N=19			N=19	N=17		
Delta GA[a]	18 (0-36)	-14 (-24- -4)	32 (11-52)	.003	2 (-11-14)	-9 (-19-1)	10 (-6-26)	.202
Every woman included once	N=24	N=22			N=24	N=20		
Delta GA[a]	15 (0-31)	-6 (-18-6)	21 (1-41)	.036	3 (-9-16)	-1 (-10-7)	4 (-11-19)	.573
No plasmapheresis performed	N=16	N=25			N=16	N=23		
Delta GA[a]	13 (-3-29)	-10 (-20-0)	23 (3-42)	.021	-2 (-11-8)	-6 (-16-5)	4 (-11-18)	.587
By early previous anemia	N=21	N=21			N=21	N=21		
Delta GA[a]	21 (4-37)	3 (-5-12)	17 (-1-36)	.063	9 (-9-27)	8 (-4-20)	2 (-20-23)	.879
By timely referral	N=21	N=18			N=21	N=16		
Hydrops	0 (0)	4 (22)	-[b]	.037[b]	0 (0)	7 (41)	-[b]	<.001[b]

Data presented in estimated mean (95% CI). Abbreviation: *EMD*, estimated mean difference; *N*, number of index pregnancies; *GA*, gestational age; *IVIg*, intravenous immunoglobulins.

[a]Difference in gestational age at onset of severe anemia between index and previous pregnancy.

[b]Due to zero counts, an odds ratio was not estimable and comparing proportions with a generalized estimated equation was not possible. A (weighted) Fisher exact test was used for this comparison.

CHAPTER 7

IMMUNOGLOBULIN FOR ALLOIMMUNE HEMOLYTIC DISEASE IN NEONATES

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Cochrane Database of Systematic Reviews, March 2018; 3.

ABSTRACT

Background

Exchange transfusion and phototherapy have traditionally been used to treat jaundice and avoid the associated neurological complications. Because of the risks and burdens of exchange transfusion, intravenous immunoglobulin (IVIg) has been suggested as an alternative therapy for alloimmune hemolytic disease of the newborn (HDN) to reduce the need for exchange transfusion.

Objectives

To assess the effect and complications of IVIg in newborn infants with alloimmune HDN on the need for and number of exchange transfusions.

Search methods

We performed electronic searches of CENTRAL, PubMed, Embase (Ovid), Web of Science, CINAHL (EBSCOhost), Academic Search Premier, and the trial registers ClinicalTrials.gov and controlled-trials.com in May 2017. We also searched reference lists of included and excluded trials and relevant reviews for further relevant studies.

Selection criteria

We considered all randomized and quasi-randomized controlled trials of IVIg in the treatment of alloimmune HDN. Trials must have used predefined criteria for the use of IVIg and exchange transfusion therapy to be included.

Data collection and analysis

We used the standard methods of Cochrane and its Neonatal Review Group. We assessed studies for inclusion and two review authors independently assessed quality and extracted data. We discussed any differences of opinion to reach consensus. We contacted investigators for additional or missing information. We calculated risk ratio (RR), risk difference (RD) and number needed to treat for an additional beneficial outcome (NNTB) for categorical outcomes. We calculated mean difference (MD) for continuous variables. We used GRADE criteria to assess the risk of bias for major outcomes and to summarize the level of evidence.

Main results

Nine studies with 658 infants fulfilled the inclusion criteria. Term and preterm infants with Rh or ABO (or both) incompatibility were included. The use of exchange transfusion decreased significantly in the immunoglobulin treated group (typical RR 0.35, 95% CI 0.25 to 0.49; typical RD -0.22, 95% CI -0.27 to -0.16; NNTB 5). The mean number of exchange transfusions per infant was also significantly lower in the immunoglobulin treated group (MD -0.34, 95% CI -0.50 to -0.17). However, sensitivity analysis by risk of bias showed that in the only two studies in which the treatment was masked by use of a placebo and outcome assessment was blinded, the results differed; there was no difference in the need for exchange transfusions (RR 0.98, 95% CI 0.48 to 1.98) or number of exchange transfusions (MD -0.04, 95% CI -0.18 to 0.10). Two studies assessed long-term outcomes and found no cases of kernicterus, deafness or cerebral palsy.

Authors' conclusions

Although overall results show a significant reduction in the need for exchange transfusion in infants treated with IVIg, the applicability of the results is limited because of low to very low quality of evidence. Furthermore, the two studies at lowest risk of bias show no benefit of IVIg in reducing the need for and number of exchange transfusions. Based on these results, we have insufficient confidence in the effect estimate for benefit of IVIg to make even a weak recommendation for the use of IVIg for the treatment of alloimmune HDN. Further studies are needed before the use of IVIg for the treatment of alloimmune HDN can be recommended, and should include blinding of the intervention by use of a placebo as well as sufficient sample size to assess the potential for serious adverse effects.

PLAIN LANGUAGE SUMMARY

Review question

Is IVIg effective in reducing the need for exchange transfusion in newborns with alloimmune hemolytic disease of the newborn (HDN)?

Background

In alloimmune HDN, maternal antibodies (circulating proteins that are produced by the immune system in response to the presence of a foreign substance) are produced against fetal blood cells. These antibodies are transferred across the placenta and destroy red blood cells, leading to fetal anemia (deficiency of red cells in the unborn baby). Intrauterine (within the womb) blood transfusion is used to treat severe fetal anemia. After birth, the antibodies persist in the infant and cause hyperbilirubinemia (a raised blood level of an orange-yellow pigment (bilirubin, a waste product of a degrading red blood cell)) with the risk of serious brain damage (kernicterus) and anemia. Traditional treatment of hyperbilirubinemia consists of (intensive) phototherapy (light treatment) and exchange transfusion (where the baby's blood is replaced with that of a donor; ET). Because ET is an invasive, high risk procedure, alternative treatments such as intravenous immunoglobulin (IVIg), have been investigated. IVIg is thought to reduce the rate of hemolysis and consequently the need for ETs.

Study characteristics

We searched the medical literature to 19 May 2017 and found nine randomized (clinical studies where people are randomly put into one of two or more treatment groups) or partly (quasi) randomized trials (including 658 participants) that assessed the efficiency of IVIg in infants with alloimmune HDN.

Key results

Analysis of all included studies showed a reduction in the need for and number of ETs in infants treated with IVIg combined with phototherapy compared to infants treated with phototherapy only. However, this was not confirmed in an analysis of the two placebo-controlled studies (where a pretend treatment was given). There was no difference in the need for or number of top-up transfusions.

Quality of evidence

The evidence from the studies was very low quality. However, two studies used a placebo, thereby minimizing bias and allowing blinding of the researchers assessing the response. These studies were consistent with each other and yielded moderate quality evidence (with a relatively small total number of participants involved (172) being the only reason to not regard the level of evidence from them as high) that IVIg was ineffective in preventing ET or top-up transfusions.

Conclusion

Based on all included studies, we could make no conclusions on the benefit of IVIg in preventing ET or top-up transfusion. However, the two placebo-controlled trials provided evidence of moderate quality that IVIg was ineffective in preventing ET or top-up transfusion, and therefore routine use in alloimmune HDN should not be recommended. However, since there was some evidence that IVIg reduced hemolysis (in laboratory studies), future high-quality studies are needed to determine whether IVIg has limited role in some infants with alloimmune HDN.

SUMMARY OF FINDINGS FOR THE MAIN COMPARISON

Intravenous immunoglobulin plus phototherapy compared to phototherapy alone for alloimmune hemolytic disease in neonates
Patient or population: neonates with alloimmune hemolytic disease
Settings: -
Intervention: IVIg + phototherapy
Comparison: phototherapy

				Anticipated absolute effects[a] (95% CI)	
Outcomes	**№ of participants (studies)**	**Quality of the evidence (GRADE)**	**Relative effect (95% CI)**	**Risk with phototherapy alone**	**Risk difference with IVIg + phototherapy**
Use of ET (≥ 1); all studies	658 (9 RCTs)	⊕⊖⊖⊖ **Very low**[1,2,3]	**RR 0.35** (0.25 to 0.49)	Study population 329 per 1000	214 fewer per 1000 (247 fewer to 168 fewer)
Use of ET (≥ 1); placebo-controlled studies	172 (2 RCTs)	⊕⊕⊕⊖ **Moderate**[4]	**RR 0.98** (0.48 to 1.98)	Study population 153 per 1000	3 fewer per 1000 (80 fewer to 150 more)
ETs performed per infant; all studies	658 (9 RCTs)	⊕⊖⊖⊖ **Very low**[1,2,3,5]	-	The mean ETs per infant for all studies was 0	MD 0.34 lower (0.5 lower to 0.17 lower)
ETs performed per infant; placebo-controlled studies	172 (2 RCTs)	⊕⊕⊕⊖ **Moderate**[4,5]	-	The mean ETs per infant for placebo-controlled studies was 0	MD 0.04 lower (0.18 lower to 0.1 higher)
Use of top-up transfusion in 1st week of life; all studies	378 (4 RCTs)	⊕⊕⊖⊖ **Low**[6,7]	**RR 1.05** (0.65 to 1.69)	Study population 130 per 1000	6 more per 1000 (45 fewer to 89 more)
Use of top-up transfusion after 1st week of life; all studies	507 (7 RCTs)	⊕⊖⊖⊖ **Very low**[1,8,9]	**RR 1.16** (0.97 to 1.38)	Study population 219 per 1000	35 more per 1000 (7 fewer to 83 more)
Maximum total serum bilirubin (µmol/L); all studies	451 (6 RCTs)	⊕⊖⊖⊖ **Very low**[10,11,12]	-	The mean maximum serum bilirubin (µmol/L) for all studies was 0	MD 25.39 lower (34.07 lower to 16.7 lower)

[a]**The risk in the intervention group** (and its 95% confidence interval) is based on the assumed risk in the comparison group and the **relative effect** of the intervention (and its 95% CI).
CI: confidence interval; **ET:** exchange transfusion; **IVIg:** intravenous immunoglobulin; **MD:** mean difference; **RCT:** randomized controlled trial; **RR:** risk ratio.
GRADE Working Group grades of evidence
High quality**:** we are very confident that the true effect lies close to that of the estimate of the effect.
Moderate quality**:** we are moderately confident in the effect estimate: the true effect is likely to be close to the estimate of the effect, but there is a possibility that it is substantially different.

Low quality**:** our confidence in the effect estimate is limited: the true effect may be substantially different from the estimate of the effect.
Very low quality**:** we have very little confidence in the effect estimate: the true effect is likely to be substantially different from the estimate of effect.
[1]In three studies, the method of randomization was not stated and there was inadequate concealment of random sequence (selection bias). In seven studies, there was no blinding of personnel (performance bias) and in five studies, no blinding of outcome assessment (detection bias). Among other potential sources of bias were that mean bilirubin levels at study entry were already higher than the threshold for the outcome (ET) in one study, differences between study groups despite randomization (one study), postrandomization withdrawals or cross-over between study groups (two studies) and criteria for ET differing between treatment arms (one study).
[2]Substantial heterogeneity: $X^2 = 34.63$, df = 8 (P = 0.0003), $I^2 = 77\%$.
[3]Four studies did not clearly specify use of intensive phototherapy (which should be a routine intervention for infants at high risk of ET).
[4]Total number of participants in these two trials was low, increasing the risk of possible bias.
[5]Only a few infants needed a second ET.
[6]In one trial, the methods of randomization and allocation concealment were not stated. In two studies, there was no blinding of personnel (performance bias).
[7]Combined studies were underpowered for use of top-up transfusion in 1st week. A total of 378 infants were enrolled in all four trials, and the overall frequency of top-up transfusion was low (13.8%).
[8]Substantial heterogeneity: $X^2 = 15.60$, df = 5 (P = 0.008); $I^2 = 68\%$.
[9]Due to small differences between treatment groups, the combined studies were underpowered for use of top-up transfusion after 1st week.
[10]Four studies used no method of blinding the intervention.
[11]Substantial heterogeneity: $X^2 = 14.82$, df = 5 (P = 0.01); $I^2 = 66\%$.
[12]Peak serum bilirubin in the control group varied 1.86-fold between studies; there was considerably greater variation between studies than between groups within studies.

BACKGROUND

Description of the condition

The use of anti-D immunoglobulin prophylaxis in D-negative women has led to a marked decline in Rh hemolytic disease of the newborn (HDN).[134] Sensitization can occur despite immunoprophylaxis, particularly if it is given too late or in insufficient dose. A proportion of HDN is caused by antibodies to antigens other than D and is, therefore, not preventable with anti-D immunoglobulin. Fetal therapy has significantly improved outcome in Rh sensitized fetuses, but it does not comprehensively prevent need for neonatal treatment (van Kamp 2004). Primary modes of postnatal therapy include phototherapy and exchange transfusion (ET) to reduce risk of mortality and kernicterus. Top- up transfusions are used to treat early and late anemia. In contemporary perinatal centers, 15% to 40% of neonates admitted for Rh or ABO HDN require at least one ET.[174,175]

The safety of ET has been reported for over 50 years. Published mortality rates vary from 0.53% to 4.7% per infant.[178-184] ET-related death is more common in sick or

premature infants than in healthy term infants.[175,178-180] Risks related to ET include adverse cardiorespiratory events; catheter-related complications; those related to the use of blood products; metabolic derangements; and other serious complications such as pulmonary hemorrhage, necrotizing enterocolitis and bowel perforation. In the last two decades, ET-related risks have been reported to be as high as 74%, although the incidence of severe adverse events is approximately 3-10%.[175,181,184,185]. Because improved perinatal care has reduced the need for ET, the complication rate could increase as clinicians become less experienced with the procedure.[175] However, Steiner 2007 reported that over a 21-year period, despite a sharp decline in the number of ETs performed, there was no increase in morbidity and mortality.

