Improving care for red blood cell alloimmunized pregnant women

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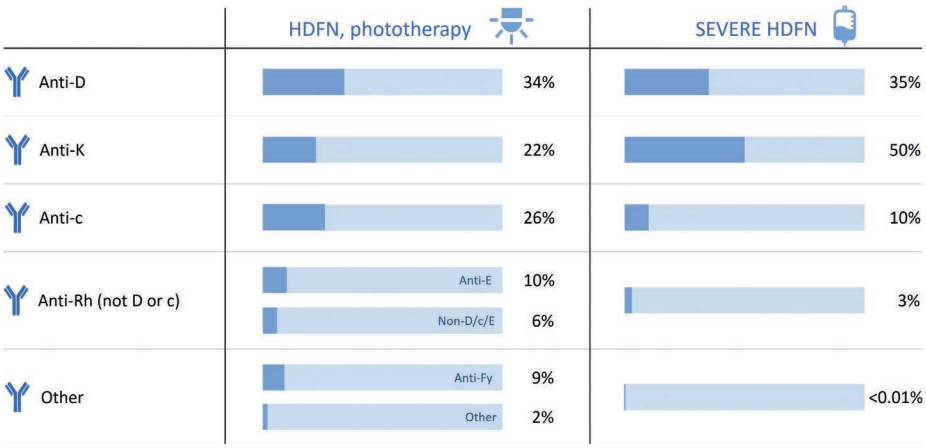
Chapter 1

Development of preventive measures to reduce occurrence of severe hemolytic disease of the fetus and newborn

General introduction

Hemolytic disease of the fetus and newborn (HDFN) is a serious complication in pregnancy, that may be life-threatening for the (unborn) child. (1-9) HDFN is caused by red blood cell (RBC) alloantibodies, developed by the mother and transferred to the fetus. Most severe cases are caused by RhD, Rhc and K antibodies (see figure 1).(1, 3-5, 10) Without treatment, HDFN may result in progressive fetal anemia, fetal hydrops, asphyxia and perinatal death (see figure 2). Even though the incidence of fetal alloimmune hydrops has declined in the last decades, this condition is still a well-known risk for adverse perinatal and long-term outcomes.(9, 11-14) After birth, neonatal hyperbilirubinemia may lead to 'kernicterus', a cause of neurodevelopmental impairment, including athetoid cerebral palsy, hearing problems and psychomotor handicaps.(12, 13, 15, 16) Primary prevention is one cornerstone in the reduction of the incidence of severe HDFN. When, despite prevention, RBC antibodies are present, early detection followed by identification of high-risk pregnancies is the second cornerstone in the prevention of adverse outcome. (17-21) If antibodies with the potency to cause HDFN are found, it is possible to genotype the fetus for the implicated blood group antigens with cell-free fetal DNA derived from maternal plasma.(22-24) If the fetus is (possibly) antigen-positive, the (relative) quantity of antibodies in the plasma and their potential hemolytic activity can be used to select pregnancies with a high risk of HDFN.(10, 18, 19, 25-27) Subsequent intermittent fetal monitoring is aimed to identify fetuses with (imminent) anemia, needing intrauterine transfusions (IUTs) and/or elective (preterm) delivery, possibly followed by phototherapy or (exchange) transfusions.(15, 28, 29)

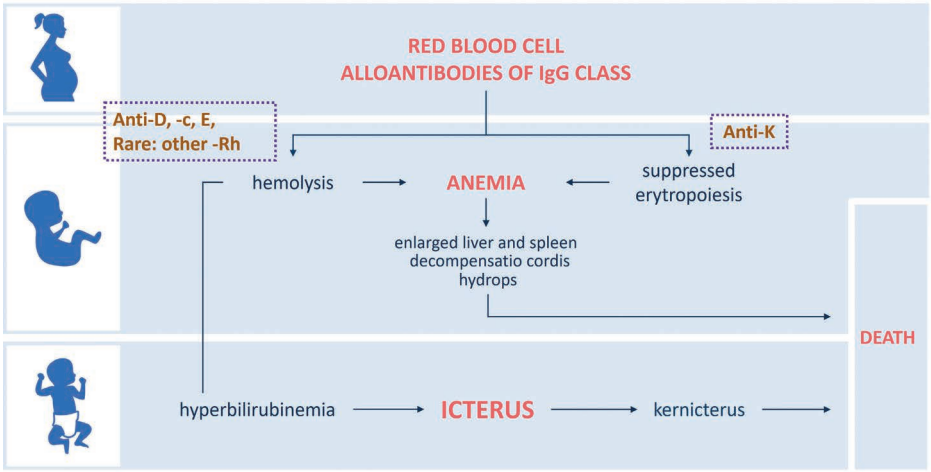
Figure 1: *Type of RBC antibody and course of disease severity.*



Koelewijn et al., Transfusion, 2008; Slootweg et al., AJOG 2018; de Winter et al., unpubished (RhD mild disease)

Primary and secondary preventive measures, such as: prenatal and postnatal RhD immunoglobulin prophylaxis (RhIg), matched blood transfusions for Rhc, RhD, RhE and K antigens to women of fertile age (<45 years) and a routine first trimester prenatal screening for RBC antibodies substantially reduced the risk on maternal alloimmunization. Also improvements in fetal monitoring and therapeutic possibilities have added significantly to a better perinatal outcome of HDFN over the past decades.(25, 30-32) However, even in countries with high standards of fetal and neonatal care, perinatal death due to HDFN still occurs. Therefore, improving the prevention of alloimmunization, the timely identification and referral of pregnancies at risk, as well as the logistic structure around the screening program, remain important keys in effectively reducing HDFN-related perinatal death.(28, 30)

Figure 2: *Pathophysiology of hemolytic disease of the fetus and newborn*



Historical scope of primary preventive measures: RhD immunization

It was already known in the 1960s that fetomaternal incompatibility in ABO blood groups affects the chance of maternal D-alloimmunization.(33) It was postulated that the mother's naturally occurring anti-A or anti-B may bind and destruct fetal RBCs, thus reducing the likelihood of D-immunization to occur in RhD-negative pregnant women with an RhD-positive fetus(34) In 1962, both a British research group led by Prof. Clarke and an American research group (Freda and co-workers) showed that male volunteers were protected against RhD immunization, if anti-D was administered prior to a test transfusion with RhD-positive blood.(35, 36) This so-called 'RhIg prophylaxis' was subsequently tested in England, America, Canada, Germany and the Netherlands, for the prevention of anti-D formation in RhD-negative women after giving birth to an RhD-positive child. The results were promising, as

it was concluded that immunization could thus be effectively prevented in 95% of cases.(33, 37-40)

In 1965, Dr Krijnen of the Central Laboratory of the Blood Transfusion Service of the Dutch Red Cross (later merged into Sanquin) produced the first batch of RhIg from the plasma of D-immunized donors.(41) Clinical studies were conducted using this batch, in order to determine the optimal dose of postnatal RhIg in the Dutch situation. Testing was performed, using different doses of RhIg (120, 180 and 250 µg), administered intramuscularly within 24 hours after delivery.(40) In this study with eighty centers participating, it was concluded that all doses were effective to prevent RhD immunization. Because RhIg was scarcely available, a dose of 200 µg RhIg was chosen. It was concluded that without RhIg prophylaxis, D-immunization occurs in 8% of RhD-negative pregnant women who gave birth to one RhD-positive child, increasing up to 17% after giving birth to two RhD-positive children. However, when using postnatal RhIg prophylaxis, only 1% of the pregnant women showed anti-D, if screened six months after giving birth.

Dr Borst-Eilers has conducted extensive and important research, in the period from 1958 to 1962, into the development and prevention of anti-D formation or “Rhesus Immunization”. Based on this research, in her thesis Dr Borst-Eilers states that, in order to implement an optimal prevention schedule with RhIg, one should first consider the following facts regarding the occurrence of RhD immunization:

1. RhD immunization can already occur before delivery;(40)
2. RhD immunization can occur during uncomplicated pregnancy and childbirth, because of not noticed fetomaternal bleedings.(42) Anti-D formation can be further prevented by performing a Kleihauer test to quantify fetal RBCs and then adjusting the RhIg dose;(43)
3. RhD immunization can occur as a result of loss of pregnancy in the first trimester;
4. A negative Kleihauer Betke test is no guarantee that immunization will not occur. (42)

Also in the current prevention program, using prenatal and postnatal administration of RhIg, these facts will still impact the outcome of this policy. Although the current prevalence of RhD alloimmunization is low (Chapter 2, this thesis),(44) it may well be that the above mentioned risk factors still lead to cases of RhD alloimmunization, preventable if only RhIg administration would be adjusted, for example by quantification of fetal RhD-positive RBCs in blood of the mother.

Developments in the prevention program: anti-D prophylaxis

In 1992, the Dutch Health Council reviewed the prevention program as it was first introduced in 1970, including the administration of RhIg to all RhD-negative women after childbirth. The Health Council concluded that significant health benefits for neonates could be achieved , by expanding the program by including the administration of RhIg prophylaxis during pregnancy.(45-47) Since RhIg is a scarce blood product, the Health Council initially proposed that only women without a living child should be given RhIg during pregnancy, because also antenatal RhIg prophylaxis predominantly contributes to prevention of severe HDFN in a next pregnancy and these women have the highest chance to have a next pregnancy. The committee assumed at that time, that "monoclonal anti-D preparations, prepared with biotechnological techniques and therefore not from the plasma of volunteers, will probably be widely available in a few years' time".(45) In the last decades, a few clinical studies have been performed with monoclonal antibody (MoAb) based RhIg prophylaxis. Still, there is no successful MoAb RhIg largely available.(48-50)

When antenatal RhIg prophylaxis was introduced, there were the following considerations of possible harm: RhIg administered antenatally can also reach the fetal circulation via active transport across the placenta and bind to the fetal red cells.(51) Indeed, after birth anti-D sensitized RBCs are observed, but the level of sensitization is too low to result in fetal hemolysis. Another adverse effect of antenatal administration of RhIg was the theoretical possibility that a very low concentration of RhIg in the mother could actually increase the chance of immunization.(52) Therefore, it was recommended in 1998 that the level should be at least 100 IU during the remaining pregnancy period after administration. In the UK, a low dose of 500 IU is given at two time points in pregnancy, week 28 and week 34, so that this lower limit is achieved over a longer period. For the Dutch situation, it has been calculated that after administration of 1000 IU in week 30, the lower limit of 100 IU is reached at a gestational age of 42 weeks. The Health Council recommended in 1992 that an extra dose of RhIg should be administered to pregnant women in week 42, and that in these cases the postnatal RhIg dose could be omitted.(44) However, this recommendation has never been incorporated into policy. In a nationwide study performed in 2004 in RhD-negative women who had one previous delivery (Parae 1), Koelewijn et al. found that post maturity is a risk factor for RhD immunization. (53) It was postulated that this finding indicated a failure of RhIg prophylaxis at advanced gestational age. It was recommended to administer an extra dose of RhIg when 42 weeks are reached or to split the prior single dose at 30 weeks into two gifts at 28 and 34 weeks. In current obstetrical practice in developed countries post maturity beyond 42 weeks has become rare, as most pregnancies are terminated before or around 41 weeks, for the benefit of fetal outcome.(54) In the group of RhD-immunized women who received complete antenatal and postnatal RhIg prophylaxis,

Koelewijn and coworkers further observed that a relatively young age at first delivery, non-spontaneous delivery (cesarean section or assisted vaginal delivery) and post maturity in the previous pregnancy emerged as independent risk factors for RhD immunization.(53) Koelewijn et al. reported that addition of antenatal RhIg to the RhD immunization prevention program halves the risk of anti-D formation in RhD-negative women and concluded that this was in line with the expected effect.(44)

Although initially, only women without a living child were eligible for administration of antenatal RhIg, in 2008 this was extended to all RhD-negative pregnant women, irrespective of their number of previous pregnancies.

Developments in the prevention program: RBC alloantibody screening.

RhD-negative women are already since the 1960's screened for the presence of anti-D (and other RBC alloantibodies) in the last trimester of pregnancy. This screening was first conducted in week 32 of pregnancy and with the introduction of antenatal RhIg prophylaxis, the blood sample was taken immediately prior to the administration of RhIg in week 30. In 1992, the Health Council stated that screening for RBC alloantibodies should become standard care during pregnancy, because besides anti-D also other type of RBC alloantibodies can cause severe HDFN.(55) An additional advice was that the laboratory testing for RBC alloantibody specificity and RBC alloantibody titers could best be centralized. Firstly, because of the required expertise in the interpretation of the results and secondly for the necessary epidemiological analysis. In 1998, these recommendations of the Health Council led to the introduction of the RBC alloantibody screening for all pregnant women before week 13 of pregnancy to be combined with the screening for infectious diseases, as part of the prevention program for screening infectious diseases and erythrocyte immunization (PSIE), nowadays being conducted by the Center for Population Research (CvB) of the National Institute for Health and Environment (in Dutch RIVM; Rijksinstituut van Volksgezondheid en Milieu). The laboratories of the University of Groningen and that of Sanquin in Amsterdam continued their role as reference laboratories for RBC antibody specification, antibody titration and determination of the hemolytic potential of the RBC alloantibodies with the antibody dependent cellular cytotoxicity assay (ADCC; only in Amsterdam).

Evaluation of RBC alloantibody screening introduced in 1998: the OPZI study

In the period 2004 to 2008, the in 1998 introduced measures (antenatal RhIg in women without living child and general RBC screening) were evaluated by the OPZI

(Investigation and Prevention Pregnancy Immunization) research group. The research team contained a mixture of expertise, with substantial immunohematological laboratory experience (prof. van der Schoot, prof. de Haas, associated with Sanquin), obstetric practical experience (dr. Koelewijn (midwife and researcher)) and epidemiological expertise (prof. Bonsel and dr. Vrijkotte). Evaluation of the effectiveness of antenatal RhIg by the OPZI team is discussed above (see under Developments in the (screening and) prevention program: anti-D prophylaxis). To evaluate the effectiveness of the screening of RBC alloantibodies before week 13 in pregnancy, a study was conducted by the OPZI team based on a nationwide sampled cohort of 400,000 pregnancies. Recommendations of this study were implemented in laboratory monitoring of RBC alloimmunized pregnancies. One of the observations was that anti-M antibodies hardly were of clinical significance and that laboratory follow-up was only necessary if anti-M was of IgG class.(10) Another recommendation was that laboratory monitoring could focus on Rh and K antibodies and that for all other type of RBC alloantibodies one additional test in last trimester would be sufficient to create sufficient awareness of the newborn being at risk to develop HDFN. These recommendations were implemented in the reference laboratories. An important observation was that 25% of severe cases of HDFN in RhD-positive women occurred unexpectedly, after a negative screening result in the first trimester. Some of the children of these unexpected cases suffered from HDFN-related handicaps caused by perinatal asphyxia or kernicterus, because fetal anemia and hyperbilirubinemia were not timely detected.(10) In contrast, all cases of alloimmunization already detected at first trimester screening were treated in a timely manner and children were healthy at the age of 1 year. All first trimester screen-negative cases of severe HDFN were caused by anti-c and/or anti-E. However, long-term sequelae were only found in anti-c cases.

The outcome of the studies performed by the OPZI team and international data formed the basis for a new advice by the Health Council in 2009 (56). The following additional measures were advised:

- Selective screening in primiparous women or women with a previous blood transfusion;
- Selective follow-up, only of women with clinically relevant antibodies (anti-D, -c, -E and -K);
- Rhc-antigen determination at 12 weeks and a second screening of Rhc-negative women around 30 weeks;
- Additional matched transfusion policy in women aged <45 years, concerning Rhc, E and K;
- Implementation of the determination of the fetal RhD-antigen at 27 weeks, followed by RhIg prophylaxis, only to women pregnant of an RhD positive fetus;

After a prospective risk analysis by the RIVM (57), in 2011, the advised selective screening in primiparous women and all women with a previous blood transfusion was rejected by the obstetric care providers because of reasons of impracticability. (56) Screening for RBC alloantibodies at the booking visit in all pregnant women continued as part of the program (see figure 3). The advice for an additional screening of all Rhc-negative women, comprising 18.7% of pregnancies, at week 27 was reviewed and implemented in order to increase the detection rate of severe HDFN with 25% (from 75% to 100%).(10) Also RIVM reviewed the feasibility and costs of fetal RHD typing in week 27 to target RhIg administration in week 30 and after delivery and it was decided by the Minster of Health to implement this measure in 2011.(24) Finally, in 2011, it was advised by the Transfusion Guideline to select Rhc, RhE and K-matched red blood cell units for girls and women if <45 years of age.(32)

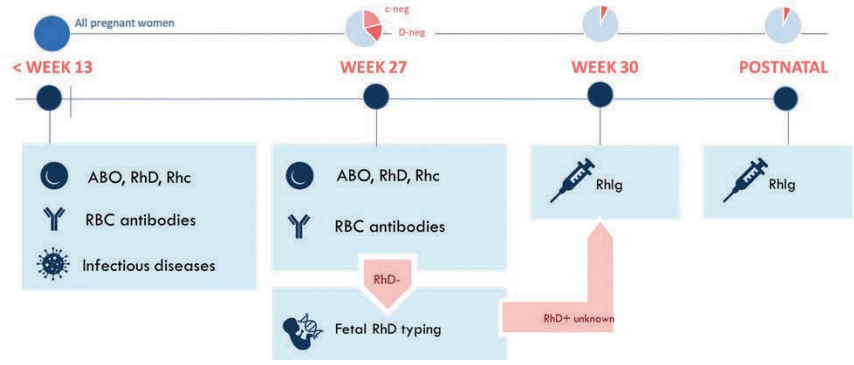


Figure 3: *Prenatal screening Infectious diseases and Erythrocyte immunization, program design since 2011.*

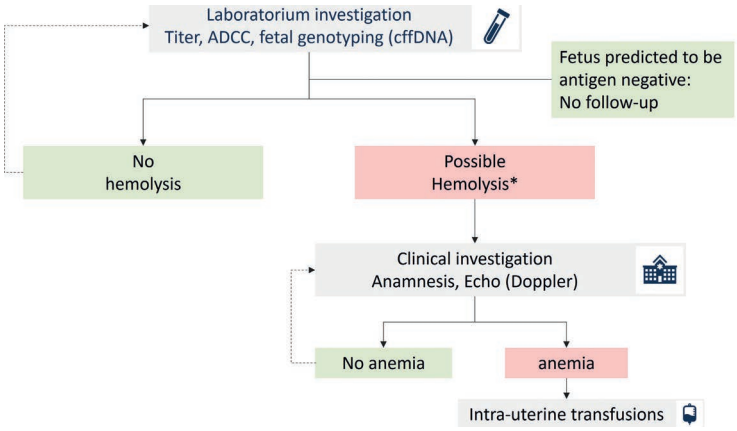
Laboratory monitoring of high-risk HDFN pregnancies

The prevention program enables timely detection of clinically relevant RBC antibodies. When detected, it is necessary to select those pregnancies at risk for severe HDFN (see figure 4). In most centers, to identify pregnancies at risk for severe HDFN, the concentration of RBC antibodies in blood plasma is quasi-quantified by determination of the RBC alloantibody titer. A titer reflects the number of times a plasma sample can be diluted before the RBC alloantibodies cannot be detected anymore, the higher the titer, the higher the concentration of RBC alloantibodies. If their RBC alloantibody titer is above a certain threshold, pregnant women are referred to a maternal-fetal medicine center for close fetal surveillance and, if needed, for fetal or neonatal treatment. In the Netherlands, the reference center is the Leiden University Medical Center. High-risk pregnancies are monitored with ultrasound and Doppler middle

cerebral artery (MCA) peak systolic velocity measurements, to predict the presence of fetal anemia.(18, 29)

In The Netherlands, fetal genotyping for the implicated RBC blood group antigen is performed with cell-free fetal DNA isolated from maternal plasma.(22) Alloimmunized pregnant women with an antigen-positive fetus are monitored by serial testing of the antibody titer and the antibody-dependent cellular cytotoxicity (ADCC) bioassay, a monocyte-based assessment of the destructive capacity of the antibodies.(58) For laboratory management of RhD-immunized pregnant women, Oepkes et al validated the cut-off values for titer and ADCC.(19) The 'critical titer' to identify pregnancies at risk for hemolysis was set on 16 for most (clinically relevant) antibodies in the Netherlands, the ADCC on 50% for anti-D and 30% for non-D antibodies.(26) An ADCC test result lower than 10% means that the pregnant woman can receive obstetric care in her own center without the necessity for referral to a specialized center. For anti-K it was concluded that a titer of 2 should already be regarded as a critical limit. In general, the guidelines in other countries have non-D/non-K alloantibodies cut-off levels of 32. (4, 21, 59, 60) In the Netherlands, pregnancies with anti-D, -c or -K are monitored every 2 weeks and other if Rh antibodies are present (anti-C/-E/-e) monitoring every 3 weeks is advised during the last trimester. In case of other antibodies (anti-Fy^a/-Fy^b, -Jk^a/-Jk^b, -S/-s and other) the laboratory testing is repeated only once in week 30. These intervals are based on the OPZI study.(10, 26, 61)

Figure 4: *Selection of high-risk pregnancies for HDFN after RBC antibody found during the screening in the 1st trimester or at 27 weeks of gestation*



**Hemolysis can be expected with a cut-off for the titer anti-D: ≥ 1:16; K: ≥ 1:2; other: ≥1:16 and a cut-off for the ADCC anti-D: ≥ 50%; K: ≥30%; other: ≥ 30%.*

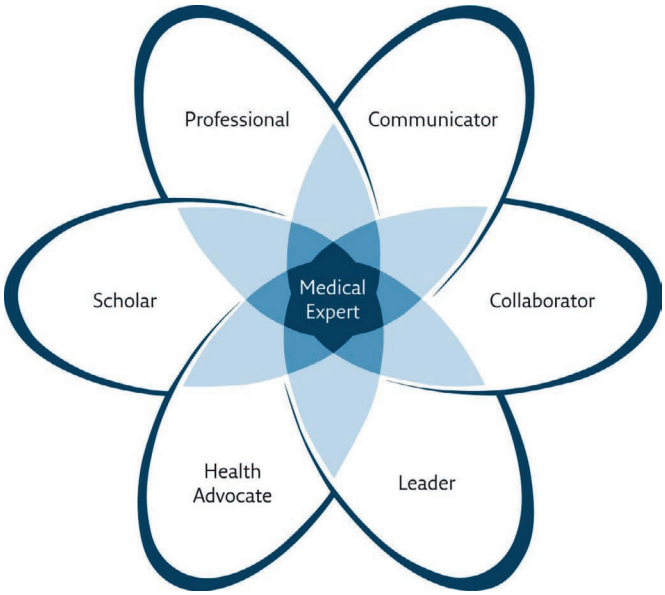
Contextual framework of the thesis

The care to pregnant women and their partners with an unborn child at risk for a rare, but potentially severe disease, such as HDFN, demands various skills from the obstetric care provider. One should not only have the knowledge of the pathology of the disease, of disease risk assessment and required laboratory and clinical tests at various moments in pregnancy, moreover one should inform and counsel the pregnant woman and her partner. Since alloimmunization in pregnancy is rare, most obstetric care providers (primary care midwives and even obstetricians) do not regularly take care of pregnant women with RBC alloantibodies. Every year, the performance of the prevention program is evaluated.(62) Currently, only 0.12-0.22% of all pregnant women in the Netherlands has clinically relevant RBC antibodies at the first trimester screening and 0.22% of RhD and Rhc negative pregnant women has clinically relevant RBC antibodies at 27th week of gestation.

Knowledge on which alloantibodies are more often associated with risks for HDFN may not easily be available. A Dutch questionnaire survey in 2004 showed that 50% to 70% of the women, particularly those with RBC antibodies, indicated that they needed more information, preferable verbally, about the consequences of the RBC alloantibodies for their child.(63) Supportive written information (e.g. folders/hand-outs) was lacking, both prenatally and postnatally.

In Canada, the Royal College of Physicians and Surgeons developed a framework that can be used for training and qualification in all these different professional roles: the so-called Can MEDS roles (Canadian Medical Education Directives for Specialists). Seven different Can Meds roles are discriminated (Figure 5). The Can MEDS framework aims to develop the competences that are of importance to improve the care system, and to provide insight in the current level of performance on the set of competences. This contextual framework may be useful to achieve high-level care to women with pregnancies complicated by the presence of RBC alloantibodies.

Figure 5: *Can Meds (Canadian Medical Education Directives for Specialists) framework for improving patient care.*



Formal adaptation by the Royal College of Physicians and Surgeons of Canada in 1996 and updated in 2005 and 2015.

Aim and outline of the thesis

The aim of this dissertation was to identify gaps in knowledge and room for improvements in certain aspects of the current system of prevention and care regarding RBC alloimmunization in pregnancy. The focus in this thesis was to evaluate the performance of new measures in the prevention program, the follow-up with laboratory monitoring in alloimmunized pregnancies and the counseling of pregnant women and their partners, in cases of RBC alloimmunization.

The need for the RBC alloantibody screening in Rhc-negative women in week 27 of pregnancy has been questioned by health care professionals in the field. Evaluation was planned to judge if the new measure led to improved care for and outcome of the expected number of 6-7 babies at risk for severe anti-c mediated HDFN (**Chapter 3**).

We postulated that the evaluation of risk factors for RhD immunization, as reported by the OPZI study group as published in 2009, created awareness for risk factors for D alloimmunization around delivery. We set out a study to investigate risk factors for D alloimmunization in the current setting with targeted RhIg administration, now based on fetal RHD typing in week 27 of pregnancy. We also planned to review the current rate of D alloimmunization (**Chapter 2**). In addition, a study has been set up to assess how women who are D immunized feel about the option of donating plasma for RhIg production (**Chapter 4**).

To further design a lean protocol for follow up laboratory testing in alloimmunized pregnancies, we conducted studies on laboratory testing in anti-K complicated pregnancies and in pregnancies with other type of RBC alloimmunization (**Chapters 5** and **6**).

Finally, since HDFN is a rare disease, we set out qualitative studies among obstetric care providers and (pregnant) women, with pregnancies at risk for HDFN, to evaluate if and how to improve the moments of contact between care providers and the women and their partners (**Chapters 7** and **8**).

Chapter 2

Risk factors for RhD immunisation in a high coverage prevention program of antenatal and postnatal RhIg: a nationwide cohort study

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Abstract

Objective: To evaluate which risk factors for RhD immunisation remain, despite adequate routine antenatal and postnatal RhIg prophylaxis (1000 IU RhIg) and additional administration of RhIg. The second objective was assessment of the current prevalence of RhD immunisations.

Design: Prospective cohort study.

Setting: The Netherlands.

Population: Two-year nationwide cohort of alloimmunised RhD-negative women.