Description of the intervention

Intravenous immunoglobulin (IVIg) is an alternative therapy that may be effective in treating alloimmune HDN. In 1987, the first report of successful treatment of late anemia due to E-incompatibility with IVIg was published.[186] Subsequent case reports and case series reported success of IVIg treatment in neonates with both Rh or ABO incompatibility.[187-189] However, Hammerman 1996a found a reduced or no response to IVIg treatment in infants with ABO incompatibility who had early and severe hemolysis. Since the early 1990s, several quasi-randomized or randomized controlled trials on the use of IVIg (including variations on timing of administration and dose) to reduce ET have been published.[173,174,190-207]

The potential benefits of IVIg over ET include that the treatment is less complicated and less labor intensive. In addition, IVIg could allow safe treatment of some infants in less sophisticated neonatal units, or avoid delaying treatment while transferring infants for ET. Comprehensive assessment of IVIg in premature infants, particularly in the treatment of sepsis, has shown that it is safe and well tolerated.[208] It is a well-established therapy for alloimmune thrombocytopenia due to maternal and fetal human platelet antigen incompatibility.[209] The risk of transmission of viral infection is extremely low.[210] Hemolysis and acute renal failure are uncommon complications of IVIg treatment.[211] One study showed an increased incidence of sepsis in premature infants receiving prophylactic IVIg.[212] Since about 2010, several cases of necrotizing enterocolitis in infants with HDN treated with IVIg have been reported.[213-215] Other rare serious adverse effects of IVIg have been described in pediatric and adult cohorts, but not in newborns.[216]

How the intervention might work

IVIg might reduce the rate of hemolysis in alloimmune HDN by nonspecific blockade of Fc-receptors on the macrophages that are thought to mediate the destruction of antibody-coated red cells.[217] Ergaz 1995 demonstrated a decline in carboxyhemoglobin levels in four of five infants treated with IVIg for alloimmune HDN.[218] Hammerman 1996b demonstrated a significant reduction in carboxyhemoglobin levels in 19 of 26 Coombs-positive infants treated with IVIg.[219] Carboxyhemoglobin levels are a sensitive index of hemolysis and hence these studies suggest that immunoglobulin could decrease hemolysis. IVIg is typically formulated in 6% to 12% solutions, so at doses of 0.5 g/kg to 1 g/kg the volume administered is 4 mL/kg to 16 mL/kg. It is possible that this is a sufficient fluid bolus to reduce bilirubin levels modestly through dilution, temporarily slowing their rate of rise and allowing more time for intensive phototherapy to have effect.

Why it is important to do this review

This is an update of a Cochrane Review first published in 2002. Although results of the previous review showed a significant reduction in the need for ET in infants treated with IVIg, the applicability of the results was limited because none of three included studies was at low risk of bias. Nevertheless, American Academy of Pediatrics (AAP) guidelines recommend the administration of 0.5 g/kg to 1 g/kg IVIg in alloimmune HDN if total serum bilirubin (TSB) is rising despite intensive phototherapy or if TSB level is within 34 µmol/L to 51 µmol/L (2 mg/dL to 3 mg/dL) of exchange level.[220] As a result of these guidelines, despite the equivocal conclusions of the previous Cochrane Review, the use of IVIg in alloimmune HDN has become widespread in many countries. However, supplies of IVIg are limited and it does present some hazards. Therefore, use of IVIg should be restricted to treatment of conditions for which it is of confirmed benefit.

OBJECTIVES

To assess the effect and complications of IVIg in newborn infants with alloimmune HDN on the need for and number of exchange transfusions.

METHODS

Criteria for considering studies for this review

Types of studies

All randomized and quasi-randomized controlled trials of IVIg in the treatment of alloimmune HDN.

Types of participants

Neonates with alloimmune HDN due to either Rh (or other red cell antigens) or ABO blood group antibodies with or without any other blood group antibodies.

Types of interventions

IVIg given for treatment of alloimmune HDN versus control (placebo or 'standard care'). Phototherapy, which is widely regarded as a safe and effective standard treatment may have been used in both IVIG and control groups. Early and late IVIg administration were defined (for this review) as IVIg started within (early) or after (late) the first 12 hours of life. Studies must have included predefined criteria for both IVIg and ET therapy.

Types of outcome measures

Primary outcomes

Efficacy:

- use of ET (proportion of infants receiving one or more ETs);
- ETs performed per infant.

Secondary outcomes

Efficacy:

- use of top-up transfusion(s) in first week of life (% of infants);
- number of top-up transfusions performed in first week of life per infant;
- use of top-up transfusion(s) after first week of life (% of infants);
- number of top-up transfusions performed after first week of life per infant;
- maximum TSB (µmol/L (mg/dL));
- duration of phototherapy (days);
- duration of hospitalization (days);
- incidence of sensorineural hearing loss (any severity);
- incidence of kernicterus;
- incidence of cerebral palsy.

Safety:

– neonatal mortality;
– incidence of adverse reactions possibly related to the use of IVIg or ET (statement of adverse events from individual trials only).

Search methods for identification of studies

Electronic searches

We performed electronic searches of the Cochrane Central Register of Controlled Trials (CENTRAL, the Cochrane Library), PubMed, Embase (Ovid), Web of Science, CINAHL (EBSCO- host), Emcare and Academic Search Premier. The subject query was applied in all databases taking into account the terminological differences between these databases. The query consisted of the combination of four subjects: immunoglobulins, alloimmune hemolytic jaundice, newborn infants and randomized controlled trials. Various synonyms and related terms for all subjects were used. Two search strategies were used: the first strategy was limited to randomized trials and systematic reviews, the second strategy included only the subjects immunoglobulins and alloimmune hemolytic disease (and synonyms and related terms for those subjects). The search was performed on 19 May 2017. The bibliographic databases yielded 1565 references in total of which titles and abstracts were screened. The complete search strategy is attached in the appendix "Complete Search Strategy." In addition to database searches, we searched the trial registers ClinicalTrials.gov and controlled-trials.com. We applied no language restrictions.

Searching other resources

We searched the reference lists of all included and excluded trials and relevant reviews for further relevant studies.

Data collection and analysis

We used the standard method of Cochrane and its Neonatal Review Group.

Selection of studies

Two review authors independently screened the titles and abstracts of all references for possible inclusion using predefined criteria for inclusion (see below). We obtained a full-text version of the article if a report appeared to meet inclusion criteria for the review, or if it was not clear based on title and abstract. We resolved any disagreements through discussion with other review authors. The inclusion criteria for this review were:

– randomized and quasi-randomized controlled trials;

- study compared IVIg with any definition of "standard care" plus placebo, or with any definition of "standard care" without placebo;
- study included neonates with alloimmune HDN due to either ABO or Rh blood group antibodies with or without any other blood group antibodies;
- study measured ETs (primary outcome) for each study arm or at least one of the secondary outcomes (see below) (or both) for each study arm;
- study used predefined criteria for both IVIg and ET therapy.

Data extraction and management

Two review authors independently extracted data using a data collection form that was pilot tested before use. We resolved any disagreements through discussion and if necessary with the help of a third review author blinded to trial author, institution and journal of publication. One review author contacted authors of studies that did not report all required data or information. One review author entered data into Review Manager 5, and at least one review author checked them.

Assessment of risk of bias in included studies

Two review authors independently assessed the risk of bias of included studies using the 'Risk of bias' tool as described in the Cochrane Handbook for Systematic Reviews of Interventions.[221] The following items for risk of bias were assessed: random sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome assessment, incomplete outcome data, selective reporting and other sources of bias. Each item was rated as 'low risk of bias', 'unclear risk of bias' or 'high risk of bias.' Any differences of opinion were discussed with a third blinded review author until consensus was reached. For selective reporting, we used the following criteria to rate a study as 'low risk of bias:'

- for studies enrolling neonates with Rh or both Rh and ABO HDN: reporting (in paper or subsequent correspondence) at least one outcome related to each of ET, bilirubin and top-up transfusion, plus adverse effects and hospitalization.
- for studies enrolling only neonates with ABO HDN: reporting (in paper or subsequent correspondence) at least one outcome related to each of ET and bilirubin, plus adverse effects and hospitalization. Top-up transfusion was not considered to be a preferred outcome measure because anemia requiring treatment is an unusual consequence of ABO alloimmune hemolysis;
- study protocols or methods section of papers should not describe an intention to report outcomes that were not subsequently reported in the paper.

Measures of treatment effect

We calculated the risk ratio (RR) and risk difference (RD) for categorical outcomes, such as the incidence of ET. We calculated the mean difference (MD) for continuous variables, such as the maximum bilirubin level. We also calculated the number needed to treat for an additional beneficial outcome (NNTB) to avoid ET, where the assumed control risk was derived from the mean baseline risk from the studies.[222] We presented 95% confidence intervals (CI).

Dealing with missing data

We contacted investigators for missing information about study design, results or both.

Assessment of heterogeneity

We assessed clinical heterogeneity by determining whether clinical characteristics of participants, interventions, outcome measures and timing of outcome measurements were similar for included studies. We assessed statistical heterogeneity using X^2 and I^2 tests. An I^2 statistic of 50% or greater was considered as substantial or considerable heterogeneity according to the Cochrane Handbook for Systematic Reviews of Interventions.[221]

Assessment of reporting biases

We contacted investigators to request missing outcome data when selective reporting bias was suspected based on the criteria described under Assessment of risk of bias in included studies. If the data remained unavailable and the absence was thought to introduce serious bias, the study was excluded.

Data synthesis

We used Review Manager 5 to synthesize the available data (RevMan 2014). Whether we used a fixed-effect model or a random-effects model depended on the level of clinical heterogeneity and the results of the X^2 test and I^2 statistic for heterogeneity.[221] If there was substantial heterogeneity, we used a random-effects model was used and examined the sources of heterogeneity. If there was no substantial statistical heterogeneity, we used a fixed-effect model.

Quality of evidence

We used the GRADE approach, as outlined in the GRADE Handbook,[222] to assess the quality of evidence for the following (clinically relevant) outcomes:

- use of ET (proportion of infants receiving one or more ETs; assessment for all studies and separately for placebo-controlled studies;
- ETs per infant; assessment for all studies and separately for placebo-controlled studies;
- use of top-up transfusion(s) in first week of life (% of infants); all studies;
- use of top-up transfusion(s) after first week of life (% of infants); all studies;
- maximum serum bilirubin; all studies.

Two review authors independently assessed the quality of the evidence for each of the outcomes. We considered evidence from randomized controlled trials as high quality but downgraded the evidence one level for serious (or two levels for very serious) limitations based upon the following: design (risk of bias), consistency across studies, directness of the evidence, precision of estimates and presence of publication bias. We used the GRADEpro Guideline Development Tool to create a 'Summary of findings' Table to report the quality of the evidence.

The GRADE approach results in an assessment of the quality of a body of evidence in one of four grades.

- High: we are very confident that the true effect lies close to that of the estimate of the effect.
- Moderate: we are moderately confident in the effect estimate: the true effect is likely to be close to the estimate of the effect, but there is a possibility that it is substantially different.
- Low: our confidence in the effect estimate is limited: the true effect may be substantially different from the estimate of the effect.
- Very low: we have very little confidence in the effect estimate: the true effect is likely to be substantially different from the estimate of effect.

Subgroup analysis and investigation of heterogeneity

Subgroup analyses were conducted to determine if effects depend on:

- population:
  - Rh incompatibility;
  - gestational age at birth (less than 37 weeks and 37 weeks or greater);
- intervention:
  - early administration of IVIg: start of IVIg 12 hours or less after birth;

- late administration of IVIg: start of IVIg more than 12 hours after birth;
- single versus multiple doses.

As in contemporary care intensive phototherapy is standard care for ABO incompatibility and therefore ETs hardly ever occur in this subgroup nowadays, no subgroup analysis was performed for ABO incompatibility only.

Sensitivity analysis

We conducted a sensitivity analysis based on whether or not the included studies used a placebo and treatment blinding (which had potential to reduce performance bias and detection bias). The two studies that used a placebo were also at low risk of other forms of bias in that they used random sequence generation, allocation concealment, reported complete outcome data for all prespecified outcomes and did not have other apparent risks of bias.

RESULTS

Description of studies

See Characteristics of included studies; Characteristics of excluded studies; and Characteristics of studies awaiting classification tables.

Results of the search

The search conducted up to 19 May 2017 identified 1565 references (see: Appendix 1). After title and abstract screening, the full text of 27 references was screened. After full text screening, nine studies were included in the meta-analysis.[173,174,190-196] Details of the studies are given in the Characteristics of included studies Table. Eleven studies were permanently excluded from this review. Details of these studies are given in the Characteristics of excluded studies Table. We found no additional studies searching reference lists of included and excluded studies and relevant reviews. A flow diagram of the study selection process is presented in Figure 1. No additional studies were included after an additional search for ongoing studies in the trial registers ClinicalTrials.gov and controlled-trials.com.

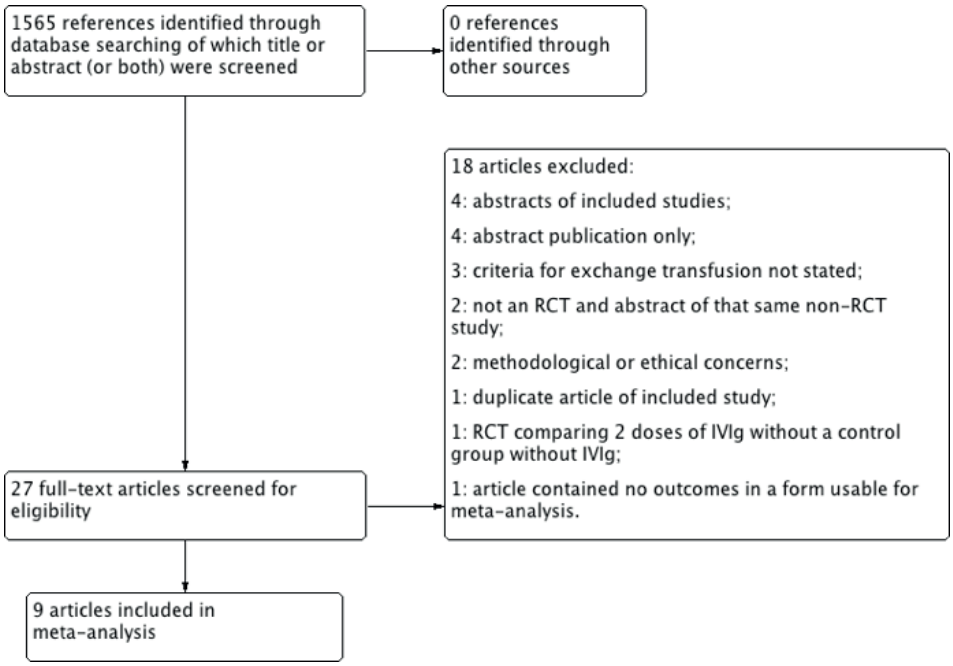


Figure 1. Flow diagram of study selection process. IVIg: intravenous immunoglobulin; RCT: randomized controlled trial.

Included studies

The review included nine randomized controlled trials published between 1992 and 2013.

Participants

The nine studies included 658 participants. Five studies included only infants with Rh incompatibility.[173,174,191,192,195] One study included only infants with ABO incompatibility.[193] Two studies enrolled mostly infants with ABO incompatibility but also some with Rh incompatibility and both ABO and Rh incompatibility.[190,194] Tanyer 2001 included 34 infants with ABO incompatibility, 18 with Rh incompatibility, two with "subgroup" incompatibility and seven with "more than one incompatibilities."[196] Only Nasseri 2006 reported results for each type of incompatibility separately and Alpay 1999 provided this information through correspondence.[190,194] Four studies enrolled only term infants of 37 weeks of gestation or greater.[190,192,194,196] None of the studies only included premature infants of less than 37 weeks of gestation. Rübo 1992 did not describe details of the gestational age at birth of enrolled infants.[195] Santos 2013 and Smits-Wintjens 2011 provided outcomes for term and preterm infants separately.[173,174]

Interventions

Seven of nine studies that met the inclusion criteria examined the effect of a single dose of IVIg in combination with phototherapy.[173,174,190-193,195] One study examined multiple doses,[194] and one study compared groups treated with a single dose or multiple doses with a control group,[196] but was inconsistent in describing which group received a single dose or multiple doses of IVIg and therefore this study was excluded from the (planned) subgroup analysis of single and multiple doses. Two studies used a placebo in addition to phototherapy for the control groups.[173,174] The intensity and topography of phototherapy fits the definition of intensive phototherapy in only three studies.[173,174,192] Tanyer 2001 used an obsolete model with three overhead lights from a single angle and Miqdad 2004 did not use a phototherapy blanket beneath the baby.[193,196] The remainder of included studies did not describe the intensity and topography of phototherapy in sufficient detail to allow a conclusion as to whether it is reasonable to describe it as intensive phototherapy. Five studies started IVIg 12 hours or less after birth,[173,174,191,192,195] and three studies started IVIg more than 12 hours after birth.[190,194,196] Miqdad 2004 started IVIg within 12 hours in nine neonates and more than 12 hours in 47 neonates, but they did not report outcomes for early and late IVIg administration separately.[193]

Since phototherapy was used in both treatment and control groups in all studies and is now considered standard of care in HDN, this review is effectively an analysis of the effectiveness of IVIg plus phototherapy versus phototherapy alone.

Outcomes

All included studies reported ET as the primary outcome. Six studies reported mean (or median) number of ETs per infant[174,194] or supplied enough data to calculate these.[191,192,195,196] The authors of four studies provided unpublished data (standard deviation or mean, or both) for ET.[173,174,190,193] Four studies reported the maximum bilirubin level.[173,174,191,195] Two studies provided unpublished data on maximum bilirubin levels.[190,192] Although all studies commented on the duration of phototherapy in their results, only seven studies reported or subsequently provided the numerical.[173,174,190,192-194,196] These studies all used predefined criteria for commencing phototherapy but not all for ceasing it. Six studies reported or subsequently provided numerical data on the duration of hospitalization.[173,174,190,192-194] Only two studies reported (after correspondence) predefined criteria for hospital discharge.[173,193] Six studies included top-up transfusion as an outcome.[174,190,191,193-195] Three studies provided additional data on top-up transfusions.[173,174,192] Smits-Wintjens 2011 did not report top-up transfusions separately for the first week and after the first week of

life, but subsequently provided this information.[174] Elalfy 2011 had a follow-up period of only one week after discharge.[192] Three studies reported predefined criteria for top-up transfusions,[174,190,194] and one study later provided data through correspondence.[173] All studies reported short-term adverse events. None of the included studies reported data on neurodevelopmental outcomes. Two studies provided additional information on neurodevelopmental outcomes.[173,193]

Excluded studies

We excluded 11 studies. One study only compared groups with a high or a low dose of IVIg,[199] and four studies were only reported in abstract form and our request for additional information was not (sufficiently) answered.[200,202,203,205] Three studies did not report predefined criteria for the primary outcome ET.[198,201,207]. One study did not report any outcome in a usable form for meta-analysis.[206] Two studies were excluded due to methodological or ethical (or both) concerns.[197,204] Details of excluded studies are given in the Characteristics of excluded studies Table.

Additional data

We attempted to contact the authors of all studies (except for the six studies that were identified for the previous review[190,191,195,196,205,206] to request further methodological information and results. We successfully contacted the authors of 11 papers[173,174,190,192,193,195,200,201,204] (including contact for the previous review)) in order to obtain additional data or to assist with the determination to include or exclude the study.

Risk of bias in included studies

For details of risk of bias of included studies, see the Characteristics of included studies Table and Figure 2.

Allocation

Only five studies reported an adequate method of randomization.[173,174,191-193] Miqdad 2004 and Elalfy 2011 provided information on randomization method only through correspondence. One quasi-randomized controlled trial allocated participants by order of admission.[196] This study was rated as high risk of bias for both random sequence generation and allocation concealment. Alpay 1999 did not state what method of randomisation was used either in the paper or in response to a query from the review authors, commenting only that that the group allocation was decided by attending neonatologists who differed from those who were conducting the study, which we

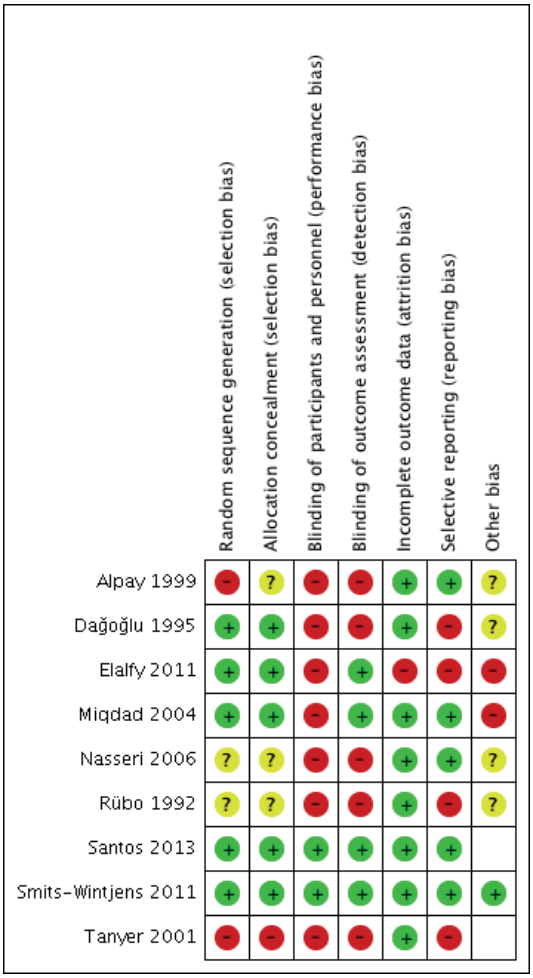


Figure 2. Risk of bias summary: review authors' judgments about each risk of bias item for each included study.

construed to mean that the allocation was not random, and that the allocation was at high risk of bias. Nasseri 2006 and Rübo 1992 stated that babies were randomly assigned to treatment groups but did not provide any detail about the method used and the allocation was therefore considered at unclear risk of bias.

Blinding

Only two studies used a placebo in the control group,[173,174] and were therefore rated as low risk for performance bias and detection bias. After correspondence with the authors of two additional studies, the risk of detection bias was rated as low; Miqdad

2004 explained that data were kept and entered to their database by personnel who were not involved in the management of the cases and Elalfy 2011 explained that the person who performed the randomization was different from the one who conducted the study and the one who analyzed the data. None of the other studies described any method of blinding of intervention after allocation and, therefore, they were rated as high risk of bias on both items.

Incomplete outcome data

Reporting of outcome data was at low risk of bias in seven studies.[173,174,190,191,193-196] For six of these studies, there were no missing data. In Rübo 1992, the amount of and reasons for missing data were similar between groups (low risk). One study was at high risk of bias because of a substantial amount of missing data on bilirubin levels.[192]

Selective reporting

Reporting bias was suspected in four studies because important outcomes were either not reported or were not reported in a form that was useable for meta-analysis, or that allowed judgment about local treatment practices (e.g. if the authors only stated that there was no significant difference between groups).[191,192,195,196] The remaining studies were at low risk of bias.

Other potential sources of bias

Elalfy 2011 had non-random cross-over after randomization and another study used an additional criterion for ET in the control group only.[192,193] These two studies were at high risk of bias. Dagoglu 1995 used post-randomization consent and although follow-up was complete for all infants for whom consent was obtained, four infants (two randomized to each arm of the study) were excluded because consent was withheld. Two infants were also excluded post-randomization in one other study because of "protocol violations" but no details were given.[195] The latter two studies were rated at unclear risk of bias because the review authors were unable to assess the impact of these withdrawals on overall outcomes. Three other studies were rated as unclear risk of bias,[190,194] or low risk of bias[174] for a potential risk of bias. For details see 'Risk of bias' tables.

Effects of interventions

See: Summary of findings for the main comparison (page 130).

Outcome or subgroup title	No. of studies	No. of participants	Statistical method	Effect size
1 Use of exchange transfusion (≥ 1)	9	658	Risk Ratio (M-H, Fixed, 95% CI)	0.35 [0.25, 0.49]
1.1 Studies without a placebo group	7	486	Risk Ratio (M-H, Fixed, 95% CI)	0.26 [0.18, 0.39]
1.2 Placebo-controlled studies	2	172	Risk Ratio (M-H, Fixed, 95% CI)	0.98 [0.48, 1.98]
2 Exchange transfusions per infant, by study quality	9	658	Mean Difference (IV, Random, 95% CI)	-0.34 [-0.50, -0.17]
2.1 Studies without a placebo group	7	486	Mean Difference (IV, Random, 95% CI)	-0.44 [-0.64, -0.25]
2.2 Placebo-controlled studies	2	172	Mean Difference (IV, Random, 95% CI)	-0.04 [-0.18, 0.10]
3 Use of top-up transfusion in 1st week by study quality	4	378	Risk Ratio (M-H, Fixed, 95% CI)	1.05 [0.65, 1.69]
3.1 Studies without a placebo group	2	206	Risk Ratio (M-H, Fixed, 95% CI)	0.71 [0.24, 2.12]
3.2 Placebo-controlled studies	2	172	Risk Ratio (M-H, Fixed, 95% CI)	1.18 [0.70, 2.00]
4 Top-up transfusions in 1st week per infant by study quality	3	262	Mean Difference (IV, Fixed, 95% CI)	0.05 [-0.07, 0.17]
4.1 Studies without a placebo group	1	90	Mean Difference (IV, Fixed, 95% CI)	0.0 [0.0, 0.0]
4.2 Placebo-controlled studies	2	172	Mean Difference (IV, Fixed, 95% CI)	0.05 [-0.07, 0.17]
5 Use of top-up transfusion after 1st week by study quality	7	507	Risk Ratio (M-H, Fixed, 95% CI)	1.16 [0.97, 1.38]
5.1 Studies without a placebo group	5	335	Risk Ratio (M-H, Fixed, 95% CI)	1.40 [1.08, 1.82]
5.2 Placebo-controlled studies	2	172	Risk Ratio (M-H, Fixed, 95% CI)	1.01 [0.80, 1.27]
6 Top-up transfusions after first week per infant, by study quality	4	316	Mean Difference (IV, Fixed, 95% CI)	-0.00 [-0.12, 0.12]
6.1 Studies without a placebo group	2	144	Mean Difference (IV, Fixed, 95% CI)	0.0 [0.0, 0.0]
6.2 Placebo-controlled studies	2	172	Mean Difference (IV, Fixed, 95% CI)	-0.00 [-0.12, 0.12]
7 Maximum total serum bilirubin (µmol/L) by study quality	6	451	Mean Difference (IV, Fixed, 95% CI)	-25.39 [-34.07, -16.70]
7.1 Studies without a placebo group	4	279	Mean Difference (IV, Fixed, 95% CI)	-29.05 [-38.32, -19.78]
7.2 Placebo-controlled studies	2	172	Mean Difference (IV, Fixed, 95% CI)	0.93 [-23.94, 25.79]
8 Duration of phototherapy (days) by study quality	7	585	Mean Difference (IV, Random, 95% CI)	-0.98 [-1.31, -0.66]
8.1 Studies without a placebo group	5	413	Mean Difference (IV, Random, 95% CI)	-1.06 [-1.41, -0.72]
8.2 Placebo-controlled studies	2	172	Mean Difference (IV, Random, 95% CI)	-0.50 [-1.24, 0.24]

Comparison 1. Intravenous immunoglobulin plus phototherapy versus phototherapy.