Methods: RhD-negative women in their first RhD immunised pregnancy were included for risk factor analysis. We compared risk factors for RhD immunisation, occurring either in the previous non-immunised pregnancy or in the index pregnancy, with national population data derived from the Dutch perinatal registration (Perined).

Results: In the two-year cohort, data from 193 women were eligible for analysis. Significant risk factors in women previously experiencing a pregnancy of an RhD positive child (N=113) were caesarean section (CS) (OR 1.7, 95% CI 1.1-2.6), perinatal death (OR 3.5, 95% CI 1.1-10.9), gestational age over 42 weeks (OR 6.1, 95% CI 2.2-16.6), postnatal bleeding (>1000 mL) (OR 2.0, 95% CI 1.1-3.6), manual removal of the placenta (MRP) (OR 4.3, 95% CI 2.0-9.3); these factors often occurred in combination. The miscarriage rate was significantly higher than in the Dutch population (35% vs 12.5% $p<0.001$).

Conclusion: Complicated deliveries, including cases of major bleeding and surgical interventions (CS, MRP) need to be recognised as risk factor, requiring estimation of foetomaternal haemorrhage volume and adjustment of RhIg dosing. The higher miscarriage rate suggests that existing RhIg protocols either need adjustment or better compliance.

Funding: This research was partly funded by a grant from Sanquin Amsterdam.

Introduction

In high-income countries, the incidence of RhD immunisation has decreased after implementing routine antenatal and postnatal Rh immunoglobulin prophylaxis (RhIg), combined with administration of RhIg after events likely causing foetomaternal haemorrhage (FMH).(26, 64, 65) This has led to a major reduction of foetuses and newborns suffering from haemolytic disease.(14, 25) However, RhD immunisation still occurs in RhD-negative women pregnant of an RhD-positive child, with an estimated incidence of 0.3 to 1.3%. (10, 66-68) RhD immunisation has a 30% risk of severe disease of the foetus or newborn.(3, 7)

Since blood transfusions are routinely RhD matched for decades, the main cause of RhD immunisation is exposure to RhD-positive red blood cells (RBC) from the foetus, due to FMH during pregnancy or around delivery.(32) Small amounts of FMH can already lead to alloimmunisation.(69) Minor FMH occurs frequently during pregnancy (44% during the third trimester and 64% at delivery).(70) A major FMH (> 5 ml of foetal cells) occurs less frequently, with an estimated range of 0.1-6% of pregnancies. (5, 70-73) If there is a risk for a major FMH, administration of extra RhIg is often indicated in guidelines.(26, 64, 65) However, the significance of possible risk factors for a major FMH, such as mode of delivery, abortion/miscarriage (spontaneous or instrumental), invasive prenatal diagnosis, external cephalic version, abdominal trauma and antenatal bleeding, is still controversial.(53, 71, 72, 74) In our previous study, non-spontaneous delivery (caesarean section or assisted delivery), post-maturity and a younger age at the previous delivery emerged as risk factors for alloimmunisation.(53)

In this study, we evaluated in a prospectively collected cohort which risk factors for RhD immunisation remain, despite adequate routine antenatal and postnatal RhIg prophylaxis (1000 IU RhIg) and, if indicated, additional administration of RhIg, as based on a guideline from the Dutch organisation of obstetricians.(26) Since 2011, routine RhIg administration is based on foetal *RHD* typing.

Methods

Setting

In the Netherlands, all pregnant women are typed for ABO, RhD and Rhc blood group antigens and screened for the presence of alloantibodies against RBCs in the first trimester of pregnancy, preferably before the 13th week of gestation.(75) RhD- and Rhc-negative women are screened again in week 27. Certified Dutch laboratories (n = 90) process the screening test according to existing national guidelines.(32) Accepted screening tests are those with a sensitivity similar or better than the bovine albumin indirect antiglobulin test (IAT) to detect clinically relevant antibodies. In daily practice, column testing is used. Sensitive techniques with addition of enzymes are not used in the screening.(3) The coverage of this screening program, monitored annually, is almost 100%.(76) Following Dutch guidelines, RhIg (1,000 IU) is given at 30 weeks of gestation and again within 48 hours after birth in case of an RhD-positive foetus, after spontaneous abortion when the pregnancy was at least 10 weeks, and following instrumental evacuation of the uterus irrespective of gestational age. An extra dose of RhIg is advised to be given, after invasive prenatal testing or external cephalic version and, after estimating FMH with a microscopic Kleihauer Betke test (KBT) or a flow cytometry-based quantitation of HbF containing red blood cells (both referred to as KBT) in case of abdominal trauma or antenatal bleeding after 16 weeks. After a delivery, only when a large FMH is suspected, quantitation (KBT) is recommended, followed, if needed, by adjustment of the RhIg dose. Guidelines to calculate the adjusted dosing are available.

When at routine screening or at any other moment in pregnancy red cell alloantibodies are detected, a maternal (and if possible paternal) blood sample is sent to one of the two national reference laboratories: Sanquin Diagnostic Services (90% of all tests) and, for the north-eastern part of the Netherlands, the laboratory of the University Medical Center Groningen (UMCG).(22, 77) Foetal RHD genotyping is routinely performed in all RhD-immunised pregnancies. This typing as well as the antibody-dependent cell-mediated cytotoxicity (ADCC) test, to determine the biological activity of RBC antibodies, is centralised at Sanquin Diagnostic Services in Amsterdam.(19)

Study design and population

This study was part of the OPZI 2.0 study (unpublished data), a nationwide cohort study on RhD immunisation in pregnancy. All pregnant women with a positive screening test for anti-D antibodies, identified at Sanquin Diagnostic Services during our study period, were eligible for inclusion. In some cases, a positive screening test

was found shortly after RhIg administration, these were excluded. The study period ranged (for practical reasons) from July 1, 2014, to March 31, 2015, and from August 1, 2015, to February 28, 2017, a total of 28 months.

Written informed consent was obtained by the obstetric care provider (OCP). Clinical data were collected using a questionnaire, sent to the OCP's. If needed, the OCP or study participants were contacted by telephone up to three times, in order to complete the data set. 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).

Data collection and outcome definitions

Maternal characteristics (age, weight, gestational age at antibody detection, pre-pregnancy blood transfusions) and relevant clinical data from all previous non-immunised and immunised pregnancies were collected in the OPZI 2.0 database. Data on all RhIg administrations and possible sensitising or boosting events during pregnancy (antenatal bleeding, abdominal trauma, invasive prenatal diagnosis, external cephalic version, twins, post-maturity) and delivery (twins, post-maturity, postnatal bleeding>1000 ml, perinatal death, caesarean section, manual removal of placenta, assisted birth and pregnancy-related RBC transfusion), were collected. Miscarriages preceding the current ongoing pregnancy were considered as possible sensitising events.

To identify risk factors for RhD immunisation, occurring despite antenatal and postnatal RhIg administration, we selected all women in their first RhD-immunised pregnancy. We excluded women with a prior delivery of an RhD-positive child who did not receive the complete RhIg prophylaxis at 30 weeks gestation and/or after giving birth. When the RHD type of the child was not registered, but the complete RhIg prophylaxis was given, the foetal RHD type was considered positive. We evaluated potential risk factors in the following three groups: the first group 'exposed to the RhD antigen' consisted of women with a previous pregnancy (> 16 weeks) of an RhD-positive child; the second group 'possibly exposed to the RhD antigen' had a previous miscarriage (< 16 weeks) without a prior pregnancy of an RhD-positive child; the third group 'non-exposed to the RhD-antigen' had neither a previous pregnancy of an RhD-positive child nor a miscarriage. Birth-related risk factors were analysed in the group of multiparous women (the RhD exposed group), whereas risk factors in the current pregnancy were analysed in the other two groups. The prevalence of potential risk factors for RhD immunisation was compared with the best available population data. These data were derived from the Dutch perinatal registration (Perined) or, when data were not available, from other nationwide studies performed in the same

period. If data concerned potential risk factors occurring in previous pregnancies, only population data from women who had a previous pregnancy (>16 weeks) were used for comparison.

To assess the prevalence of both newly detected and already existing RhD immunisations, we used data from the year 2016, collected in the OPZI 2.0 cohort. The denominators to assess the prevalence of RhD immunisation were derived from the monitor of the National Institute of Public Health and Environment of 2016.(78)

Statistical analysis

The associations between potential risk factors and the occurrence of RhD-alloimmunisation were described as Odds Ratios and 95% confidence intervals (categorical variables) or as mean difference with 95% confidence intervals (normally distributed continuous variables) according to Altman, 1991.(79) All statistical analyses were performed with the Statistical Package for the Social Sciences (SPSS) 26.0 and medcalc.org (https://www.medcalc.org/calc/odds_ratio.php). Risk factors were tested univariately. The mutual interrelation of univariate significant risk factors was depicted in a vector diagram.

Results

Prevalence of RhD immunisation

The prevalence of newly detected RhD immunisations in 2016 was 0.31% (79/25,170) of RhD-negative pregnant women in the Netherlands. Pregnancies from women who were likely immunised before immigration to the Netherlands were excluded (N=15). In 0.18% of RhD-negative women anti-D was newly detected at the screening early in pregnancy and in 0.13% during routine screening in week 27 of pregnancy. The prevalence of all RhD immunisations (including immigrants) in 2016 was 0.09% of all pregnant women (158/171,727) and 0.63% of all RhD-negative pregnant women.

Selection of the study population

During the study period, 304 RhD-immunised pregnant women were eligible for inclusion in the OPZI 2.0 study. Figure 1 shows the selection and the composition of our study population, used for the analysis of risk factors for RhD-immunisation despite RhIg prophylaxis. After exclusion, 193 women remained, 65 of whom were nulliparous (33.7%) and 128 multiparous (66.3%). Of this group 113 women were

exposed to the RhD antigen, 28 were possibly exposed and 52 were non-exposed, respectively. Only one woman carried an RhD variant (in the 'possibly exposed group'). She had not received previous transfusions. Additional RBC antibodies were found in 53 (27.5%) women; the most common antibodies were anti-RhC (19.7%) and anti-RhE (3.1%) (Table S1).

General risk factors for RhD immunisation

When compared with the Dutch pregnant population, multiparous women were significantly overrepresented in our study group (66% vs 55.3% P=0.002), but still a large number of women were in their first ongoing pregnancy (Table 1, details population rates Table S2). We found a higher miscarriage rate in RhD-immunised women compared to the general Dutch population (21% vs 12.5% p<0.001). A total

of 40 women had a miscarriage preceding the RhD-immunised pregnancy (25 nulliparous and 15 multiparous women). Eleven out of sixteen (69%) women who had a miscarriage past 10 weeks' gestation or a curettage did not receive the advised RhIg (Table S3).

First detection of anti-D after a negative first trimester screening concerned 44% (86/193) of all cases (Table 1). Mostly these antibodies were found at the routine third trimester screening: 36% (41/113) of the women from the 'exposed group', 43% (12/28) of the women from the 'possibly exposed' group and 60% (31/52) of those from the 'non exposed group'.

Figure 1 *Composition of the study population*

The dark blue box reflects the number of eligible women before exclusion. Red boxes reflect exclusions and the green box the number of included women, eligible for the risk factor analysis. We stratified into three groups: exposed to RhD, most likely non-exposed to RhD and possibly exposed to RhD in history.

* RhD-antigen previous child unknown (n=21), Antenatal and postnatal RhIg prophylaxis was given, therefore the child was considered to be RhD positive

Table 1 *Baseline characteristics of 193 RhD-immunised pregnant women.*

	Cases		General pregnant prevalence	
	Mean (SD)	N (%)	Mean (SD)	(%)
Maternal age at delivery before the immunised pregnancy (y) (N=113)	27.4 (4.0)		29.5 (4.5)	
Pre-pregnancy weight (kg) (n=155)[1]	71.2 (13.5)		70.4 (12.6)	
Blood transfusion in history		32 (16.5)		-
Nulliparous		65 (33.7)		44.7
Multiparous		128 (66.3)		55.3
Miscarriage[2@]		40 (20.7)		12.5
Moment of detection of RhD-antibodies				
Before current pregnancy*		2 (1)		
Early first trimester screening[$]		102 (53)		-
First screening 20th- 27th week		3 (2)		-
Routine third trimester (27th week) screening[#]		84 (43)		-
Around delivery		2 (1)		-

Variables with other comparable evidence than the Dutch perinatal registration: [1]Pre-pregnancy weight, Bakker et al 2011, Miscarriage, [2]Dutch general practitioner's guideline "Miscarriage", for comparison a mean miscarriage rate of 10-15% was used.(80, 81)
In 2015, the number of women delivered in the Netherlands was 166.733, of which 73,121 were nulliparous
[@]Nulliparous or multiparous with one or more miscarriages before immunised pregnancy
**Pre transfusion screening*
[#]Foetal RHD typing result was positive in all cases

Risk factors for RhD immunisation in previously RhD-exposed women

As shown in table 2, caesarean section, manual removal of the placenta, post-partum bleeding >1000 mL, delivery at gestational age ≥42 weeks and perinatal death in history were significant risk factors for RhD immunisation in the 'exposed' group, when compared with the reference population ($p<0.05$). One third (37/113, 33%) of all 'exposed' women experienced none of the analysed risk factors in the previous pregnancy. In 61% of these cases, anti-D was detected during the first trimester. Of the women whose RhD immunisation was first detected at the 27th week screening, foetal RHD typing was positive in all cases. In the 'exposed group', who all had

a previous pregnancy with an RhD-positive foetus, 10.6% (12/113) women had a miscarriage in between the previous and the current pregnancy. This miscarriage rate was not different from the population rate of 12.5%.(81)

The incidence of vaginal blood loss before 16 weeks could only be compared with one prospective cohort study, performed in two US general hospitals, since our national Perined database does not collect these data.(82) This study reported a 21.5% incidence, while we found an incidence of 5.3% in our group.

For antenatal bleeding after 16 weeks, we could use the Dutch perinatal registration data.(83) None of the risk factors currently regarded as indication to administer (extra) RhIg prophylaxis (abdominal trauma, antenatal bleeding after 16 weeks and cephalic version), occurred more frequently in women of the 'exposed group' compared to the general population.

Combined parturition-related risk factors

Figure 2 shows that some parturition-related risk factors occurred in combination, hence some of these could be considered as confounders. Post-partum bleeding > 1000 mL occurred in 8 out of 12 (67%) pregnancies in combination with other risk factors, most often with manual removal of the placenta. One more case of excessive post-partum bleeding occurred in combination with a perinatal death (not depicted in Figure 2). Delivery from 42 weeks onwards was an isolated risk factor only once. Caesarean section was an isolated risk factor in 30 out of 32 (94%) pregnancies.

Risk factors for RhD immunisation in 'non-exposed' or 'possibly RhD-exposed' women

In the combined group of 'non-exposed' and 'possibly exposed' women (n=80), we analysed possible sensitising moments, occurring either before or during the current pregnancy (Table 3). Twenty-eight women (35%) had a miscarriage preceding the current pregnancy, in which anti-D was first detected, whereas the population rate of miscarriage is only 10-15% (OR 4.3; 95% CI 2.7-6.8). In half of the women with a miscarriage in their history, anti-D was not identified until the third trimester of the subsequent pregnancy with an RhD-positive child (table S3). There was only one woman with a miscarriage in her history who had an additional incident (antenatal bleeding <16 weeks) during the current pregnancy, before anti-D was detected in the third trimester. Twenty percent of women (16/80) reported a blood transfusion in their history, unrelated to pregnancy. There are no comparable population data on incidence of non-pregnancy related blood transfusions in the history of women of fertile age.

Figure 2 *Association of significant parturition-related risk factors for RhD immunization.*

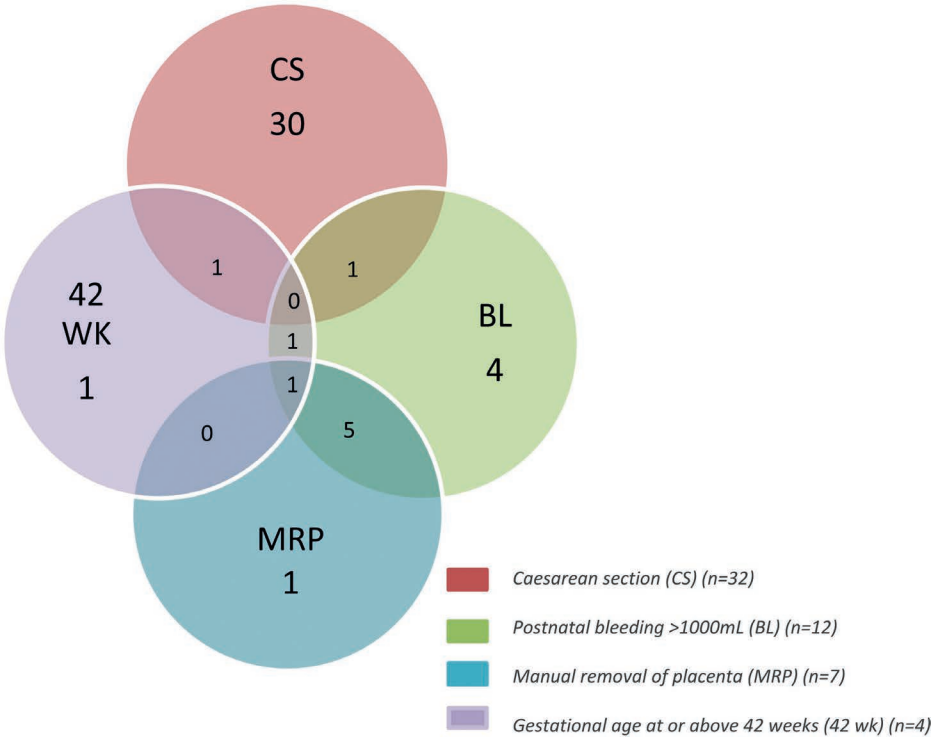

Table 2 *Potential risk factors for RhD immunisation in multiparous women exposed to the RhD-antigen in previous pregnancy >16 weeks.*

Prevalence				
Risk factors	Cases (N=113)	Population prevalence	Odds ratio 95%CI	P-value
	N (%)	%		
Risk factors around previous delivery, ongoing pregnancies above 16 weeks				
Caesarean section	32 (28.3)	18.7	1.7 (1.1-2.6)	0.009
Assisted birth	18 (15.9)	16.4	1.0 (0.6-1.6)	0.89
Manual removal placenta	7 (6.1)	1.5	4.3 (2.0-9.3)	<0.001
Twins	3 (2.7)	1.1	2.4 (0.8-7.7)	0.13
Gestational age delivery >=41 weeks	21 (18.6)	14.5	1.3 (0.8-2.2)	0.22
Gestational age delivery >=42 weeks	4 (3.5)	0.6	6.1 (2.2-16.6)	<0.001
Perinatal death	3 (2.7)	0.8	3.5 (1.1-10.9)	0.03
Postnatal bleeding >1000 ml[1]	12 (10.6)	5.9	2.0 (1.1-3.6)	0.02
Blood transfusion[2]	8 (7.1)	3.9	1.9 (0.95-4.0)	0.07
Male gender (N=103)	62 (60.2)	51	1.4 (0.98-2.2)	0.07
External cephalic version[6#]	5 (4.4)	2.4	1.9 (0.76-4.61)	NS
Risk factors during current pregnancy, before detection of RhD immunisation in week 27				
Invasive prenatal testing[3]	1 (0.9)	1.7	0.52 (0.07-3.75)	NS
Antenatal bleeding <16 weeks[4]	7 (5.3)	21.5	0.27 (0.13-0.59)	0.001
Antenatal bleeding >16 weeks	2 (1.8)	1.3	1.4 (0.3-5.6)	NS
Abdominal trauma[5*]	6 (5.3)	6	0.87 (0.39-2.0)	NS

Variables with other comparable evidence than the Dutch perinatal registration:
[1,2]Postnatal bleeding >1000 mL and blood transfusion pregnancy related - van Stralen et al 2016, [3]Prenatal diagnosis - WPDT and Liefers 2015, [4]Antenatal bleeding prior 16 weeks - Hossain et al 2007, [5]Abdominal trauma - Cheng et al 2012, [6] External cephalic version - Vlemmix et al 2010. (82, 84-88)
[#]Abdominal trauma without RhIg N=3.
**External cephalic version without RhIg N=1 and unknown N=1.*
Number of delivered women in the Netherlands in 2015 is 166.733, number of nulliparous was 73,121.

Table 3 *Potential risk factors for RhD immunisation before or during pregnancy in women previously non-exposed or possibly exposed to the RhD-antigen.*

Primigravid women, nulliparous women with a miscarriage in history and multiparous women with an RhD-negative child and with or without miscarriage in history (n=80)				
	Cases (n=80) N (%)	Population prevalence (%)	Odds ratio 95%CI	P-value
Miscarriage*	28 (35.0)	10-15	4.3 (2.7-6.8)	<0.001
Blood transfusion non pregnancy related	16 (20.0)	-	-	-
Blood transfusion pregnancy related	4 (5.0)	3.9	1.7 (0.69-4.22)	NS
Invasive Prenatal testing~	2 (2.5)	1.68	1.52 (0.37-6.19)	NS
Antenatal bleeding < 16 weeks#	4 (5.0)	21.5	0.19 (0.07-0.52)	0.001
Abdominal trauma&	3 (3.8)	6	0.61 (0.19-1.93)	NS

**Miscarriage after 10 weeks gestation without or unknown RhIg N=10, curettage without RhIg N=1, ~Invasive prenatal testing without RhIg N=2, #Antenatal bleeding without RhIg N=4, &Abdominal trauma without RhIg N=2*

Discussion

Main findings

In this study, we found the following risk factors for RhD immunisation to remain, despite adequate routine antenatal and postnatal RhIg prophylaxis of 1,000 IU as per our national guideline: caesarean section, manual removal of the placenta, excessive post-partum haemorrhage (1000 ml), delivery at or past 42 weeks and perinatal death. These risk factors occurred often in combination.

The prevalence of both newly detected and of all RhD-immunisations in RhD-negative pregnant women has nowadays reached unprecedented low percentages of 0.31% and 0.63% respectively. This is in line with previously reported figures of large studies.(44, 89, 90) With a frequency of 15% of RhD-negative women, RhD immunisation now concerns only 0.09% of all pregnant women in the Netherlands. Half of the RhD immunisations were detected in the first trimester of pregnancy.

Caesarean section was the main and most often single risk factor for RhD immunisation in our cohort, confirming findings from our earlier study.(53) The second risk factor, post-partum haemorrhage >1000ml, was in the majority of the cases (9/12) associated with one (or more) of the other risk factors we observed, including manual placental removal (6/7 cases), and perinatal death (1/3), suggesting a cascade of possibly immunising events. Post-maturity (delivery $\geq$42 weeks) was a less common risk factor, associated with excessive post-partum bleeding and caesarean section in three out of four cases.

The overall miscarriage rate in our study was significantly higher than that in the Dutch population (21% vs 10-12.5% $p<0.001$). This finding can be fully attributed to the high miscarriage rate (35%) in the group of women in their first ongoing pregnancy with an RhD positive baby. In most cases, these women did not have a positive RhD antibody screen during the first trimester, but only at the 27-week test, as has been described before.(10, 91)

Strengths and limitations

This is the largest study to date on risk factors for RhD immunisation in pregnant women participating in a high-coverage RhD immunisation prevention program. A strength of our study is that we were able to collect national data on all RhD-immunised women and their previous non-immunised and immunised pregnancies.

This created the opportunity to evaluate all potential obstetrical and non-obstetrical incidents that may induce RhD immunisation.

A limitation of this study design is that we could not include a control group. We had to compare our findings with published data in other populations or Dutch national registry data. The current data set substantiates the outcome of our previous prospective study on risk factors in a smaller but more defined group of primigravidae, in which a control group was included.(53)

Interpretation

In our study, we found caesarean section to be a significant risk factor for RhD immunisation, having almost no interrelations with other events potentially increasing FMH. These findings confirm data reported by other smaller studies.(53, 71-74, 82)

Current Dutch guidelines recommend estimating the volume of FMH by performing a KBT after caesarean section and, depending on the results, to increase the RhIg dose.(26, 64, 65) This is however no obligation. In some countries, a KBT is routinely performed after delivery or in case of risk factors related to increased FMH.(64, 92) In some prophylaxis programs, a higher dose of RhIg of 1,500 IU is used routinely, in order to reduce the risk of RhD immunisation. Our data support the concept that a caesarean section should be regarded as a risk for RhD immunisation. We hypothesise that making FMH testing mandatory might further reduce the number of RhD immunisations. Alternatively, a double dose of RhIg could be given after caesarean section, especially in settings were FMH testing is not easily available.

Previously, we hypothesised that post-maturity may lead to a failure of antenatal RhIg prophylaxis, due to the long interval between the administration of prophylaxis and delivery.(53) The current study however suggests that immunisation in post-maturity is mostly related to complications during delivery. In current obstetrical practice in developed countries, post-maturity past 42 weeks has become rare, as most pregnancies are nowadays induced before or around 41 weeks.(93) In this context, adjustment of RhD-prophylaxis in post-term pregnancies is no priority.

Postnatal excessive bleeding will always be a sign of a more complex delivery with an additional risk of a larger FMH, increasing the risk of alloimmunisation in RhD-negative women. In addition, perinatal death appeared to be associated with a higher risk of RhD immunisation. Therefore, if these risk factors occur, estimation of FMH volume and adjustment of RhIg dosing is advised. Surprisingly, in one third of women who previously had given birth to an RhD positive baby, none of the high-risk features that we found to be related to RhD immunisation were reported. Possibly, a

larger but subclinical FMH than could be covered by the RhIg prophylaxis occurred, as has been reported earlier.(94) An alternative explanation would be that some women respond more strongly to a relatively low amount of foetal blood entering their circulation around delivery.

The miscarriage rate in the combined non-exposed and possibly exposed group was almost three times higher than in a comparable age group.(81) Half of the RhD immunisations in ongoing pregnancies after a miscarriage were first detected in the third trimester. This finding confirms the theory that the miscarriage may be a primary sensitising event, however with such a low level of RhD antibodies that these are still undetectable in the first trimester of the subsequent pregnancy. Only after renewed contact with foetal RhD-positive red cells, the antibody levels increase and may become first detectable at the 27-week screening.(69, 95, 96) Our observations regarding current guidelines to administer RhIg prophylaxis in cases of miscarriage or abortion suggest insufficient adherence. Further studies are needed to explore the effectiveness of RhIg in preventing immunisation after all spontaneous or induced (including instrumental) abortions.(64, 65)

Overall, we did not find evidence that potential antenatal risk factors for FMH in the current pregnancy were associated with RhD immunisation. These events (invasive diagnostic procedures, twin pregnancy, antenatal bleeding and abdominal trauma) are relatively rare and there is likely sufficient awareness of the prophylactic measures that need to be taken.(26, 64, 65) In case of antenatal bleeding in pregnancies before 16 weeks, extra RhIg is currently not recommended, and based on our findings, we would not advise to change this policy.