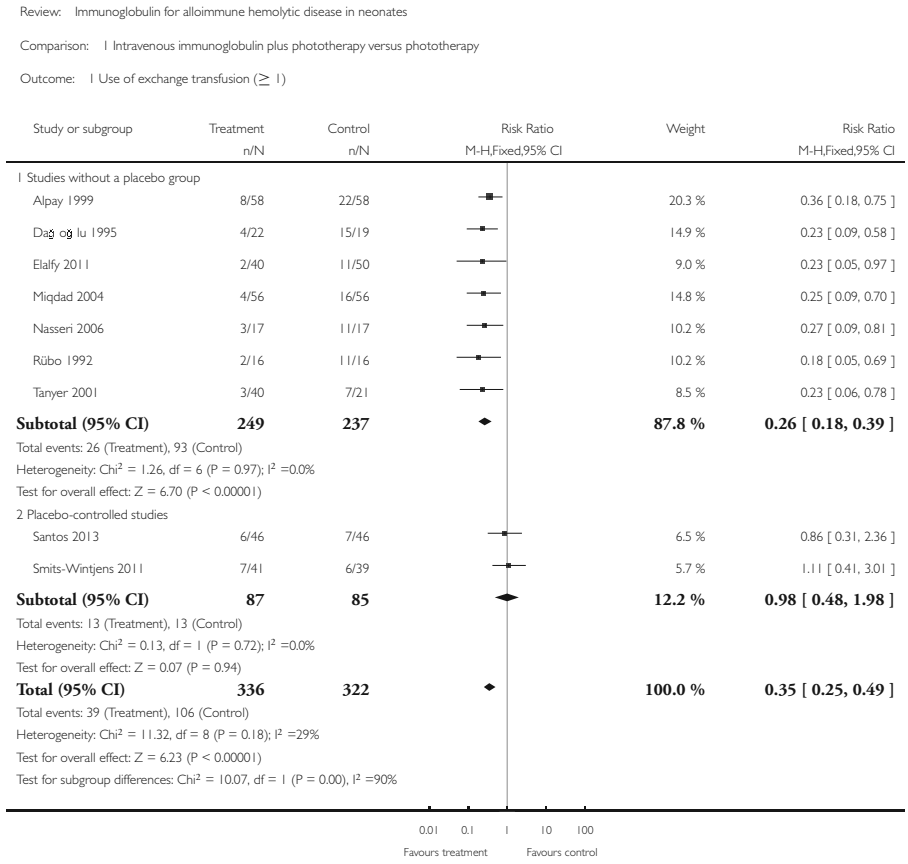
Intravenous immunoglobulin plus phototherapy versus control (phototherapy only)

Primary outcomes

Exchange transfusion

The results of nine included studies could be entered into the meta-analysis.[173,174,190-196] Most studies found a statistically significant reduction in the use of ET for IVIg treated infants.[190-196] Two studies concluded that the use of (one or more) ETs was not reduced despite using early IVIg in combination with phototherapy.[173,174] The meta-analysis of all nine studies (658 participants) showed that IVIg reduced the need for an ET (typical RR

0.35, 95% CI 0.25 to 0.49; typical RD -0.22, 95% CI -0.27 to -0.16; NNTB 5) (Analysis 1.1). However, overall, we rated this as very low quality evidence, because, although it was derived from randomized trials, there was very serious risk of bias in most trials, and moderate heterogeneity and serious indirectness, related to the fact that some trials did not use intensive phototherapy, which would be considered standard practice.



Analysis 1.1. Comparison 1. Intravenous immunoglobulin plus phototherapy versus phototherapy, Outcome 1 Use of exchange transfusion (≥ 1).

Subgroup analysis of infants with only Rh incompatibility supported a reduction in the use of ET with IVIg treatment (371 participants, typical RR 0.38, 95% CI 0.25 to 0.58; NNTB 5) (Analysis 2.1).[173,174,190-192,194,195]

In only those infants born at 37 weeks of gestation or greater, IVIg reduced the use of ETs (391 participants, typical RR 0.39, 95% CI 0.25 to 0.61; NNTB 6) (Analysis 6.1).[173,174,190,192,194,196] In the subgroup of infants born at less than 37 weeks of gestation, IVIg did not reduce the use of ETs (82 participants, typical RR 0.77, 95% CI 0.31 to 1.91; NNTB 20) (data not shown).[173,174]

Five studies found reductions in the use of ET where IVIg was used 12 hours or less after birth (335 participants, typical RR 0.41, 95% CI 0.26 to 0.66; NNTB 6) (Analysis 3.1).[173,174,191,192,195] Reductions were also found in the three studies which used IVIg more than 12 hours after birth (211 participants, typical RR 0.31, 95% CI 0.18 to 0.53; NNTB 4) (data not shown).[190,194,196] Subgroup analyses of infants receiving a single dose of IVIg and infants receiving multiples doses of IVIg supported a reduction in the use of ET with IVIg treatment, although there was insufficient evidence to support a dose-response effect (single dose of IVIg: 563 participants, typical RR 0.37, 95% CI 0.26 to 0.53; NNTB 6; Analysis 4.1;);[173,174,190-193,195] multiple doses of IVIg: 34 participants, RR 0.27, 95% CI 0.09 to 0.81; NNTB 1; Analysis 5.1).[194]

However, despite these apparently promising results, analysis of the only two placebo-controlled studies at low risk of all forms of bias showed no reduction in the use of ET (172 participants, typical RR 0.98, 95% CI 0.48 to 1.98) (Analysis 1.1.2).[173,174]

Furthermore, when all studies were considered, heterogeneity was moderate for use of ET (X^2 = 11.32, degrees of freedom (df) = 8 (P = 0.18); I^2 = 29%) and was high for ETs per infant (Tau^2 = 0.04; Chi^2 = 36.77, df = 8 (P < 0.0001); I^2 = 78%), whereas the results of both these outcomes for the placebo-controlled trials were highly consistent (I^2 = 0% for both). We rated the quality of evidence from the two placebo-controlled studies as moderate, downgrading it only for imprecision because of the low total number of participants.

Overall, immunoglobulin treatment also led to a reduction in the mean number of ETs per infant (658 participants, MD -0.34, 95% CI -0.50 to -0.17). We assessed the level of evidence from the whole group of studies as very low, again downgrading the evidence from randomized trials because of very serious risk of bias, high heterogeneity, indirectness and imprecision. In contrast, analysis of the two placebo-controlled studies were consistent with each other and when considered alone, yielded moderate quality of evidence that IVIg did not reduce the number of ETs (172 participants, MD -0.04, 95% CI -0.18 to 0.10) (Analysis 1.2.2).

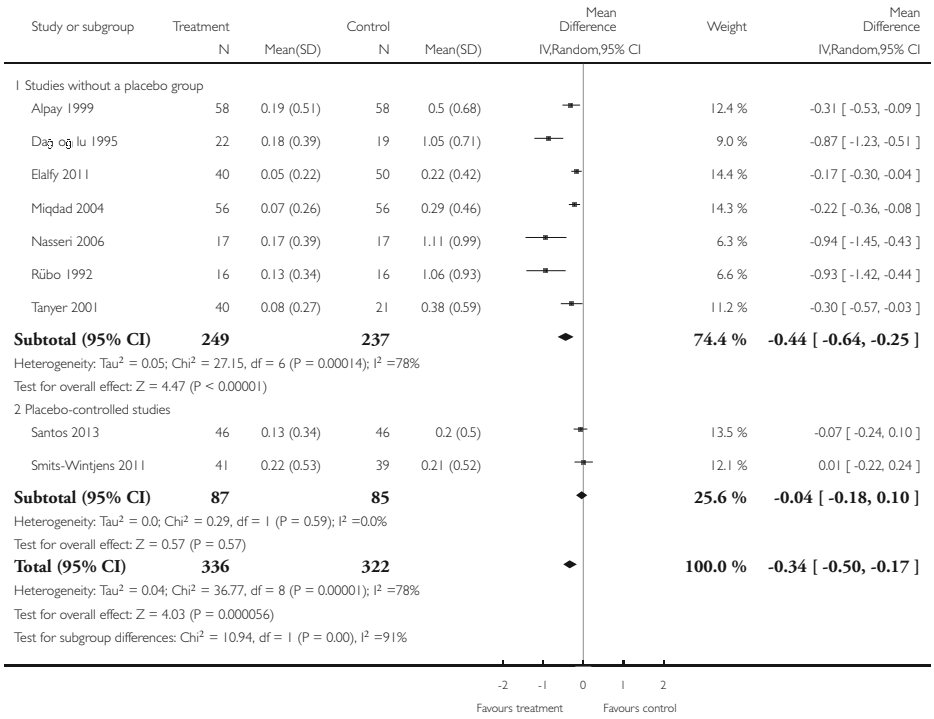
Secondary outcomes

Top-up transfusions during and after the first week

The results of four studies could be entered in the meta-analysis of the use of top-up transfusions in the first week of life[173,174,190,192] and of seven studies for the use of top-up transfusions after the first week of life.[173,174,190,191,193-195] IVIg did not increase the need for top-up transfusions during the first week (378 participants, typical RR 1.05, 95% CI 0.65 to 1.69) (Analysis 1.3) or in the period after the first week (507 participants, typical RR 1.16, 95% CI 0.97 to 1.38) (Analysis 1.5). IVIg also did not increase the need for top-up transfusions in the first week and after the first week of life in the following subgroups: infants with Rh incompatibility only (first week: typical RR 1.08, 95% CI 0.65 to 1.77 (Analysis 2.3); after first week: typical RR 1.09, 95% CI 0.92 to 1.28 (Analysis 2.5)); infants born 37 weeks or more of gestation (first week: typical RR 0.91, 95% CI 0.48 to 1.74 (Analysis 6.3); after first week: typical RR 1.18, 95% CI 0.81 to 1.71 (Analysis 6.5)); infants born less than 37 weeks of gestation (first week: typical RR 1.39, 95% CI 0.70 to 2.73; after first week: typical RR 1.24, 95% CI 0.93 to 1.67 (data not shown)); infants treated with IVIg 12 hours or less after birth (first week: typical RR 1.18, 95% CI 0.70 to 2.00 (Analysis 3.3); after first week: typical RR 1.04, 95% CI 0.89 to 1.22 (Analysis 3.5)); and in infants treated with a single dose of IVIg (first week: typical RR 1.05, 95% CI 0.65 to 1.69 (Analysis 4.3); after first week: typical RR 1.13, 95% CI 0.95 to 1.33 (Analysis 4.5)). Although the need for top-up transfusions during the first week of life was not increased for the subgroup of infants treated with IVIg more than 12 hours after birth (typical RR 0.71, 95% CI 0.24 to 2.12) (data not shown), the need for top-up transfusions after the first week of life was increased with late IVIg treatment (typical RR 8.00, 95% CI 1.03 to 62.26) (data not shown). However, the CIs were very large and the lower CI limit was nearly one. For infants treated with multiple IVIg doses, the use of top-up transfusions after the first week of life was not increased (typical RR 5.65, 95% CI 0.25 to 126.87) (Analysis 5.3) and not estimable for the first week of life.

For the subgroup of infants included in placebo-controlled studies only, at low risk of all forms of bias, the need for top-up transfusions in the first week of life and thereafter was also not altered in infants treated with IVIg (first week: 172 participants, typical RR 1.18, 95% CI 0.70 to 2.00 (Analysis 1.3.2); after first week: typical RR 1.01, 95% CI 0.80 to 1.27 (Analysis 1.5.2)).[173,174]

Smits-Wintjens 2011 and Santos 2013 were the only studies included in the analysis of the number of top-up transfusions per infant. In the first week of life and thereafter, the



Analysis 1.2. Comparison 1. Intravenous immunoglobulin plus phototherapy versus phototherapy, Outcome 2 Exchange transfusions per infant, by study quality.

number of top- up transfusions was not altered in IVIg treated infants (first week: MD 0.05, 95% CI -0.07 to 0.17 (Analysis 1.4); after first week: MD -0.00, 95% CI -0.12 to 0.12 (Analysis 1.6)).

When all studies reporting these outcomes were considered, there was low to very low quality evidence (downgraded for risk of serious to very serious bias, and serious imprecision) that IVIg did not alter the risk of early or late top-up transfusion. These results were consistent with the findings of the placebo-controlled trials.