Conclusion

We advocate to be strict in the policy of recognising risk factors, determination of FMH volume and adjustment of RhIg dosing, especially in pregnancies with complicated deliveries, including cases of major bleeding and surgical interventions, such as caesarean section and manual (surgical) removal of the placenta. Our data suggest that miscarriage may be an additional risk factor for RhD immunisation, requiring further studies, and possible to reconsider the current RhIg policy. For future research, we recommend to critically and prospectively evaluate any adjustments to the RhD immunisation prevention program made.

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Table S1. *Additional antibody specificities in women with RhD- immunization.*

Additional antibody specificities			N	(%)
C			38	19.7
E			6	3.1
K			1	0.5
G			2	1.0
Jk(a)			1	0.5
Fy(a)			1	0.5
C	E		3	1.6
C	E	K	1	0.5
	Total		53	27.5

Table S2. *Content of evidence, other than perinatal registration Netherlands.*

Variable	Reference	Year	Population (N)*
Prenatal diagnosis	WPDT/ Liefers	2015	2796:56,685
Blood transfusion pregnancy related	Van Stralen	2016	93,864:2,406,784
Postnatal bleeding >1000 ml	Van Stralen	2016	142,000:2,406,784
Miscarriage	NHG Guideline	2015	20,842:166,733
Maternal weight	Bakker	2011	8,623

**numerator: denominator*

Table S3. *Details of previous miscarriages in the groups of Possibly RhD exposed and RhD Exposed women*

	Possibly exposed (N=28) N (%)	Exposed (N=12) N (%)
Median duration pregnancy (days)	53 (35-76)	63 (51-72)
Gestational age <10 weeks	13 (46)	7 (58)
No anti-D or unknown	11 (85)	6 (86)
Gestational age >10 weeks and/curettage	11 (39)	5 (42)
No anti-D or unknown	8 (73)	3 (60)
Gestational age unknown	4 (14)	0
No anti-D	4 (100)	
Screen positive 1st trimester	14 (50)	4 (33)
Screen positive 3rd trimester	12 (43)	8 (67)
Screen positive delivery/operation/blood transfusion	2 (7)	0

Possibly exposed and exposed women to the RhD antigen with a miscarriage before the RhD immunization was detected (N=40)

Chapter 3

Third trimester screening for alloimmunisation in Rhc-negative pregnant women: evaluation of the Dutch national screening programme

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Abstract

Objective: To evaluate the effect of red blood cell (RBC) antibody screening in the 27th week of pregnancy in Rhc-negative women, on detection of alloimmunisation, undetected at first trimester screening ('late' alloimmunisation), and subsequent Haemolytic Disease of the Foetus and Newborn (HDFN); to assess risk factors for late alloimmunisation.

Design: Prospective cohort and nested case-control study.

Setting: The Netherlands. **Population:** Two-year nationwide cohort.

Methods: Prospectively inclusion of Rhc-negative women with negative first trimester screening and of screen-negative controls. **Main outcomes measures:** Late alloimmunisation, HDFN.

Analysis: Assessment of incidence and Numbers Needed to Screen (NNS) of late alloimmunisation and HDFN; logistic regression analysis to establish risk factors for late alloimmunisation.

Results: Late alloimmunisation occurred in 99/62,096 (0.159%) of Rhc-negative women, 90% had c-/E-antibodies, 10% non-Rhesus-antibodies. Severe HDFN (foetal/neonatal transfusion) occurred in 2/62,096 (0.003%) of Rhc-negative women and 2% of late alloimmunisations; moderate HDFN (phototherapy) occurred in 20 children (22.5%;95%-CI:13.8-31.1%). Perinatal survival was 100%. The NNS to detect one HDFN case was 2,823 (31,048 for severe, 3,105 for moderate HDFN). Significant risk factors were former blood transfusion OR 10.4;95%-CI:1.14-94.9), parity (P-1 OR 11.8;95%-CI:3.00-46.5; P:>1 OR 7.77;95%-CI:1.70-35.4) and amniocentesis/chorionic villus sampling during current pregnancy (OR 9.20;95%-CI:1.16-72.9).

Conclusion: Additional screening of Rhc-negative women improved detection of late alloimmunisation and HDFN, facilitating timely treatment, with a NNS of 2,823. Independent risk factors for late alloimmunisation were blood transfusion, parity and chorionic villus sampling/amniocentesis in the current pregnancy. The occurrence of most factors before the current pregnancy suggests a secondary immune response explaining most late alloimmunisations.

Keywords: alloimmunization, screening, Rhc-negative, risk factors, incidences.

Introduction

Haemolytic Disease of the Foetus and Newborn (HDFN) is caused by maternal alloimmunisation against paternally inherited foetal red blood cell (RBC) antigens. HDFN may lead to foetal anaemia, hydrops, asphyxia, perinatal death, and neonatal hyperbilirubinaemia, that may cause 'kernicterus'. Kernicterus can result in neurodevelopmental impairment with athetoid cerebral palsy, hearing problems and psychomotor handicaps.(4, 6-9, 20, 97) Most severe HDFN cases are caused by RhD-, Rhc- and Kell-antibodies (hereafter called anti-D, anti-c, etcetera).(4, 6, 7, 10, 20, 97) Timely detection of maternal alloimmunisation facilitates foetal monitoring, aimed to identify foetuses with severe disease needing intrauterine transfusions (IUT) and/or preterm delivery followed by phototherapy or (exchange) transfusions. These therapies have all contributed to a considerable decrease in HDFN-related perinatal death and long-term sequelae.(13, 98)

Most Western countries have maternal alloimmunisation screening programmes. A wide variation in design of these programmes exists between and within countries, ranging from several screenings in all pregnant women to a single screening of RhD-negative women only.(17, 67, 97, 99-101)

In the Netherlands, all pregnant women are screened for RBC antibodies at the booking visit; screening is repeated in week 27 for RhD-negative women, and since July 2011 also for Rhc-negative women.(57, 102) Implementation of screening in Rhc-negative women, comprising 18.7% of pregnancies(103), was based on a nationwide study in 400,000 pregnancies, showing that 25% of severe HDFN cases in RhD-positive women occurred unexpectedly, after a negative screening result in the first trimester. Some of these unexpected cases suffered from HDFN-related handicaps due to perinatal asphyxia or kernicterus, because foetal anaemia and hyperbilirubinaemia were not timely detected. In contrast, all cases of alloimmunisation detected at first trimester screening were timely treated and children were healthy at the age of one year.(10) All first trimester screen-negative cases of severe HDFN were caused by anti-c and/or anti-E. However, long-term sequelae were only found in anti-c cases. (10) Based on this outcome an additional screening of all Rhc-negative women in week 27 was set-up to increase the detection rate of severe HDFN cases with 25% (from 75 to 100%). Undetected, these cases might result in severe anaemia, hydrops, death or (too) late treatment of icterus.

So far, a few smaller studies showed no advantage of a second screening in RhD-positive women.(66, 104-107) In the current large nationwide study, we set out to assess the incidence of HDFN after a positive antibody screening in week 27 in Rhc-negative pregnant women and evaluated whether implementation of this third trimester screening improved timely diagnosis and treatment of HDFN. In addition, we

aimed to identify risk factors for alloimmunisation first recognized late in pregnancy, in order to provide insight in the causative mechanism in order to be able to develop strategies for the prevention and timely detection of late alloimmunisation.

Methods

Setting and Prevention programme in the Netherlands

In the Netherlands, all pregnant women are typed for ABO, RhD and Rhc blood group antigens and screened for RBC antibodies at the first trimester booking visit. All RhD- and Rhc-negative women, without RBC antibodies at the initial screening, are screened again in week 27.(57) This repeated screening is centralised in the laboratory of Sanquin Diagnostics in Amsterdam. When clinically relevant RBC antibodies are detected, i.e., antibodies with the potency to destroy foetal RBC's, the antibody titre and the Antibody Dependent Cellular Cytotoxicity Test (ADCC) are performed, in order to assess the ability of these antibodies to cause foetal haemolysis. The father of the foetus is typed for cognate antigen(s) and in case of heterozygosity, non-invasive typing on foetal DNA in maternal plasma is offered (for *RHD*, *RHC*, *RHc*, *RHE* and *K*).(22) If the foetus does not have the cognate antigen(s), further monitoring of the pregnancy is not necessary. If the foetus is diagnosed as antigen-positive, the pregnancy is frequently monitored by laboratory testing. In the presence of non-RhD RBC antibodies, an antibody titre ≥ 1:16 and/or ADCC test ≥30% indicates a major risk for HDFN, and foetal anaemia is monitored with middle cerebral artery (MCA) Doppler measurements.(29, 61) Severe foetal anaemia is treated with intrauterine transfusion(s) (IUT's) at the Leiden University Medical Centre (LUMC), which is the national Dutch referral centre for management and treatment of pregnancies complicated by maternal red cell alloimmunisation. In the Netherlands this study design does not require formal approval of the Medical Ethical Committee.

Study design

To assess the occurrence of HDFN in Rhc-negative women diagnosed with newly detected RBC antibodies (cases) at week 27 of pregnancy ('late alloimmunisation'), we prospectively collected data on all these women and their offspring in the Netherlands between October 1^{sts} 2011 and October 1st, 2013.

The association between potential risk factors for late alloimmunisation and the occurrence of late alloimmunisation among Rhc-negative pregnant women was examined in a case-control study comprising Rhc-negative women with (the cases)

and without (the controls) late alloimmunisation, sampled between October 1stst 2011 and October 1st, 2012. Our planned study period was one year. To obtain a more reliable estimation of the incidence of severe HDFN we extended the study period with one year. We did not prolong the case-control study.

Cases and controls were identified at Sanquin Diagnostics Amsterdam. For each case, three controls were selected. These were the first three Rhc-negative women that were screened negative, directly following the alloimmunised Rhc-negative woman.

Outcomes

The primary outcome was the incidence of severe and moderate HDFN in the offspring of Rhc-negative pregnant women with antibodies first detected at 27 weeks gestation. Severe HDFN was defined as alloimmune disease with the need for intrauterine transfusion and/or neonatal exchange or blood transfusions in the first week of life. Moderate HDFN was defined as the need for treatment of neonatal jaundice with phototherapy only. Long-term sequelae are all long-term impairments, most likely associated with the severe HDFN, such as kernicterus and/or perinatal asphyxia.

Potential risk factors

We hypothesized that late in pregnancy detected alloimmunisations may emerge from a primary immune response during the current pregnancy or from a secondary immune response, triggered by foetomaternal (micro-)transfusions (FMT) of antigen-positive RBCs.(17, 105) Data on known risk factors for red cell alloimmunisation, including risk factors for FMT during the current pregnancy were collected in cases and controls.

Data collection

For inclusion of cases and controls, two of the researchers (YS, JK) contacted the obstetric care provider (midwife, general practitioner and/or obstetrician) to explain our study. The obstetric care provider asked the pregnant woman for consent for data collection and collection of cord blood, to be sent to our laboratory by post.

During the first year of the study, data on potential risk factors were collected during pregnancy, immediately after consent was given, from the obstetric care provider

and/or from the pregnant woman. Potential risk factors comprised both general risk factors and in-pregnancy risk factors. General risk factors included factors of general history (RBC transfusions, surgery, haematological diseases), as well as gravidity and parity. 'In-pregnancy risk factors' were factors within the previous pregnancy (gender child, caesarean section, surgical removal of placenta and postpartum haemorrhage (>1L), and factors during the current pregnancy until week 27 (vaginal bleeding, abdominal trauma and invasive diagnostic and therapeutic interventions).(73, 108-110)

To assess the occurrence of mild or severe HDFN in the study group, we collected the results of laboratory monitoring during pregnancy from Sanquin Diagnostics, data of clinical monitoring and IUT treatment during pregnancy, if applicable, from the LUMC, and neonatal outcome data about treatment with blood transfusion(s) or phototherapy from the obstetric care provider, from the paediatrician, from hospital laboratories and/or from the mothers, within two months after birth.

All data were collected by questionnaires, which were completed by phone, e-mail or by post.

Data analyses

We assessed the incidence of late alloimmunisation as proportion of all screened Rhc-negative women at 27 week of gestation and the occurrence of severe and moderate HDFN in association with late immunisation. The cases with HDFN were classified by antibody specificity. When multiple antibodies were present, the antibody specificity for which the paternal antigen was positive and/or with the highest estimated risk for development of HDFN was considered as 'dominant' antibody.

We calculated the Number Needed to Screen (NNS) to detect one case with severe HDFN timely, assuming that none of these cases would have been detected without the third trimester screening programme in Rhc-negative women. We also calculated the NNS to detect one case with moderate HDFN and to detect one case of 'late alloimmunisation'. The NNS were calculated as 1/ (0-incidence of severe/moderate HDFN/late alloimmunisation in Rhc-negative women, screened in the third trimester).

Dichotomous outcomes were described as number and percentage, normally distributed

continuous variables as mean and standard deviation and not-normally distributed continuous variables as median and range.

The association between potential risk factors and the occurrence of late alloimmunisation was examined with logistic regression, firstly by univariate and secondly by multivariate analysis. Potential 'general' risk factors and in-pregnancy risk factors during the current pregnancy were included in the first logistic model. Potential in-pregnancy risk factors originating from the previous pregnancy were included in a second logistic model. Interactions between the covariates were tested formally. All statistical analyses were performed with the Statistical Package for the Social Sciences (SPSS) 21.0.