Maximum total serum bilirubin

Six studies reported results for maximum serum bilirubin. [173,174,190-192,195] The meta-analysis of all six studies showed that the mean maximum serum bilirubin decreased by 25.39

μmol/L in infants receiving IVIg (MD -25.39 μmol/L, 95% CI - 34.07 to -16.70) (Analysis 1.7). Furthermore, subgroup analyses showed that IVIg decreased maximum bilirubin levels in infants with Rh incompatibility, infants of more than 37 weeks of gestation, infants treated early or late, and infants treated with a single dose of IVIg. However, subgroup analyses of the only two placebo- controlled studies[173,174] and of infants born at less than 37 weeks of gestation[173,174] showed that IVIg did not reduce maximum serum bilirubin (placebo-controlled trials: MD 0.93 μmol/L, 95% CI - 23.94 to 25.79 (Analysis 1.7.2); infants born at less than 37 weeks of gestation: MD -18.91 μmol/L, 95% CI -54.49 to 16.68 (data not shown)). The quality of evidence regarding maximum serum bilirubin was very low, with evidence from six randomized controlled trials downgraded for risk of bias and serious inconsistency; (heterogeneity: $X^2 = 14.82$, df = 5 (P = 0.01); $I^2 = 66\%$). Of note, the peak serum bilirubin in the control groups varied nearly two-fold between studies, indicating that there were likely to be very different thresholds for ET between the studies.

Duration of phototherapy

Results of seven studies could be included in the meta-analysis of the duration of phototherapy.[173,174,190,192-194,196] Although all studies gave criteria for commencing phototherapy, only five studies described or provided predefined criteria for ceasing phototherapy.[173,174,190,192,196] Analysis of all seven studies showed that duration of phototherapy decreased by 0.98 days with IVIg treatment (MD -0.98 days, 95% CI -1.31 to -0.66) (Analysis 1.8). All subgroup analyses showed a decrease in duration of phototherapy in IVIg-treated infants varying from a mean decrease of 1.12 days in infants treated with a single dose of IVIg (MD -1.12 days, 95% CI -1.30 to -0.94) (Analysis 4.8) to 1.24 days in infants treated with IVIg 12 hours or less after birth (MD -1.24 days, 95% CI -1.44 to -1.03 (Analysis 3.8)). However, as for maximum bilirubin levels, analyses of the two placebo-controlled studies and of infants born less than 37 weeks of gestation showed no reduction in duration of phototherapy (placebo-controlled trials: MD -0.50 days, 95% CI -1.24 to 0.24 (Analysis 1.8.2); infants born less than 37 weeks of gestation: MD -0.91 days, 95% CI -1.96 to 0.14) (data not shown)).

Duration of hospitalization

Results of six studies could be entered in the meta-analysis.[173,174,190,192-194] None of these studies described predefined criteria for hospital discharge and only two studies provided them through correspondence.[173,193] The analysis showed that IVIg treatment shortened duration of hospitalization by 1.34 days (MD -1.34 days, 95% CI -1.60 to -1.09)

(data not shown). All subgroup analyses showed a shorter duration of hospitalization with IVIg treatment (data not shown).

Incidence of adverse reactions

All studies reported or subsequently provided data on adverse reactions, although for most of the trials, we did not know any details of what protocols were used to identify adverse events or how they were defined. Nine studies reported that there were no adverse reactions of IVIg treatment.[173,174,190-196] None of the adverse reactions were necrotizing enterocolitis. In the study by Alpay 1999, two control infants receiving ET developed hypoglycemia and hypocalcemia after ET. In the study by Rübo 1992, one control infant who required ET developed sepsis and one control infant who required ET developed inspissated bile syndrome. However, the authors stated that a causal relationship with ET could not be established in either infant. In the study by Dagoglu 1995, one control infant developed inspissated bile syndrome. Miqdad 2004 described that "no immediate adverse effects related to IVIg were noted, including fever, allergic reactions, volume overload or hemolysis;" however, they also stated that "ten of the babies who had ET, from both groups, had to be treated for blood culture-positive or clinical sepsis." In the study by Smits-Wintjens 2011 one infant from the IVIg group developed a Bacillus cereus sepsis with brain abscesses a few days after ET. Sterility tests on the used IVIg batches and cultures of all donor blood products used for intrauterine transfer (IUT) and ET were sterile. The sepsis may have been related to the umbilical venous catheterization and ET. A case report provided information on this exceptional case.[174]

Long-term outcomes

Only two studies had a relatively long follow-up period of one year[173] and two years.[193]. In both studies, there were no cases of kernicterus, deafness or cerebral palsy. All participants of the Smits-Wintjens 2011 study were included in a subsequent long-term follow-up study and neurodevelopmental outcome in children of at least two years of age was equal in children treated with IVIg and children treated with placebo.[223] The authors stated that their findings may have been limited by a small sample size.

Neonatal mortality

None of the studies reported neonatal mortality data.

Incidence of adverse reactions possibly related to the use of intravenous immunoglobulin or exchange transfusion

None of the studies reported Incidence of adverse reactions possibly related to the use of IVIg or ET.

DISCUSSION

Summary of main results

Data from nine studies with 658 participants provided limited evidence that IVIg treatment in neonates with alloimmune HDN reduced the need for ET. Although this review update showed a significant reduction in the need for ET, most of the included studies were at high risk of bias. IVIg treatment was also associated with a significant reduction in maximum bilirubin level and duration of phototherapy when all included studies were analyzed and for most of the subgroup analyses based on type of alloimmunization, gestational age at birth, and timing and number of doses of IVIg. Duration of hospitalization was significantly reduced when analyzing all studies that reported this outcome and for almost all subgroup analyses, including the analysis of studies at low risk of bias only. Although there was some evidence that IVIg reduced hemolysis and shortened hospital stay, these results should be interpreted with considerable caution because the studies reporting these benefits were not blinded, only two studies used predefined criteria for hospital discharge, and criteria for stopping phototherapy were not reported in most studies. In addition, since the late 1980s, guidelines for phototherapy have recommended using it more promptly for infants at risk of hemolysis.[224] In many hospitals, the quality of phototherapy has also improved over the years. Nevertheless, the quality/intensity of phototherapy can still vary today, especially in low-resource settings and if good quality control is not applied. The incidence of late top-up transfusions is an important outcome, especially in areas where follow-up of infants is difficult or where supply of safe blood for transfusion is limited. However, as thresholds for top-up transfusions in neonates vary widely, this outcome is susceptible to bias, particularly in unblinded studies. Seven of nine studies were included in the analysis of the incidence of top-up transfusion after the first week of life.[173,174,190,191,193-195] However, only five of the seven studies used predefined criteria for top-up transfusions.[173,174,190,193,194] In addition, the predefined criteria varied between studies, thus conclusions were limited. Data on adverse events of IVIg seemed to indicate that it can be used safely. Although we found reports of a higher incidence

of NEC in infants with HDN treated with IVIg in the literature,[213-215] there were no cases of NEC in the current meta-analysis.

Importantly however, subgroup analysis of the only two studies that were placebo-controlled, blinded, at low risk of all forms of bias, including 172 participants, were very consistent with each other and showed that IVIg treatment had no effect on the need for ET or the number of ETs per infant.[173,174] As for ET, analysis of these two studies at low risk of bias demonstrated no difference in maximum bilirubin level and duration of phototherapy.

Overall completeness and applicability of evidence

This review included all (quasi-) randomized controlled trials on the use of IVIg in alloimmune HDN. We identified 27 trials, of which nine trials, comprising 658 infants, fulfilled inclusion criteria for the review. The only two included studies that were placebo-controlled comprising a total of 172 infants, enrolled only infants with Rh HDN and the intervention consisted of a single dose of 0.5 g/kg to 0.75 g/kg IVIg administered within four to six hours after birth.[173,174] Santos 2013 included infants of 32 gestational weeks or greater and Smits-Wintjens 2011 included infants of 35 gestational weeks or greater. Criteria for phototherapy and ET were similar in both studies. Evidence from subgroup analysis of these two studies with 172 participants showed that early administration of IVIg in a single dose of 0.5 g/kg to 0.75 g/kg did not reduce ETs or had other benefits in the treatment of Rh HDN. There was no clear evidence from this review that a higher dose improved efficacy. The only randomized controlled trial comparing the effect of two doses of IVIg in Rh HDN showed that 0.5 g/kg and 1 g/kg had a similar effect on the duration of phototherapy, duration of hospitalization and ET requirements.[199] However, this study was not powered to detect a difference in the need for ET. Only two studies examined long-term neurodevelopmental outcome, which found no cases of kernicterus, deafness or cerebral palsy in a follow-up period of one year[173] and two years.[193] All participants of the Smits-Wintjens 2011 study were included in a subsequent long-term follow-up study and neurodevelopmental outcome in children of at least two years of age was equal in children treated with IVIg and children treated with placebo.[223]

American Academy of Pediatrics guidelines of 2004 recommend the administration of 0.5 g/kg to 1 g/kg IVIg in alloimmune HDN if TSB is rising despite intensive phototherapy or if TSB level is within 34 µmol/L to 51 µmol/L (2 to 3 mg/dL) of exchange level.[220] Based on the results of this review and because IVIg administration is not completely without

risks,[211-213] and supplies of IVIg are limited, we do not recommend routine use of IVIg. However, since there is some evidence that it reduces hemolysis and it appears safe in infants with alloimmune HDN, it might be reasonable to consider using it in special circumstances, such as during transfer of an infant to a location that can perform an ET, where the risk of ET is considered to be much higher than usual, such as in very or extremely low birth weight infants, or in the context of a future research study.

Quality of the evidence

The quality of included studies ranged from fulfilling none of the 'risk of bias' criteria to fulfilling all criteria (see 'Risk of bias' section of Characteristics of included studies Table, and Summary of findings for the main comparison). Only two of nine trials ful-filled all criteria to be rated as high-quality studies. We made the decision to evaluate the quality of evidence using GRADE criteria separately for the seven studies at high risk of bias and the two studies at low risk of bias, because evaluation of the seven studies at high risk of bias as a group also demonstrated other concerns including inconsistency and indirectness. For the outcomes of use and number of ETs in the first week, the quality of evidence from the seven studies at high risk of bias was very low, whereas the evidence from the two studies at low risk of bias was moderate (down- graded only for small number of participants). For the outcome of top-up transfusions after the first week, we evaluated the level of evidence only for Rh HDN and only for the two studies at low risk of bias, because we deemed this outcome to usually be irrelevant for infants with ABO incompatibility (who are at much lower risk of late anemia) and because of incomplete reporting of data in other studies. The evidence was of very low quality. Analysis of the effect of IVIg on the need for ET in infants with ABO incompatibility included only three studies at high risk of bias,[190,193,194] because other studies only enrolled infants with Rh HDN, did not use predefined criteria for top-up transfusion or did not provide sufficient detail to separate Rh- and ABO-affected infants. The quality of evidence for IVIg for ABO incompatibility was very low (GRADE analysis not shown). For several of the secondary outcomes of the review, the RR (or other relevant statistic) was not estimable for included studies (no events in either intervention or control groups), highlighting the extent to which these studies were seriously underpowered. In summary, we considered that the evidence from the two trials at low risk of bias provided a sufficient quality of evidence to guide practice.

It was unclear why placebo-controlled, high-quality trials yielded such different results. Possibilities included that when administration of IVIg was not compared with use of a

placebo administered in similar dose and over similar duration, there were differences in timing of the next bilirubin measurement, meaning that in IVIg-treated infants, there was longer exposure to phototherapy before the decision about ET was made. Another possibility was that there was bias in the decision to perform an ET, influenced by knowledge of group allocation. A third possibility was that rather than a specific immunomodulatory effect, IVIg (and where used, the placebo solution) has a sufficient non-specific dilutional effect to change the rate of rise of bilirubin, altering duration of exposure to phototherapy and decision making about ET.

Potential biases in the review process

We tried to minimize bias by working with two review authors who independently assessed eligibility for inclusion of trials, extracted data and assessed risk of bias. However, we were aware that these parts of the review process were based on personal judgment because reviewing research is influenced by prior beliefs. In addition, one included trial was performed by two of the five review authors. Nevertheless, we attempted to review all studies in a similar way. In addition, we were unable to contact authors of all potentially eligible studies and, therefore, we could not include all available data. While the translator of the Turkish included study was a medical doctor from Turkish parents, he may have missed some details regarding the risk of bias of that study.

Agreements and disagreements with other studies or reviews

The overall findings of this review were consistent with previous systematic reviews. Louis 2014 included 12 studies (813 participants) and concurred with our finding that high-quality studies found no effect of IVIg, whereas low-quality studies found IVIg effective in HDN. Gottstein 2003 included three studies that were also included in our review,[190,191,195] and one study that was excluded from our review.[206] They concluded that with IVIg treatment significantly fewer infants required ET. Duration of hospitalization and phototherapy were also significantly reduced in their review. However, based on our judgment, none of their included studies was of high quality. Two Chinese systematic reviews[225,226] also found a reduction in ET requirements, duration of phototherapy and hospitalization but concluded that well-designed trials with a larger sample size were required for further evaluation of the efficacy and safety of IVIg. Until the date we conducted our search, our review was the most recent, extensive and up-to-date review of all randomized and quasi-randomized trials on the effect of IVIg in alloimmune HDN.

AUTHORS' CONCLUSIONS

Implications for practice

Based on the overall outcomes of the review, there is insufficient evidence to conclude that intravenous immunoglobulin (IVIg) is beneficial in neonates with hemolytic disease of the newborn (HDN). We gave particular weight to the results of the only two studies that provide evidence at sufficient low risk of bias to guide routine clinical practice, and that show no reduction in the use of exchange transfusion (ET), or improvement in any other important outcomes of the review. In addition, IVIg has risks that have been identified in other contexts of treatment. Therefore, we believe routine use of IVIg for HDN should not be recommended.

The effect of IVIg plus phototherapy compared to phototherapy alone on eventual neurodevelopmental outcomes remains unknown, although there was no difference in neurodevelopmental outcome between these groups in a (small) long-term follow-up study.[223] However, since there is some indirect evidence that IVIg reduces hemolysis and because it appears safe in neonates with alloimmune HDN (acknowledging that the combined sample size of all studies is insufficient to assess uncommon, put potentially serious adverse effects), it may have a limited role in special circumstances, such as where ET is impossible, or is considered particularly high risk. Nevertheless, undertaking preparations for ET, including ensuring earliest possible use of intensive phototherapy, and birth at or transfer to a center that can perform ET, would seem to be strongly indicated in high-risk infants, and should not be abandoned in the expectation that IVIg will be efficacious.

Implications for research

Future research into the role of IVIg in the early treatment of alloimmune HDN may be warranted, especially for infants for whom ET carries particularly high risks. Such a trial should examine the safety and efficacy of IVIg by recording both short-term outcomes such as the need for transfusion therapy and the incidence of adverse events and also long-term neurodevelopmental outcomes. Both ETs and (late) top-up transfusions should be recorded because reduction of ETs can increase the number of top- up transfusions.[227] Consideration should also be given to including additional measures to assess the severity of hemolysis such as carboxyhemoglobin or end tidal carbon monoxide. Based on evidence from the two placebo-controlled trials at low risk of all forms of bias, the conclusion of the review authors is that IVIg is of very limited usefulness

in Rh HDN. However, neither of these placebo-controlled studies enrolled infants with severe established jaundice due to ABO incompatibility. In contrast to Rh incompatibility, ABO incompatibility mainly results in hyperbilirubinemia without significant anemia. This is primarily due to the relatively few group A and B antigenic sites on neonatal red blood cells.[228] Furthermore, infants with ABO- mediated hemolysis often present for neonatal care when they already have severe jaundice. Due to these differences between Rh and ABO incompatibility it is conceivable that IVIg has a different effect in anti-A or anti-B-mediated jaundice. Due to the relative rarity of severe jaundice caused by ABO incompatibility in many countries and the fact that this condition almost always resolves with phototherapy alone,[229] exploring the use of IVIg to treat established jaundice would require a multicenter randomized controlled trial. Based on the discordance of results in this review between trials that were conducted with and without careful blinding of the intervention using a placebo, we recommend that any future trials should either use a placebo or a robust alternative method for blinding of treatment and outcome assessment. Future trials should be well designed and give priority to establishing guidelines for the "conventional" management of alloimmune HDN, focusing on the criteria for performing both top-up and ETs and on the role of intensive phototherapy.

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CHARACTERISTICS OF INCLUDED AND EXCLUDED STUDIES

Available through the Cochrane Database of Systematic Reviews.

DATA AND ANALYSES

The main comparison 'Intravenous immunoglobulin plus phototherapy versus phototherapy' (Comparison 1) and the forest plots of the primary outcome (use of exchange transfusion, Analysis 1.1, and Exchange transfusions per infant, Analysis 1.2) are shown in this chapter. The remainder of the 'Data and Analysis' section is available through the Cochrane Database of Systematic Reviews.

WHAT'S NEW

Available through the Cochrane Database of Systematic Reviews.

HISTORY

Available through the Cochrane Database of Systematic Reviews.

DIFFERENCES BETWEEN PROTOCOL AND REVIEW

In addition to analysis of all included studies, analyses were performed of all placebo-controlled studies. The search method, inclusion criteria and criteria to measure risk of bias were more extensively described in the review than in the protocol.

For the 2017 update, we updated the search and included seven new studies. We updated the background to include contemporary literature. The eligible participants were further specified (from "Neonates with isoimmune hemolytic disease" to "Neonates with alloimmune HDN due to either Rh or ABO blood group antibodies with or without any other blood group antibodies." The primary and secondary outcomes were adjusted to more relevant outcomes in the current era. Previously, the subgroup analysis for timing of IVIg treatment was divided in 'prophylactic use' and 'treatment of established jaundice'. For the current review, this was changed to within 12 hours of life or later. Due to the lack of definitions and the possibility of incomplete reporting in regard to adverse events, the adverse events of individual trials were stated in the current review, rather than combined raw outcomes of all included studies.

A sensitivity analysis for risk of performance or detection bias (or both) was added. Furthermore, we incorporated the GRADE criteria and added a 'Summary of findings' Table for the most important outcomes.

SUMMARY AND DISCUSSION

SUMMARY AND GENERAL DISCUSSION

EPILOGUE

NEDERLANDSE SAMENVATTING

SUMMARY AND GENERAL DISCUSSION

Hemolytic disease of the fetus and newborn (HDFN) is a serious condition that, if remained untreated, may result in fetal hydrops or even death. In the past decades, much has changed in the prevention, screening, monitoring and (intrauterine) treatment of HDFN. Outcome data of pregnancies complicated by even the severest forms of HDFN, at least in specialized tertiary care centers, are considered excellent, and generally regarded to have reached an 'as good as it gets' level. However, even in the best centers, fetal and neonatal death due to HDFN still occurs.

The aim of this thesis was to review and critically analyse current management for this disease and to assess factors that contributed to reaching this point, in order to provide specific tools to further improve outcome.

Chapter 1 provides an overview of the current state-of-the-art setting, technique and outcome of intrauterine blood transfusions (IUTs) for HDFN. Additionally, we summarize the available literature on two alternative (or additional) prenatal non-invasive treatment options: therapeutic plasma exchange and intravenous immunoglobulin treatment. The chapter concludes that IUT is nowadays considered a safe procedure in experienced hands, although complication rates of IUTs performed in early gestation remain relatively high. Maternal treatment with plasma exchange or intravenous immunoglobulins are thought to lower the pathogenic alloantibody levels in mother and child. From several case series these treatments have been postulated to delay the onset of fetal anemia and, hence, to postpone the necessity of early IUTs. To evaluate whether IVIg indeed may improve outcome in these cases, we performed an international cohort study, further described in Chapter 6 of this thesis.

Pathogenesis and prevention

HDFN is caused by maternal antibodies against fetal red cell antigens, usually provoked by fetomaternal hemorrhage during pregnancy or delivery. HDFN is most frequently caused by D (formerly known as RhD) alloantibodies, although alloantibodies with other Rh specificities (c, C, E, e) or non-Rh alloantibodies (K, Fy, Jk) may also induce fetal hemolysis. As a result of several preventive measures, of which anti-D immunoprophylaxis (RhIg) is the most important, both Rh and non-Rh alloimmunization in pregnancies is more and more becoming rare. Interestingly, the working mechanisms of RhIg remain partly unclear to date.

In 2016, 14.6% of the Dutch pregnant population (N=172,414) was D-negative and thus at risk for D antibodies. Only 156 pregnant women, out of this total pregnant population (D-negative and D-positive,) indeed had D antibodies. 94 were new immunizations; the prevalence was thus 0.09%, the incidence 0.05% (rates obtained by cohort study described in chapter 3, unpublished data). The prevalence of all types of RBC alloantibodies in pregnant women, including D, was 0.29-0.41%.[14] Importantly, apart from c-, E- and Kell-matching of red cell transfusions to women of reproductive age, no preventive measures are available to prevent these other immunizations. The pivotal observation in the 1940s that pregnancies of ABO incompatible couples were less often complicated by D immunization, led to a better understanding of the pathogenesis of D immunization and eventually to the development of RhIg as an important preventive measure.[4,131] However, as it is unknown whether this is also the case for non-D immunizations, we addressed in **Chapter 2** both the pathogenesis of these immunizations and the working mechanism of RhIg, by evaluating whether ABO incompatibility and RhIg also prevent the occurrence of non-D antibodies. We identified women with newly detected non-D antibodies, immunized during their first pregnancy and/or delivery. Of these 232 women (cases), 11.9% had a possible ABO incompatible pregnancy, significantly less than the expected 19.4% in the Dutch pregnant population (controls). We furthermore found that the subgroup of 99 women with only non-Rh antibodies had significantly less often received RhIg in their first pregnancies. These findings suggest that both ABO incompatibility and RhIg not only reduce the risk of D immunization, but also of non-D and non-Rh immunizations, respectively, implying that antibody-mediated immune suppression (AMIS) by RhIg is not antigen-specific. In our opinion, future attempts to manufacture the second generation of anti-D immunoprophylaxis, not dependent on the decreasing human donor pool, could focus on a fetal RBC-specific target. By doing so, a universal prophylaxis against the fetal red blood cell, to prevent all types of RBC alloimmunization, might be achievable.

An additional, perhaps more realistic, future perspective on immunoprohylaxis concerns combining the non-invasive prenatal testing (NIPT) for syndromal abnormalities with an extensive maternal and fetal antigen typing in the first trimester. Subsequently, a personalized tailor-made mix of engineered monoclonal antibodies can be administered based on the found incompatibilities. As we found that the AMIS response seems to be not antigen- specific, it might even be redundant to administer a prophylaxis for all incompatibilities separately.

Disease severity

Preventing red blood cell alloimmunization is of utmost importance, as this condition may have serious sequelae. A decade ago, Koelewijn et al. performed a nationwide Dutch cohort study amongst pregnant women with D immunization and found that in primiparae with first detected D antibodies, 19% of children needed an intrauterine transfusion (IUT), a neonatal exchange transfusion, or even died.[5] **In Chapter 3,** we investigated the expert opinion that disease severity increases in subsequent D alloimmunized pregnancies with D-positive children (thus at risk for HDFN), in a new nationwide Dutch cohort study. We found that this was indeed true, as most disease characteristics were more severe in second pregnancies at risk for HDFN, compared to the index (first immunized) pregnancy. For example, second pregnancies at risk showed higher titer and ADCC results, more fetuses treated with IUT, more births induced prematurely, lower neonatal hemoglobin and higher bilirubin levels and more children treated with phototherapy and neonatal red cell transfusions. It seemed that antibodies first detected at 27th week screening ('late') is in fact a sign of strong maternal respondership to a small fetomaternal hemorrhage during pregnancy, rather than a weak response to a previous birth. Although the group in which antibodies were already found at first trimester screening were most often immunized after giving birth to a D-positive child (relatively large hemorrhagic event) and had the entire extend of the pregnancy to develop substantial disease severity, the severity in the index pregnancy was equal to the late immunized group. Furthermore, disease severity did increase only in the group with late antibody formation, also indicating a more active immune response. It is not completely known why women are low or high-responders, although authors have postulated associations with certain HLA-DRB1 types.[154,230] Identification of 'high responders' early in pregnancy in the future might enable the administration of additional early RhIg, before antibody formation can occur. The antibody dependent cell-mediated cytotoxicity (ADCC) test result showed predictive for severe disease: if it did not exceed 10% in the index pregnancy, 94% of second D-positive children at risk did not need intrauterine transfusion(s). Based on the moment of antibody detection, antibody characteristics as reflected by ADCC test results and the disease severity in the index pregnancy, parents can now be properly counselled on the risk of severe disease in a subsequent pregnancy.

Already in the second pregnancy complicated by D immunization, almost a third of patients were in need for antenatal intervention by intrauterine transfusion (IUT). The Leiden University Medical Center has served as the national referral center for

fetal therapy since the introduction of intraperitoneal intrauterine transfusion in the Netherlands in the 1960s. In that era, no routine first trimester antibody screening was performed and patients referred for IUT often presented with (severe) fetal hydrops,[49] a condition associated with poor outcome on both the short and the long term.[31,32] The intraperitoneal IUT was gradually replaced by the ultrasound-guided intravascular IUT from 1987 onwards and routine early screening was implemented in 1998. **Chapter 4** describes how this and other measures have led to the near disappearance of severe alloimmune hydrops in the complete 30-year cohort of 645 fetuses treated with intravascular IUT in the Netherlands. Since routine screening before week 13 was introduced in 1998, fetuses at risk for anemia are identified and treated more timely. This is reflected by the finding that the proportion of fetuses first presenting with hydrops significantly declined from 40 to 16%. Strikingly, during the last six years of the study, only one fetus presented with severe hydrops. Thus, if hydrops occurs nowadays, it is usually mild. As mild hydrops is known not to be associated with impaired outcome,[31] survival of fetuses with and without hydrops is currently almost equal, rising up to or above 95%. This is a major gain in the outcome of fetuses suffering from HDFN in the Dutch setting. We postulate that this improvement is likely the result of a cascade of measures, including: the introduction of early screening for alloantibodies in all pregnancies, use of national guidelines and the availability of both national reference laboratories and the pooling of knowledge and expertise in a single referral center for fetal therapy.

Intrauterine transfusion

Another important step forward in the survival of IUT treated fetuses was to optimize transfusion techniques in order to lower complication rates. In **Chapter 5** we found, in approximately the same cohort as in Chapter 4, that the procedure-related fetal loss rate is currently as low as 1.8%. This rate decreased significantly over time and was relatively low compared to that of most other centers (this thesis, Table 2 of Chapter 1). An important factor probably influencing this decrease is the large number of IUTs performed annually in our center, as a result of the Dutch approach to centralize fetal therapy. Thus all IUTs, a critical and skill-demanding procedure, that are needed in the Dutch pregnant population of approximately 180,000 pregnant women annually, are performed in our national referral center. These large volumes both enable the identification of technical risk factors, such as transfusing into a free loop of cord or refraining from applying fetal paralysis, to optimize the methods used, and the existence of a highly experienced team of operators, sonographers and specialized nurses, to increase safety and efficacy of these complex procedures.[95] Therefore, we advocate

centralization of fetal therapy in larger volume centers to profit from the 24/7 availability of an optimally trained team to consult and refer to and to benefit from up to date knowledge and care.