Results

Study population and response

From October 1st, 2011, till October 1st, 2013, 62,096 Rhc-negative women, without RBC antibodies in the first trimester of pregnancy, were screened again in week 27 of gestation. Of these, 99 (0.16%;95-CI 0.13-0.19%) had newly detected clinically relevant RBC antibodies (Figure 1). During the first year of the study, 168 controls were selected (matched to 54 cases), of which 104 (62%) gave consent to collect data. The proportions of nulliparae, primiparae and multiparae in the control group were 47.1% (95%-CI 34.1-60.1%), 35.6% (95%-CI 24.3–46.9%)

and 18.5% (95%-CI: 2.7–34.3%) respectively, compared to proportions of 44.9%, 35.9% and 19.2% respectively in the Netherlands in 2012.(111)

From the newly immunised pregnant women, 10% (10/99) refused participation in the study. None of these women had either titres or ADCC values above the cut-off to select high-risk cases, or was referred to the LUMC, the national referral centre for severe alloimmunized pregnancies. Therefore, the occurrence of severe foetal haemolytic disease in the non-consent group is very unlikely, although severe neonatal HDFN cannot be completely ruled out. Therefore, incidences for severe HDFN are described in the whole group, but for moderate HDFN only in the group with consent.

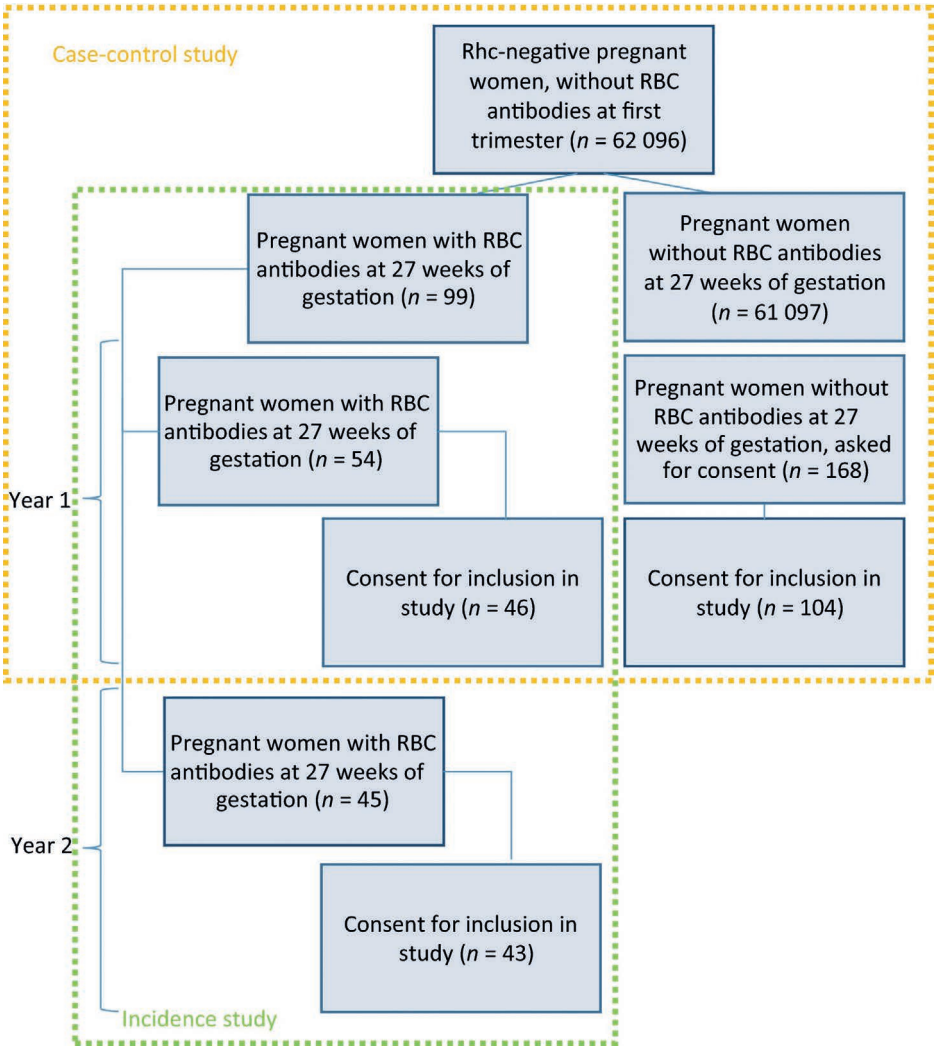

Figure 1: *Flowchart of inclusions and exclusions of cases and controls*

Incidence of late alloimmunisation

From the 99 late alloimmunisations, anti-c was the most frequently detected alloantibody (65/99;66%), in 20 cases anti-c was present in combination with anti-E and in seven cases with other antibodies. Anti-E was present in 45/99 (45%) cases, in 25 as a single antibody specificity. In 54 cases with anti-c and 36 with anti-E the father was tested for the cognate antigen(s) and was found to be positive in 53 and 35 cases, respectively. For the remaining 17 antibody specificities, the father was

typed in 14 cases and appeared positive for the cognate antigen(s) in 5 cases (Table 1). The NNS to detect one late alloimmunisation was 628 (Table 2).

Table 2. *Calculation Numbers Needed to Screen (NNS) to detect late alloimmunisation in Rhc-negative women and subsequent disease.*

	Screened Rhc-negative women 1/10/2011 – 1/10/2013 N=62,096			**Numbers Needed to Screen to detect one case***
	n	**% (95%-CI)**	**% (95%-CI)**	**n**
		of Rhc-negative women	of cases with late alloimmunisation	
Late alloimmunization	99	0.159 (0.128-0.191)		628
HDFN	22	0.035 (0.021-0.050)	22.22 (12.94-31.51)	2 823
- severe	2	0.003 (0-0.008)	2.02 (0-4.82)	31 048
- moderate	20	0.032 (0.018-0.046)	20.20 (11.35-29.06)	3 105

CI, confidence interval; HDFN, haemolytic disease of the fetus and newborn.
**Assumption for the calculation of NNS: timely detection without screening program = 0%. NNS calculated as: 1/ (0 _ incidence in Rhc negative women).*
Formulae for the calculation of the 95% confidence intervals: p ± 1.96 9 √ [p (1- p)/n]. p, proportion of alloimmunized women (0.16%); n, number of screened women (62 096).

Table 1. *Newly detected clinically relevant RBC antibodies in week 27 in Rhc-negative pregnant women*

Antibody specificity		N (%)		Phenotype father antigen dominant antibody*			Severe HDFN	Moderate HDFN	HDFN in lab tests > cut-off
Dominant antibody*	**Additional antibodies**	**N**	**%**	**negative**	**positive**	**?**	**IUT/ (exchange) transfusion**	**Phototherapy only ****	
c	-	38	38.4	1	30	7	1	9/34***	7/13
E	-	25	25.3	1	19	5	0	3/24	2/3
c	E	14	14.1	0	11	3	0	2/12	1/3
E	c	6	6.1	0	5	1	1	3/5	3/3
c	K	1	1.0	0	1	0	0	1/1	1/1
c	K+Fy^{a}	1	1.0	0	1	0	0	0/0	0/0
c	Jk^{a}	3	3.0	0	3	0	0	1/3	1/1
c	Jk^{b}	1	1.0	0	1	0	0	0/1	0/0
c	Wr^{a}	1	1.0	0	1	0	0	1/1	1/1
K	-	1	1.0	1	0	0	0	0/1	0/0
Jk^{a}	-	2	2.0	0	2	0	0	0/2	0/0
s	-	1	1.0	0	1	0	0	0/1	0/0
C^{w}	-	5	5.1	5	0	0	0	0/4	0/0
Total		99	100	8	75	16	2	20/89	16/25

HDFN, haemolytic disease of the foetus and newborn; IUT, intrauterine transfusion; ?, unknown phenotype of the father for the dominant antibody

** Dominant antibody if multiple antibodies are present: antibody specificity for which the paternal antigen is positive and/or with the highest estimated risk for development of HDFN.*

*** Denominators for phototherapy: cases with known outcome.*

**** In one antigen-positive child only a maximum bilirubin level of 289 µmol was known, but data about phototherapy treatment were missing; this case was classified as moderate HDFN.*

Incidence of HDFN

Severe HDFN due to RBC antibodies first detected at 27 weeks, occurred in two of the 62,096 Rhc-negative pregnancies screened and 2.0% of screen positive pregnancies (Table 2). One severe case was caused by the combination of anti-c and anti-E, mostly by anti-E (titre 1:256). During this pregnancy, one IUT (pre-transfusion Hb 9.0 g/dL) was performed at 30+3 weeks, followed by induction of labour at 36 weeks. The Hb and Ht levels postpartum were 12.4 (g/dL) and 0.42, respectively. Phototherapy was given during seven days. An exchange transfusion was needed after two operations for pyloric stenosis, carried out after the first week of life. Two months postpartum this child was confirmed to be in a good condition. The other severe case was caused by anti-c only. No intrauterine transfusion was given. Labour was induced at 36 weeks + 4 days; Hb and Ht at birth were 13.3 (g/dL) and 0.42, respectively. The lowest Hb was 9.8 (g/dL), five top-up transfusions were given, no exchange transfusions were needed.

Phototherapy was given in 20 cases (12 anti-c, 5 anti-E and 3 anti-c and anti-E), resulting in an incidence of moderate HDFN of 0.032% of all screened Rhc-negative women (Table 2) and 20.20% of screen-positive pregnancies. In cases with known outcome (n=89) the incidence of moderate HDFN was 22.5%(95%-CI:13.8-31.1%).

The NNS to detect one case of severe HDFN was 31,048 and to detect one case of moderate HDFN 3,105.

Six cases of moderate HDFN occurred in association with laboratory test results below the cut-offs.

Forty-nine children of the 90 pregnancies with anti-c and/or anti-E, were antigen-positive for the cognate antigens (based on antigen typing of the child (n=26) or homozygosity of the father for the antigens concerned (n=23)), five were antigen-negative and in 36 cases the antigen-typing was unknown. We calculated those 17 children with unknown antigen-typing should have been antigen-positive (Box S1), resulting in a risk for moderate HDFN in antigen-positive foetuses/children from c-/E-immunised pregnancies of 30.35% (20/66;95%-CI 24.6-36.0%).

Interventions for maternal alloimmunisation

Preterm induction of labour was performed in both severe cases. In addition, 13 term inductions were performed at least in part based on the presence of RBC antibodies (Figure S1), without signs of foetal anaemia on ultrasound or Doppler. Five of the six cases with antibody titres and/or ADCC test results above the cut-off values used in

the Netherlands to indicate high-risk cases needed phototherapy treatment. None of the seven cases of induced labour, with laboratory testing results below the cut-offs, needed treatment for HDFN. Two of the phototherapy cases were born prematurely (gestational age 28 and 34 weeks respectively), which was not associated with the maternal alloimmunisation. Twenty-four children were admitted to the neonatal ward, of which 20 were treated with phototherapy only. This concerned almost one third of anti-c cases, 14% of only anti-E cases, and none of the cases with other antibodies.

Risk Factors for late alloimmunisation

A history of RBC transfusion, major surgery, previous parity, maternal age was, as well as amniocentesis/chorion villus sampling in the current pregnancy were univariately associated with the occurrence of late alloimmunisation in Rhc-negative women (Table S1).

Potential risk factors within previous pregnancies were not associated with late alloimmunisation.

RBC transfusion, parity and amniocentesis/chorion villus sampling in the current pregnancy were statistically significant independent risk factors for late alloimmunisation (Table 3).

Table 3. *Associations between risk factors and late alloimmunisation*

		Cases N (%)	Controls N (%)	Crude OR (95%-CI)	Adjusted OR* * (95%-CI)
General risk factors		N=46*	N=104		
Age	25-29	8 (17)	33 (32)	Ref	Ref
	<25	4 (9)	15 (14)	1.10 (0.29-4.23)	1.38 (0.27-6.99)
	30-34	18 (39)	37 (36)	1.90 (0.72-4.96)	1.21 (0.39-3.71)
	>=35	16 (35)	19 (18)	3.47 (1.25-9.63)	1.78 (0.54-5.83)
Parity	0	3 (7)	49 (47)	Ref	Ref
	1	30(65)	37 (36)	13.2 (3.75-46.7)	11.81 (3.00-46.5)
	>2	13(28)	18 (17)	11.8 (3.01-46.3)	7.77 (1.70-35.4)
RBC transfusion		6 (13)	1 (1)	15.45 (1.80-132.4)	10.39 (1.14-94.9)
Major Surgery		18 (40)	21 (20)	2.64 (1.23-5.66)	2.37 (0.96-5.86)
In-pregnancy risk factors in current pregnancy: Chorionic villus sampling/ amniocentesis 6 (13)		2 (2)	7.65 (1.48-39.5)	9.20 (1.16-72.9)	

CI, confidence interval; OR, odds ratio; RBC, red blood cell.

** Proportions determined in group with known data; missing data maximum 1.*

*** Adjusted for maternal age, parity, RBC transfusion, major surgery and chorionic villus sampling/ amniocentesis*

Goodness of fit tests showed no evidence of lack of fit (p=0.90); explained variance 36.7% (Nagelkerke Chi-square)

Discussion

Main findings

Late alloimmunisation, detected at 27th week screening, occurred in 0.16% of all pregnancies of Rhc-negative women. Within the group of late alloimmunisation, the risk for severe HDFN was 2% and for moderate HDFN 22.5%. Most new immunisations and all HDFN cases were caused by anti-c and/or anti-E. Amniocentesis or chorionic villus sampling in the current pregnancy, as well as parity and a history of RBC transfusion were independent risk factors for alloimmunisation detected late in pregnancy.

Strengths and limitations

To our knowledge this is the first prospective nationwide study on the effect of a second antibody screening in Rhc-negative women. Our study provides a reliable estimation of the incidence of late alloimmunisation and subsequent HDFN. Although outcome data of 10% of the cases were missing, severe HDFN is very unlikely in these cases, because laboratory results were not above the cut-off values indicating high-risk for HDFN and no cases needed monitoring in the national referral centre. Moreover, in some cases it was impossible to separate the contribution of alloimmunisation from other causes for hyperbilirubinaemia, for example in two prematurely born children. This may have caused an –at most slight- overestimation of the incidence of moderate HDFN.

One third of the controls did not participate in our study, which may have caused selection bias in our risk factor analysis. Most common reasons for non-participating were a language barrier, social problems and declined cooperation of the obstetric caregiver, reasons unlikely associated with risk factors for alloimmunisation. This was supported by the distribution of parity, a strong risk factor, in our control group, which did not differ from national data.

Some risk factors showed wide confidence intervals, due mainly to limited numbers. We consider it unlikely that with increased numbers and thus narrowed confidence intervals, the risk estimations would turn out different.

Previous findings and interpretation

The incidence of late alloimmunisation in Rhc-negative women was in line with expectations following our former evaluation of the Dutch screening programme for non-RhD antibodies.(10) No studies are available yet in which only Rhc-negative women were screened for late alloimmunisation. A small Dutch study in which RhD-positive women underwent a second screening reported higher incidences of late alloimmunisation, which might at least partly be explained by the fact that this study was performed in a population of parous women, at increased risk for alloimmunisation.(112) Studies including 3,000-70,000 RhD-positive pregnant women reported incidences of late alloimmunisation varying between 0.06 and 0.43%, in line with our data.(113) The incidence of late alloimmunisation in Rhc-negative women might be somewhat higher than in all RhD-positive women, since anti-c and anti-E, the most frequent newly detected antibodies in all studies, are found especially in Rhc-negative women.

Remarkably, the incidence of severe HDFN in cases with late alloimmunisation was considerably lower than expected, resulting in a NNS to detect one severe HDFN case of 31,048. Based on the 0.002% incidence of severe HDFN by late alloimmunisation, found in our study in 2003-2004,(10) a NNS of about 9,000 was expected. An explanation for this decreased incidence might be that timely detection of cases at risk for foetal haemolysis, followed by labour induction in week 37, as advised in the Dutch Guideline on maternal alloimmunisation, preventing the development to severe HDFN in some cases.(26) This explanation is supported by the shorter median gestational age in cases with labour induction, followed by phototherapy treatment, than in the missed severe HDFN cases in our former study (265 versus 274 days). Moreover, the increased availability of intensive phototherapy combined with the introduction of a new guideline in 2008 including a more conservative approach concerning the use of exchange transfusions to lower bilirubin levels, will have reduced the use of exchange transfusions.

Both severe cases of HDFN in our study, were probably not detected without the screening programme. These were uncomplicated pregnancies and normally developed foetuses. Current standard of care for such pregnancies in The Netherlands does not include routine ultrasound in the third trimester. Even if ultrasound would be done, without a high index of suspicion specific anaemia detection by middle cerebral artery Doppler would not have taken place. Clinically, only reduced foetal movements and hydrops on ultrasound would be detected, which are very late stages of disease associated with a significant perinatal death risk. Therefore, we hypothesize that the remarkable decrease of the incidence of severe HDFN by late alloimmunisation, for which no other explanation can be given, is a benefit of the implementation of third

trimester screening in Rhc-negative women, a benefit that highly exceeds the benefit as suggested by the NNS of 31,048.

A possible negative feature of screening might be several relatively early inductions of labour because of maternal alloimmunisation, despite laboratory test results being below the cut-offs, as was the case in 50% of term inductions. It should be kept in mind that in these cases, factors other than maternal alloimmunisation may have contributed to the decision to induce labour. It was however reassuring that the induction rate in cases was comparable with national figures (17.2 versus 21.4%). (111)

One severe HDFN case occurred in a pregnancy complicated by low anti-c and high anti-E levels, while three moderate cases were due to anti-E only. This raises the question whether also women with an Rhc-positive but RhE-negative phenotype (CcDee (35%) or ccDee (1,6%)(105) should be offered a second screening. Our former evaluation showed only one missed case during two years with the CcDee phenotype, while all cases with long term sequelae were caused by anti-c.(10) Therefore, expanding the screening to all RhE-negative women will most likely not significantly improve the detection of severe HDFN cases. Registration of screen-undetected cases with HDFN would be helpful to clarify this issue.

We identified risk factors before as well as during the current pregnancy. Parity and blood transfusion were identified in our former study as risk factors for early alloimmunisation.(21) These findings are in accordance with the hypothesis that the primary immune response occurred already in, or following, a previous pregnancy. Antibody levels then fall too low to be detected at first trimester screening and rise again after renewed contact during pregnancy of the maternal immune system with foetal red cells. This might have occurred after amniocentesis or chorionic villus sampling, when these cases also had one or more risk factors before the current pregnancy. The contribution of each of the risk factors is difficult to be estimated

in this relatively small study. In the risk factor analysis only cases from the first year of the study with consent to collect data on risk factors (n=46) were included. We did not match for potential confounders, because, as described by Altman (1991), any variable used for matching cannot be investigated as a possible risk factor for maternal alloimmunisation.(79) As this is the first study on risk factors for late alloimmunisation, we aimed to investigate all possible risk factors instead of collecting variables, known as risk factors for maternal alloimmunization detected at first trimester screening only.

Our analysis underlines a restrictive blood transfusion policy, as well as the use of Rhc- and RhE-matched donor blood, according to current Dutch guidelines. (32) Moreover, invasive diagnostic procedures are associated with foetomaternal haemorhage (109), which can cause a primary or secondary immune response, the latter with a rapid rise of maternal RBC antibody levels. This underlines the importance of non-invasive prenatal testing (NIPT).(114)

Theoretically, third trimester screening in Rhc-negative women may be restricted to women with risk factors, 62% of the pregnant women in our control group. However, subgroup first trimester screening, as advised by the Dutch Health Council(102), was not implemented, because of practical objections of the obstetric care workers. Our study confirms the usefulness of the additional third trimester screening for RBC alloantibodies in all Rhc-negative women.

Our previously published economic analysis showed that the extra costs of the expanded screening programme in the Netherlands are about 1.4 M€/year. As we detected two severe cases during two years, this means 1.4 M€/case, which is lower than the estimated life time costs of a surviving child with long term sequelae, which are about 3 M euro, when this person reaches the age of 60 years.(115) We also showed that the psychological burden of antibody screening is small and balanced with the benefits.(63)

Conclusion

A repeated RBC antibody screening in week 27 of pregnancy in Rhc-negative women contributes to the timely detection and treatment of severe HDFN and most likely also leads to a decrease of the incidence of severe HDFN. An optimal management eventually results in less severely compromised cases and a reduction in the long-term morbidity and mortality associated with severe HDFN.

Acknowledgements

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Cases and controls were identified at Sanquin Diagnostics Amsterdam (Dr. C. Folman and Ms. H. Woortmeijer are acknowledged for making data of their laboratory registries available for the study).

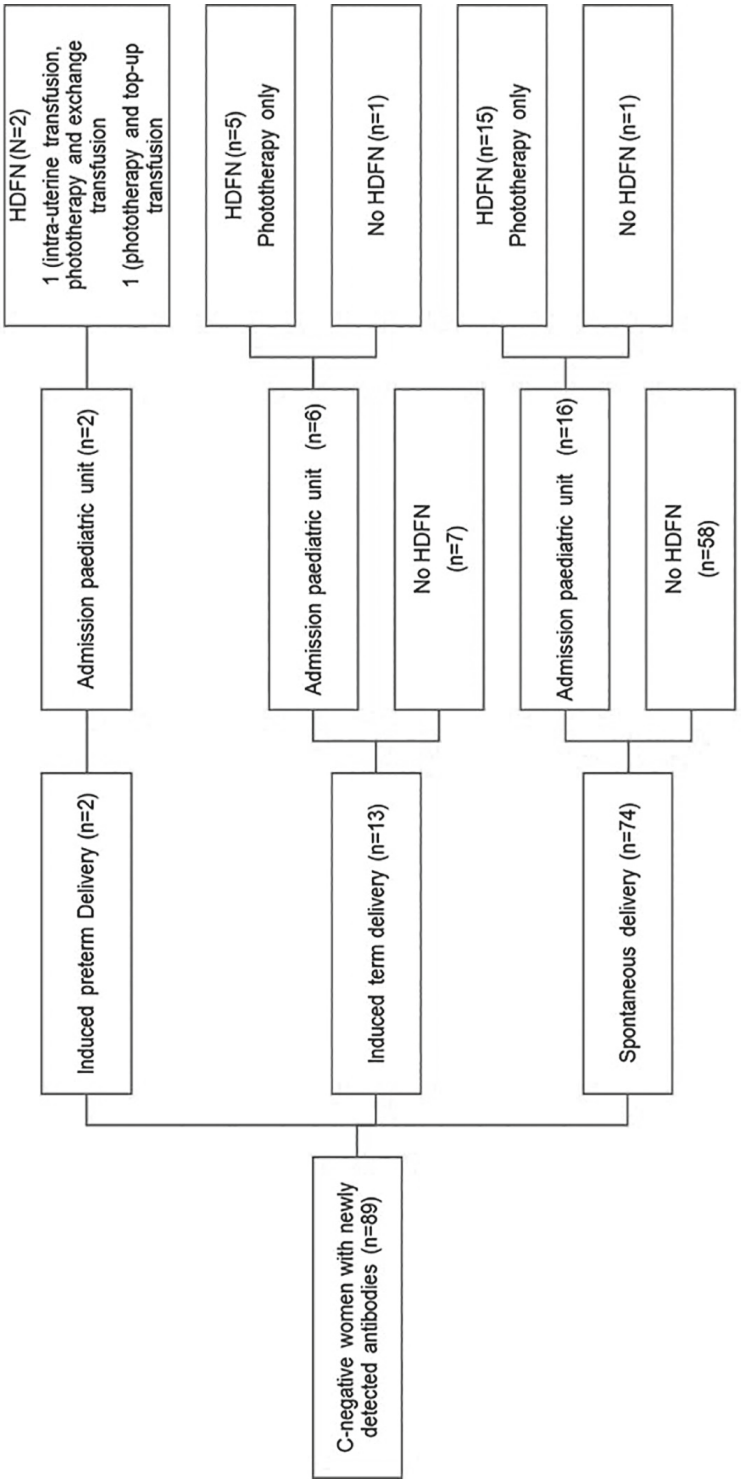

Figure S1: *Interventions because of RBC antibodies in cases with known outcome (n=89)*

Table S1. *Univariate risk factors for the presence of newly formed RBC antibodies at screening in week 27*

Variable	Cases n (%)	Controls n (%)	p
General risk factors:	n=46*	n=104*	
Only small surgery	7 (16)	22 (21)	0.43
Major Surgery	18 (40)	21 (20)	0.012
Blood transfusion	6 (13)	1 (1)	<0.003
Platelet transfusion	0 (0)	1 (1)	1.00
Haematological disease	1 (2)	2 (2)	1.00
Parity			<0.001
0	3 (7)	49 (47)	
1	30 (65)	37 (36)	
>=2	13 (28)	18 (17)	
Abortion (spontaneous/induced) in history	12 (26)	24 (23)	0.69
Maternal age (mean, SD)	32.7 (4.8)	30.4 (4.5)	0.005
In-pregnancy risk factors during previous pregnancy (>=16 weeks):	n=43*	n=55*	
Male child*	15 (43)	23 (45)	0.84
Caesarean section	7 (16)	7 (13)	0.62
Instrumental delivery	3 (7)	4 (7)	1.00
Surgical removal placenta and/or curettage within 28 days post partum	4 (9)	5 (9)	1.00
Haemorrhage post-partum (>1L)	6 (14)	2(4)	0.13
In-pregnancy risk factors during current pregnancy:	n=46	n=104	
Vaginal bleeding	4 (9)	12 (12)	0.78
Abdominal trauma	2 (4)	2 (2)	0.58
Chorionic villus sampling/amniocentesis	6 (13)	2 (2)	0.011

** proportions determined in group with known data; missing data maximum 1, except for gender previous child. A previous male child was previously shown to be a risk factor.(108)*
*** missing data in 8 cases and 4 controls*

Box S1: *Calculation antigen distribution in children with unknown antigen typing*

Antibody-specificity	Father heterozygous	Risk antigen-positive child	Paternal typing unknown	Risk antigen-positive child**
Anti-c	8	50%	3	32.3%+49.2*0.5%=56.8%
Anti-E	13	50%	5	2.4%+26.3*0.5%=1.2%
Anti-c + anti-E*	5	75%	2	(100%-(1-56.8%)*(1-1.2%)) = 57.3%
Total (n)	26	14.3	10	2.9

**All fathers were heterozygous for c-antigen and E-antigen*
*** Antigen distribution in Caucasians: c-antigen 32.3% homozygous, 49.2% heterozygous; E-antigen 2.4% homozygous, 26.3% heterozygous (103)*

Chapter 4

Facilitators and barriers for RhD-immunized women to become and remain anti-D donors

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Abstract

Background: The successful introduction of prophylaxis with anti-RhD Ig has resulted in a significant decline of pregnancy-related RhD immunizations, but also in decreasing availability of naturally immunized women as (new) anti-D donors. An influx of new donors is necessary to maintain a sufficient pool of anti-D donors. We investigated motivators, barriers and predictors for anti-D donorship in RhD-immunized women.