The safest transfusion techniques, as described in Chapter 5 and other studies, are transfusing into the placental cord insertion or the intrahepatic part of the umbilical vein.[89] In **Chapter 4** an additional advantage of the intrahepatic technique was presented: the possibility to leave an intraperitoneal blood deposit, in order to potentially prolong the inter-procedure interval. If performed at the last intrauterine transfusion, such a deposit was associated with a higher neonatal hemoglobin level.

Our finding that current monitoring and treatment of HDFN affected fetuses has reached a 'as good as it gets' state, does not apply to fetuses in need for early transfusion. It is known, and confirmed in Chapter 5, that transfusions performed before 20-22 weeks' gestation carry a substantially higher risk for adverse outcome.[96-99] Probably due to the technical difficulties of transfusion at this early gestational period, fetal death rate in this group unfortunately did not decrease significantly over time in our cohort. Therefore, evidence based alternative treatment options to postpone or entirely preclude the need for IUT are urgently required.

Alternative treatment options

As summarized in Chapter 1, therapeutic plasma exchange (TPE) and/or intravenous immunoglobulins (IVIg) are frequently proposed as alternatives for early intrauterine transfusion. The current experience mostly concerns case series with promising yet contradicting results, prospective trials performed on this topic are lacking. Probably, this results from the fact that such severe HDFN is very rare, troubling the sample size of single center studies. We therefore performed an international multicenter study among women with a history of severe HDFN. The results of this PETIT study (Postponing Early intrauterine Transfusion with Intravenous immunoglobulin Treatment) are described in **Chapter 6**.

Pregnancies of mothers with a previous first IUT before 24 weeks or a previous HDFN-related fetal or neonatal death that were treated with IVIg in the next pregnancy, were compared to non-IVIg treated mothers with a similar history. As clinical reasons undoubtedly have influenced the choice whether or not to treat these women with IVIg, we aimed to adjust for this 'confounding by indication' by performing propensity analysis.

We found that IVIg treatment was associated with less fetal hydrops and less neonatal exchange transfusions. Furthermore, treatment with IVIg postponed the first IUT with 15 days, compared to that in the previous pregnancy, and with a non-significant adjusted 4 days compared to the non-IVIg group. If IVIg was started early, before 13 weeks' gestation, the effects were more profound. In general, the estimates of all outcomes pointed in the same direction: a possible clinically relevant effect of IVIg on the course and severity of disease in this severely affected group of patients. A prospective, multicenter and preferable randomized trial (RCT) is needed to truly settle the dispute whether IVIg postpones the onset of alloimmune anemia and thus the need for early transfusions. Based on our results however, it is unlikely that IVIg will completely preclude the need for treatment with IUT and therefore other immunomodulatory agents should additionally be evaluated.

The reasons that we advocate performing a randomized trial on the effect of IVIg, although our results already indicate a beneficial effect, are described in **Chapter 7** of this thesis. This chapter contains a Cochrane systematic review on the use of neonatal IVIg to prevent the need for postnatal exchange transfusion. Although IVIg appeared favourable in case series and even in small randomized trials, such a beneficial effect of IVIg administration to the newborn was not found in two high quality randomized and double blinded RCT's. No differences were seen in the need for exchange transfusions or in the number of exchange transfusions per infant in these two studies at low risk of bias. Furthermore, IVIg is expensive and concerns have been raised on a possible correlation between neonatal IVIg treatment and necrotizing enterocolitis. Lastly, the use of high quality intensive phototherapy gradually precludes the need for exchange transfusions in developing countries. The review concludes that there is insufficient evidence of the efficacy of neonatal IVIg for HDFN and therefore the neonatology department of the Leiden University Medical Center advocates against the routine use for this indication.

EPILOGUE

Again, I want to take you back to the 1960s with the quote of professor Jack Bennebroek Gravenhorst, the pioneer of intrauterine transfusions in the Netherlands:

'Hoofdzaak is het bestrijden van de anemie. Bij het voortschrijden van de technische mogelijkheden en door uitgebreidere toepassing van de laatstgenoemde methode zal ongetwijfeld een elegantere methode gevonden worden voor de toediening van het bloed, waardoor bezwaren, die thans ongetwijfeld bestaan, uit de weg geruimd zullen worden.' [35]

In English, this quote would be: *'The main issue is to counter the anemia. The broadening of technical possibilities and more extensive application of the abovementioned method will undoubtedly lead to a more distinguished method for the administration of blood. Hereby the objections, that at present surely exist, will be eliminated.'*[35]

More than 50 years later, times have changed drastically for patients with HDFN. The primary goal (*'hoofdzaak'*) is evolving. It is no longer solely to treat fetal anemia (*'bestrijden van anemie'*), but also preventing it. Preventing red cell alloimmunization with RhIg and matched blood transfusions, preventing the resultant fetal anemia with immunomodulatory agents and preventing hydrops with early antibody screening. Evaluation and adjustment of intrauterine transfusion techniques (*'voortschrijden van de technische mogelijkheden'*) and extensive team experience (*'uitgebreidere toepassing'*) have led to a tremendous reduction in procedure risks. Professor Bennebroek Gravenhorst was certainly right, most of the hesitancies about IUT that were still abundant in the early years, have disappeared. HDFN has become a rare disease, with a reliable standard therapy. The future is exciting: the development of new immunomodulatory therapies might eventually even lead to the complete eradication of invasive intrauterine treatment.

NEDERLANDSE SAMENVATTING

Rode bloedcellen, die zuurstof door het lichaam transporteren, hebben op hun oppervlak bepaalde kenmerken (antigenen) die van persoon tot persoon kunnen verschillen. De meest bekende kenmerken of bloedgroepen zijn A, B en AB. Een ander bloedgroepsysteem is het Rhesus-systeem. Het komt nagenoeg nooit voor dat alle bloedgroepkenmerken van twee personen exact overeenkomen. Wanneer het bloed van iemand zonder een bepaald bloedgroepkenmerk ('antigeen-negatief') in aanraking komt met bloed waarin dit bloedgroepkenmerk wel aanwezig is ('antigeen-positief'), kan de antigeen-negatieve persoon antistoffen maken tegen het onbekende bloedgroepkenmerk. Dit proces heet alloimmunisatie.

Alloimmunisatie kan ontstaan als gevolg van een bloedtransfusie of een orgaantransplantatie, of tijdens een zwangerschap of na de bevalling van een antigeen-positief kind. Het kind draagt in dat geval bloedgroepkenmerken die onbekend zijn voor de moeder, omdat deze geërfd zijn van de vader. De door de moeder gemaakte antistoffen worden actief over de placenta naar het kind getransporteerd en kunnen daar de foetale rode bloedcellen afbreken (hemolyse). Het gevolg is dat de foetus bloedarmoede kan ontwikkelen, een ziekte die bekend staat als hemolytische ziekte van de foetus en pasgeborene (HZFP). Indien de foetus nog te prematuur is om geboren te worden, kan de bloedarmoede intra-uterien worden gecorrigeerd door middel van een intra-uteriene bloedtransfusie (IUT). De meeste gevallen van HZFP hebben echter geen prenatale gevolgen, maar uiten zich na de geboorte door een hoge concentratie van het afbraakproduct van rode bloedcellen, het bilirubine (hyperbilirubinemie ofwel geelzucht) en/of bloedarmoede.

De preventie en behandeling van HZFP heeft in de afgelopen decennia grote ontwikkelingen doorgemaakt. Met kennis en ervaring van nu lijken de immunisatiecijfers en het risico van IUT's in ontwikkelde landen en centra met een ervaren team voor management en behandeling van HZFP tot een minimum beperkt zijn. Dit proefschrift bevat een samenvatting en kritische analyse van de huidige stand van zaken, geeft nieuwe inzichten in de pathogenese en verduidelijkt welke factoren hebben bijgedragen aan het optimaliseren van de zorg voor de foetus en pasgeborene met HZFP.

In **Hoofdstuk 1** van dit proefschrift hebben we het huidige beleid voor de behandeling van zwangere vrouwen met HZFP samengevat en geëvalueerd. Tegenwoordig overleeft

ongeveer 95% van de foetussen die IUT's nodig hebben. Dit geldt helaas niet voor foetussen die al voor 20 tot 22 weken zwangerschapsduur een transfusie moeten ondergaan. In deze groep met vroege en dus zeer ernstige HZFP is het risico op perinatale sterfte verviervoudigd. Het evalueren van non-invasieve therapeutische mogelijkheden om deze risicovolle vroege IUT's uit te stellen is derhalve van levensbelang. De toediening van intraveneuze immunoglobulinen voor dit doel wordt in Hoofdstuk 6 verder beschreven.

Pathogenese en preventie

HZFP is meestal het gevolg van antistoffen tegen het Rhesus-D (RhD) antigeen, maar antistoffen tegen andere Rh-antigenen (RhC, Rhc, RhE, Rhe), anti-Kell, anti-Duffy en anti-Kidd kunnen ook ernstige HZFP veroorzaken. Om RhD-immunisatie te voorkomen, wordt in ontwikkelde landen aan alle vrouwen die zwanger zijn van een RhD-positief kind rond de 30^{e} week en na de bevalling anti-D immunoprofylaxe toegediend (anti-D). Deze immunoprofylaxe is zeer effectief gebleken, het risico op RhD-immunisatie na een bevalling van een D-positief kind is hierdoor afgenomen van 5% naar 0.3%. Voor het voorkomen van andere antistoffen is echter geen immunoprofylaxe beschikbaar. Wel worden bloedtransfusies aan vrouwen in de vruchtbare leeftijd preventief zo uitgekozen dat deze wat betreft de Rhc, RhE en Kell bloedgroepen overeenkomen met die van de vrouw.

In de jaren 60 van de vorige eeuw merkte Levine en collega's op dat RhD-zwangerschapsimmunisatie minder vaak voorkwam bij stellen die ook verschilden in hun ABO bloedgroep (ABO-incompatibel). Dit leidde tot een beter begrip van hoe RhD-immunisaties ontstaan en uiteindelijk tot de ontwikkeling van anti-D profylaxe. In **Hoofdstuk 2** van dit proefschrift hebben we daarom onderzocht of ABO-incompatibiliteit ook beschermt tegen andere immunisaties dan het RhD-type. Bovendien is de beschermende werking van anti-D tegen non-Rh (Kell, Duffy, Kidd, etc.) immunisaties geëvalueerd. We vonden dat in zwangerschappen waarin non-RhD-antistoffen voorkwamen, de ouderparen minder vaak ABO-incompatibel waren dan in de rest van de bevolking. Dit suggereert dat in ABO-incompatibele zwangerschappen, net als RhD-antistoffen, ook non-RhD-antistoffen minder snel ontstaan, wijzend op een beschermende werking van ABO-incompatibiliteit. Ook viel op dat vrouwen met non-Rh antistoffen minder vaak dan verwacht anti-D hadden gehad in een vorige zwangerschap. Deze bevinding impliceert een niet-antigeen specifieke werking van anti-D profylaxe, waardoor anti-D niet alleen beschermt tegen de vorming van RhD-antistoffen, maar

ook van antistoffen van het non-Rh type (bijvoorbeeld Kell, Duffy en Kidd). Deze nieuwe inzichten ondersteunen het ontwikkelen van een universele immunoprofylaxe gericht tegen een antigeen dat alleen voorkomt op de foetale rode bloedcel, waardoor maximale preventie van alloimmunisatie theoretisch haalbaar is.

Ziekte-ernst

Wanneer ondanks bovengenoemde preventieve maatregelen toch alloimmunisatie optreedt, ziet men een grote variatie in ziekte-ernst. Al decennia lang heerst de aanname dat HZFP in een volgende zwangerschap ernstiger zal verlopen dan in de voorafgaande geïmmuniseerde zwangerschap. Deze veronderstelling werd getoetst in **Hoofdstuk 3** van dit proefschrift. Er werd een nationaal cohortonderzoek uitgevoerd, waarin alle zwangere vrouwen met RhD-antistoffen in Nederland werden geïncludeerd. De verzamelde informatie over de huidige en alle voorgaande zwangerschappen van deze geïmmuniseerde vrouwen bracht ons tot de conclusie dat de HZFP inderdaad meestal ernstiger verloopt in een volgende zwangerschap, maar niet altijd. Vooral die vrouwen waarbij de antistoffen zich pas laat in de eerst geïmmuniseerde zwangerschap ontwikkelden, kregen in de volgende zwangerschap vaker een kind met ernstigere HZFP. Dit impliceert dat late antistofvorming juist geassocieerd is met een sterke immuunrespons op een incompatibiliteit in de huidige zwangerschap, terwijl dit eerder werd geduid als een zwakke respons op de foetale bloedgroepantigenen in de vorige zwangerschap. Deze nieuwe hypothese wordt ondersteund door onze bevinding dat de kinderen uit zwangerschappen met late antistofvorming (en dus relatief korte blootstelling) even ziek waren als kinderen uit eerst geïmmuniseerde zwangerschappen waarin al vroeg, en dus gedurende de hele zwangerschap, antistoffen aanwezig waren. Als de groep vrouwen die laat, maar wel nog voor hun routine anti-D profylaxe bij 30 weken, antistoffen aanmaken, in de toekomst al vroeg geïdentificeerd kunnen worden, kan alloimmunisatie mogelijk door een extra vroege anti-D gift worden voorkomen.

In de tweede opvolgende zwangerschap die door HZFP gecompliceerd wordt, heeft bijna een derde van de foetussen een IUT nodig. Deze therapie is in Nederland gecentraliseerd in één gespecialiseerd centrum voor foetale therapie, het Leids Universitair Medisch Centrum (LUMC). In de jaren zestig werden hier voor het eerst IUT's uitgevoerd, toen nog met transfusie in de foetale buikholte. In die periode werd niet routinematig op antistoffen gescreend in de zwangerschap, met het gevolg dat een groot deel van de foetussen ten tijde van hun eerste IUT ernstige anemie hadden, zich uitend in hydrops (vochtophoping). Bij foetale hydrops is de anemie zo ernstig geworden, dat

alle compensatiemechanismen tekort schieten. Het hart faalt en er hoopt vocht op in de foetale buikholte, rond het hart, de longen en/of in de huid. Ingrijpen voor het ontstaan van deze preterminale conditie door middel van IUT is dus van levensbelang. Door de ontwikkeling van echoscopie werd het vanaf 1987 in Nederland mogelijk direct in de foetale bloedvaten (intravasculair) te transfunderen. In 1998 werd een routine antistofscreening voor de 13e zwangerschapsweek voor alle zwangeren ingevoerd, waardoor zwangerschappen 'at risk' voor ernstige HZFP en foetale hydrops nu vroeg kunnen worden geïdentificeerd. **Hoofdstuk 4** beschrijft, in een 30-jarig cohort van 645 foetussen die met intravasculaire IUT zijn behandeld in Nederland, hoe deze screening en andere beleidswijzigingen ertoe hebben geleid dat ernstige hydrops tegenwoordig bijna niet meer voorkomt. Het percentage hydrops (mild en ernstig) ten tijde van de eerste transfusie is aanzienlijk gedaald, van 40% voor de invoering van vroege antistofscreening tot 16% daarna. Opvallend is dat hydrops, als het toch voorkomt, veelal mild verloopt. Deze positieve ontwikkeling heeft ertoe geleid dat foetussen met en zonder hydrops tegenwoordig gelijke overlevingskansen hebben, namelijk beiden meer dan 95%. De afname van hydrops lijkt het gevolg van een cascade van veranderingen: de invoering van vroege antistofscreening, landelijke richtlijnen voor diagnostiek en verwijzing, centralisatie van laboratoriumtesten en foetale therapie en innovatie in neonatale opvang en behandeling.

Intra-uteriene transfusie

Een andere aanzienlijke stap voorwaarts in de overleving van foetussen met HZFP is het verbeteren van de intra-uteriene transfusietechniek, met het doel het complicatierisico te verlagen. In **Hoofdstuk 5** vonden we, in hetzelfde cohort als in Hoofdstuk 4, dat het percentage foetussen dat sterft als gevolg van een IUT tegenwoordig nog maar 1.8% is. Dit percentage heeft een indrukwekkende daling doorgemaakt in de afgelopen jaren, en is relatief laag vergeleken met andere centra voor foetale behandeling (Tabel 2 van Hoofdstuk 1). Het grote aantal procedures dat jaarlijks in het LUMC wordt verricht, als gevolg van het centraliseren van alle foetale therapie in één Nederlands centrum, speelt hierin waarschijnlijk een grote rol. Deze clustering van alle IUT's die nodig zijn voor een populatie van 175.000 zwangere vrouwen per jaar maakt het mogelijk in Hoofdstuk 5 potentiële risicofactoren voor IUT complicaties te identificeren en zo de uitkomsten te optimaliseren. Voorbeelden hiervan zijn transfusies in een losse navelstrenglus en het verrichten van een IUT zonder de foetus eerst een spierverslapper toe te dienen (zodat deze stil blijft liggen tijdens de procedure). Bovendien maakt de centralisatie van deze complexe ingreep het mogelijk om 24/7 een gespecialiseerd team van operateurs,

echoscopisten en gespecialiseerde verpleegkundigen beschikbaar te hebben voor acute zorg. Dit, en het feit dat IUT's door voldoende aantallen jaarlijkse ingrepen per operateur op kwalitatief hoog niveau verricht kunnen worden, zijn argumenten die het pleidooi ondersteunen om wereldwijd de HZFP zorg te bundelen in grote gespecialiseerde centra.

In Hoofdstuk 5 werden tevens de twee veiligste routes voor transfusie in een foetaal bloedvat geïdentificeerd: de insertieplaats van de navelstreng op de placenta en transfusie in een grote ader in de foetale lever (zie Afbeelding 2 van de General Introduction). Een voordeel van de tweede methode is de mogelijkheid om ook bloed achter te laten in de foetale buikholte, met het doel het interval tussen twee transfusies te verlengen. Bovendien blijkt een bloeddepot in de buikholte na de laatste IUT geassocieerd met een hoger neonataal hemoglobinegehalte (Hoofdstuk 4).

De bevinding dat de huidige complicatierisico's zeer laag zijn, mogelijk zelfs zo laag als met de huidige middelen redelijkerwijs mogelijk is, geldt niet voor foetussen die al voor 20-22 weken zwangerschapsduur een IUT nodig hebben. De bekende hogere sterftecijfers na vroege transfusies (Hoofdstuk 1) werden in Hoofdstuk 5 wederom bevestigd en zijn waarschijnlijk te wijten aan de technische moeilijkheden bij de zeer jonge foetus. In deze meest ernstig aangedane groep is het risico op IUT-gerelateerde sterfte in de periode van onze studie dan ook niet significant afgenomen in de tijd, hetgeen de urgentie van onderzoek naar evidence based alternatieve behandelmogelijkheden groot maakt.

Alternatieve behandelopties

Zoals eerder aangegeven in Hoofdstuk 1, is een veelbesproken en frequent onderzochte alternatieve behandelmethode het wekelijks maternaal toedienen van intraveneuze immunoglobulinen (IVIg). De tot heden gepubliceerde resultaten van studies naar IVIg therapie met het doel vroege transfusies te voorkomen zijn veelbelovend, hoewel ook tegenstrijdig. Deze studies zijn vaak retrospectieve verzamelingen van IVIg cases uit één behandelcentrum. Mede door de zeldzaamheid van gevallen van zeer vroege en ernstige HZFP is de studiepopulatie van alle studies veelal te klein is voor het trekken van harde conclusies. Ons antwoord op dit dilemma is de internationale PETIT studie ('Postponing Early intrauterine Transfusion with IVIg Treatment'), uiteengezet in **Hoofdstuk 6**.

In deze studie werden uit 12 gespecialiseerde centra voor foetale therapie alle cases van zeer ernstige HZFP, gedefinieerd als een vorige zwangerschap met HZFP-gerelateerde sterfte of met een IUT voor 24 weken zwangerschapsduur, verzameld. Patiënten met en

zonder IVIg behandeling in de volgende zwangerschap zijn onderling vergeleken door middel van propensity analyse. Dit is een statistische manier om te corrigeren voor de mogelijkheid dat patiënten waarbij voor IVIg is gekozen ernstiger ziek waren dan de patiënten zonder IVIg.

In onze PETIT studie wezen alle uitkomsten in dezelfde richting: een mogelijk voordelig effect van IVIg in patiënten met zeer ernstige HZFP. We vonden significant minder hydrops bij de eerste IUT en minder postnatale wisseltransfusies in de IVIg groep. Vergeleken met de vorige zwangerschap stelde IVIg de eerste IUT met 15 dagen uit, een niet-significant gecorrigeerd verschil van 4 dagen ten opzichte van de groep zonder IVIg. Bij vroege IVIg toediening, voor 13 weken zwangerschapsduur, was het effect duidelijker aanwezig. Om definitief een eind te kunnen maken aan de discussie of IVIg wel of niet de eerste IUT uit kan stellen, is een prospectieve gerandomiseerde multicenter studie nodig. Omdat zelfs in het LUMC, een landelijk verwijscentrum met grote aantallen IUT's, slechts één of twee patiënten per jaar voor een dergelijke studie in aanmerking zouden komen, zou dit een grote logistieke uitdaging zijn. Bovendien lijkt het op basis van onze resultaten onwaarschijnlijk dat IVIg de noodzaak van IUT's compleet kan doen verdwijnen. Om die reden zou ook verder onderzoek naar andere immunomodulatoire therapieën gedaan moeten worden.

In **Hoofdstuk 7** wordt een Cochrane systematische review betreffende de neonatale behandeling met IVIg ter preventie van wisseltransfusies beschreven. Net als prenatale IVIg behandeling, leek neonatale IVIg behandeling veelbelovend in verschillende case series en in kleine gerandomiseerde studies (RCT's). Nadere analyse toonde gebreken in de opzet van deze RCT's, wat de betrouwbaarheid van deze studies negatief beïnvloedt. De enige twee RCT's in onze Cochrane review met laag risico op bias lieten geen positief effect zien van het neonataal toedienen van IVIg op het aantal wisseltransfusies. Deze uitkomst, tezamen met de kosten van IVIg en de mogelijke bijwerkingen, maakt dat de neonatologie afdeling van het Leids Universitair Medisch Centrum tegen het routinematig gebruik van IVIg voor de preventie van wisseltransfusies pleit.

Conclusie

Er is in de afgelopen decennia veel verbeterd in de zorg voor patiënten met HZFP. Aanvankelijk liep men vaak achter de feiten aan: er werd niet gescreend op de aanwezigheid van alloantistoffen in de zwangerschap en als de moeder werd verwezen

in verband met foetale anemie, was er veelal sprake van ernstige hydrops. Ook al werd therapie alsnog gestart, de uitkomst van deze zwangerschappen bleef suboptimaal.

Tegenwoordig ligt de nadruk in de HZFP ketenzorg vooral op preventie. Preventie van het ontstaan van HZFP door het toedienen van anti-D profylaxe aan RhD-negatieve moeders die zwanger of bevallen zijn van een RhD-positief kind. Preventie van hydrops door routinematige antistofscreening vroeg in de zwangerschap, landelijke richtlijnen voor optimale diagnostiek van foetale anemie en tijdige verwijzing naar een gespecialiseerd behandelcentrum. Preventie van complicaties door optimalisatie van de IUT techniek en door toediening van IVIg aan moeders met een voorgeschiedenis van zeer ernstige HZFP. Met behulp van al deze maatregelen is HZFP, ooit de belangrijkste oorzaak van perinatale sterfte, een zeldzame ziekte geworden, met een grote kans op overleving.

APPENDICES

PUBLICATIONS

CURRICULUM VITAE

DANKWOORD

LIST OF ABBREVIATIONS

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Zwiers C, Lindenburg ITM et al. Complications of intrauterine intravascular blood transfusions: lessons learned after 1678 procedures. October 2016. Ultrasound in Obstetrics and Gynaecology.

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CURRICULUM VITAE

Carolien Zwiers werd op 15 februari 1990, op haar vaders verjaardag, thuis geboren in Capelle aan den IJssel. Als jongste van drie kinderen bracht zij haar jeugd door in Schiedam.

Na in 2007 eindexamen te hebben gedaan aan het Stedelijk Gymnasium in Schiedam, begon zij aan de studie Gezondheidswetenschappen aan de Vrije Universiteit in Amsterdam. Gelukkig werd Carolien in 2008 wél ingeloot voor de studie Geneeskunde, tevens aan de Vrije Universiteit. Tijdens een vrijwillige stage in Trujillo (Peru) tussen de bachelor- en masterfase van haar studie ontstond haar passie voor het vak Gynaecologie en Verloskunde.

In 2014 vertrok Carolien naar Zuid-Afrika voor de laatste maanden van haar studie. Haar wetenschappelijke stage naar Tweeling-Transfusie syndroom, uitgevoerd aan de Stellenbosch University onder leiding van professor Lou Pistorius, wekte haar interesse voor Foetale Therapie. Dit maakte dat Carolien, na haar afstuderen in mei 2015, met succes solliciteerde bij professor Dick Oepkes in het Leids Universitair Medisch Centrum, voor een promotietraject naar Hemolytische Ziekte van de Foetus en Pasgeborene. Dit traject werd gefinancierd door Sanquin Bloedvoorziening.

Dit promotietraject werd afgewisseld met een baan als ANIOS Gynaecologie in het Medisch Centrum Haaglanden, locatie Westeinde. Daar werkte zij negen maanden, alvorens zij zich in oktober 2016 weer fulltime aan haar onderzoek wijdde. In die periode presenteerde zij haar resultaten op verschillende internationale congressen. Een presentatie van de PETIT studie, beschreven in dit proefschrift, werd beloond met de Best Oral Presentation award.

Een grote droom werd realiteit toen Carolien in oktober 2017 werd aangenomen voor de opleiding Gynaecologie en Verloskunde aan het Leids Universitair Medisch Centrum (opleider Prof Dr. J.M.M. van Lith). Na het afronden van haar proefschrift startte zij haar opleiding in oktober 2018, in het Haaglanden Medisch Centrum, onder leiding van opleider dr. M.J. Kagie.

Tijdens haar junior coassistentschap Gynaecologie en Verloskunde werd Carolien bij toeval gekoppeld aan Rogier. Inmiddels wonen zij al enkele jaren samen in Amsterdam.

DANKWOORD

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LIST OF ABBREVIATIONS

ADCC - antibody-dependent cell-mediated cytotoxity assay

ET – exchange transfusion

GEE – generalized estimating equations.

HDFN – hemolytic disease of the fetus and newborn

HZFP – hemolytische ziekte van de foetus en pasgeborene

IUFD – intrauterine fetal death

IUT – (intravascular) intrauterine transfusion

IVIg – intravenous immunoglobulins

LUMC – Leiden University Medical Center

MCA-PSV – middle cerebral artery peak systolic velocity

NPR – non procedure-related

PR – procedure-related

RBC – red blood cell

Rh – Rhesus

RhIg – anti-D prophylaxis

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