Study design and methods*:* A mixed-methods design was applied, including focus group discussions and questionnaires. The focus groups (two, including 11 women) served as input for the questionnaire.

Results: 47.6% out of 750 anti-D donors and potential donors completed the questionnaire (50.4% donors; 38% non-donors; 11.6% ex-donors). Almost 70% of the non-donors would have become a donor if they had known about the possibility. (Travel) time investment was reported as disadvantage; half of the donors mentioned no disadvantages. Motivators for anti-D donorship were 'doing something in return' (31.2%) and 'preventing others having a sick child or losing a child' (33.9%). In multivariable analysis, living single (OR 5.8;p=0.02) and living partnered without resident children (OR 7.9;p=0.03), compared with living partnered with children, were predictors for anti-D donorship. Not being registered as organ donor (OR 0.25;p< 0.001) predicted not being an anti-D donor.

Conclusion: The main barrier for anti-D donorship was a lack of knowledge. Positive predictors of anti-D donorship were living without resident children, altruism and being registered as an organ donor. A blood bank should develop targeted recruitment strategies with the focus on spreading knowledge about anti-D donorship among RhD-immunized women.

Key Words: donors, intravenous immunoglobulin, HDN

Introduction

Before the introduction of anti-D immunoprophylaxis, RhD immunization was a major cause of perinatal death.(1, 7, 20) Since the 1960s, RhD-negative pregnant women in developed countries have received anti-D immunoglobulin (anti-D) within 48 hours after delivery or in situations during pregnancy creating a risk of fetomaternal hemorrhage (FMH).(77) In the Netherlands, postnatal anti-D prophylaxis was introduced in 1969.(45) Routine antenatal anti-D prophylaxis in the 30th week was introduced in 1998. From 2011 onwards, fetal RhD genotyping in maternal plasma has been performed first, restricting prophylaxis to women pregnant with an RhD-positive foetus.(24, 44) These preventive measures have together substantially reduced the risk of RhD alloimmunization and subsequent hemolytic disease of the foetus and newborn (HDFN). HDFN is known as Rhesus disease in the Netherlands. Nowadays, in the Netherlands, the number of newly immunized women is estimated to be about 50 per year (data from registration of alloimmunized pregnancies at Sanquin Diagnostic Services, national reference center).

To safeguard the anti-D prophylaxis program in the Netherlands, anti-D immunoglobulins are partly obtained from the plasma of RhD-immunized donors and partly imported from abroad.(57) In the Netherlands, most anti-D donors are RhD-negative women between 45 and 70 years old, who are immunized naturally after pregnancy and delivery of an RhD-positive child.(116) Some – both male and female – donors are intentionally immunized by administering a small amount of RhD-positive erythrocytes. To meet the national demand for anti-D prophylaxis, approximately 32,000 vials are needed, corresponding to 3200 donations per year – one donation being sufficient for ten products.(117) Assuming an average of five donations per donor per year, 640 donors would be required to reach self-sufficiency in the Netherlands.(116, 118) However, the group of active anti-D donors has decreased over the last years from 501 in December 2010 to 406 in December 2015 because dropout of donors exceeds influx of new donors, the negative result of a successful prophylaxis program. An important dropout reason concerns anti-D donors who were immunized by a pregnancy and delivery before introduction of anti-D prophylaxis in 1969 and reached the maximum age for donation of 70 years. The proportion of old-age stoppers reached its peak in 2014 and is now stabilized at 2–7% yearly.(116) As fewer women are newly immunized by pregnancy and delivery it becomes more important to recruit a higher proportion of newly or already immunized women to become anti-D donors in order to increase and stabilize the donor population.

Recruiting naturally immunized women has some advantages compared with intentionally immunized males. Firstly, recruiting naturally immunized women prevents future problems in intentionally immunized donors, when they need a transfusion themselves. The presence of RhD antibodies can delay the process of

preparing suitable donor blood, especially when people are travelling to Asia, where there are fewer RhD-negative people than in Western countries.(119) Secondly, voluntary unpaid blood donation is recommended by all international authorities (WHO/ Council of Europe/ ISBT/ EBA) because they are the best way to strive for self-sufficiency in blood products of all kinds, while maintaining an optimal level of quality and safety for recipients as well as for donors.(120-122)

Although much is known about the behavior and motivations of whole blood donors,(123) less research has been focused on the motivational and psychological factors associated with plasma donor behavior.(124) In the specific group of anti-D plasma donors, to the best of our knowledge, no research has been performed. Factors playing a role in the intention to donate whole blood are educational level, age, gender and marital status.(125) Several studies pinpointed motivators positively associated with becoming and remaining a donor. These factors include a positive attitude towards donating blood, social pressure to donate, perceived behavioral control or self-efficacy, the importance of being a blood donor, altruism and feeling an obligation.(123) In addition, donor career influences return behavior: the longer donors actively remain donating, the more likely they become committed donors.(126) Between whole blood donors and plasma donors few recognizable differences exist. Plasma donors have a higher donation intention, self-efficacy, attitude and conscientiousness, and a lower anxiety than whole blood donors.(124) It is not unlikely that anti-D donors may also differ in some ways from whole blood and nonspecific plasma donors. They emerge from a special group of women which has potentially experienced severe disease of their unborn or newborn child, or maybe even loss of a child through HDFN. These confrontational memories might influence their intention to donate both positively and negatively.

Based on knowledge about RhD-immunized women and their considerations to donate anti-D, targeted recruitment strategies and retention interventions could be developed to guarantee a continuous supply of anti-D plasma from voluntary, immunized, unpaid donors in the Netherlands. To this end, we investigated motivators, barriers and predictors, and appreciated recruitment strategies for anti-D donorship in RhD-immunized women, who are potentially eligible to become an anti-D donor.

Materials and Methods

Design

We applied a mixed-methods study design, combining qualitative and quantitative approaches. Qualitative data were collected by means of focus groups, quantitative data by a questionnaire. The main objective of the qualitative approach was to

identify key themes central to motivations and barriers of (potential) anti-D donors, serving as input for the development of a quantitative questionnaire. We chose to use focus group discussions so that the effect of mutual interaction on the motivation for anti-D donorship and the relation with experiences and preferences could be more easily identified. This study was part of a larger project to gain more insight into the willingness of obstetric care providers to play a role in the recruitment of new anti-D donors. The opinion of obstetric care providers will be elaborated in another paper. The Medical Advisory Council of the Leiden University Medical Center (LUMC) approved the study.

Participants

Participants were anti-D donors and potential new anti-D donors, i.e. naturally RhD-immunized women between 43 and 65 years of age. Age limits were defined based on the ability to be hyperimmunized (after 45 years of age) and the age limit for donating (70 years of age). Participants were selected from the database of anti-D donors at the Sanquin Department of Donor Relations and from the database of the LUMC, the reference center for the management and treatment of pregnancies with severe RhD immunizations in the Netherlands.

For the focus group discussions, 100 RhD-immunized women were selected from the LUMC database. These were the patients seen most recently at the LUMC mixed with some older women from their neighborhood. They received an invitational letter and informed consent form from the obstetric care providers of the LUMC. Consenting women were contacted to make an appointment for the focus group discussion by the first author. The focus groups were put together using purposive sampling. In each focus group, active and potential anti-D donors were included, and variation in age and severity of offspring HDFN was pursued. The groups consisted of four to seven women and were organized (if possible) in the neighborhood of the participants. Focus groups were organized until data saturation was achieved.

For the questionnaire, RhD-immunized women from the LUMC database received a letter on behalf of their obstetric care provider with a link to the online questionnaire. Current anti-D donors were approached via the Sanquin Department of Donor Relations by email.

Data collection procedure

Focus groups

We conducted two focus group discussions. A skilled moderator guided the participants through an open discussion, stimulating and influencing their thinking to finally generate a maximum number of different ideas and opinions. The discussion was structured around a set of carefully predetermined open questions (Appendix 1) based on evidence about donor motivation and fueled by the researchers' expertise on the topics of blood donor behavior, RhD immunization and the problems of HDFN. The moderator ended the discussion when new ideas and opinions were no longer put forward. The discussion was video-recorded and notes were taken. Each focus group session was transcribed verbatim, also including relevant non-verbal cues. After the first focus group the verbatim protocol was analyzed to identify central topics to be discussed in the following sessions. Participants in the focus groups were offered travel expense refunds and a small gift.

Questionnaire

Based on the core themes identified in the focus groups (including also the motivators and barriers mentioned), the questionnaire was developed. Specific questions on motivation and donation barriers for (potential) anti-D donors were also included.

The dependent variable was anti-D donor status, asked as: '*Are you currently an anti-D-donor?*' (yes/no/past-donor).

Independent variables:

Obstetric medical history: pregnancies (yes/no, number, year of last pregnancy), spontaneous/induced abortions (<16 weeks, number), severity of HDFN per pregnancy (yes/no perinatal death due to HDFN, prenatal and/or postnatal transfusion, exchange transfusion, phototherapy). The severity of HDFN was classified into four categories, based on the most severe HDFN the women experienced during one or more pregnancies: 1 fetal demise, 2 prenatal fetal transfusion, 3 postnatal neonatal (exchange) transfusion and 4 neonatal phototherapy.

Knowledge about/attitudes towards anti-D donorship: '*Do you know what an anti-D injection is and what it is for?*', '*Have you ever heard about anti-D donorship?*' (yes/no, string value for explanation in own words) and for donors: '*How did you come up with the idea to become anti-D donor?*' Motivators: '*I want to do something in return*',

'*It does not cost me much trouble and it delivers much*', '*I want to prevent others having a sick child or losing a child*', '*Anti-D donors are needed*', '*other*' (yes/no/string value for explanation in own words) and 'the most important value to become an anti-D donor' (above-mentioned categories, single answer). Negative factors: '*Time*', '*Travel time*', '*Travel cost*', '*Health*', '*Confrontations with memories of HDFN*', '*Negative experience of blood donation*', '*Fear of needles*', '*No negative factors*', '*Other*' (yes/no/string value for explanation in own words). Recruitment: '*mode of recruitment*' (social media, magazines, newspapers, door to door flyers, via obstetric care provider/other health care provider, via obstetric care provider at LUMC, via other blood donors) and timing of contact (during pregnancy, short time after delivery, 6 weeks after delivery, 6 months after delivery, a few years after delivery, approximately … years).

Demographics: we included a set of standardized measures from the Donor InSight Study (DIS).(117) The questions concerned age (years), postal code (to check for double responses), ethnicity, religion, level of education, working status, family income and family situation (marital status, family composition). Answering categories and descriptive statistics are presented in Table 3.

Pro-social values and behavior: we included questions concerning being a registered organ donor (yes/no/choice to relatives) and volunteer work (yes/no). We measured pro-social value orientation differentiated by three scales (answer categories on a five-point Likert scale, 'totally agree' to 'totally disagree'); sum scores were calculated. The first scale, 'trust', comprised two items referring to generalized social trust (maximal total score 10) included in the statements developed by Rosenberg.(127) The second scale, 'altruism', comprised five items (maximal total score 25) referring to altruism, constructed by Gordon and translated into Dutch by Drenth and Kranendonk.(128, 129) The third scale, 'empathic concern', comprised four items (maximal total score 20) referring to empathy, developed by Davis and modified by Bekkers.(130, 131)

Analysis

Qualitative data were analyzed using thematic content analysis.(132) Coding schemes identifying key categories in facilitators and barriers were revised and expanded, resulting in core themes.

We described the attitude towards anti-D donorship in terms of motivators and negative factors of anti-D donorship. Motivators were mentioned by anti-D donors as well as non-donors who indicated the intention to become a donor. Negative factors were mentioned by anti-D donors and non-donors who were unwilling to become a donor.

For analysis of the quantitative data, we compared motivators and barriers and potential predictors for anti-D donorship between current donors and non-donors, disregarding women who were currently not anti-D donors but had been in the past.

Dichotomous outcomes were described as number and percentage, normally distributed continuous variables as mean and standard deviation, and non-normally distributed continuous variables as median and P25–P75. Differences between non-donors and current donors were tested univariably by means of Pearson's chi-square test (dichotomous variables), and Student's *t*-test (normally distributed continuous variables) or the Mann–Whitney U-test (not normally distributed continuous variables). All variables with a p-value of <0.20 were included in a multivariable logistic regression analysis, to estimate the association between potential predictors and anti-D donorship. The strengths of the associations were expressed as odds ratios (OR) and their corresponding 95% confidence intervals (95% CI). Age (continuous) was included in the model as a potential confounder. Variables with a significant ($p<0.05$) association in the regression analysis were included in a prediction model, predicting anti-D donorship. Data were analyzed using SPSS Statistics version 23.

Results

Results from the qualitative focus group interviews

From the 100 RhD-immunized women invited for the focus group discussions, about one in five was an anti-D donor. Twenty-four women gave consent to participate in a focus group. After two focus groups, including a total of 11 women, data saturation was achieved. The remaining 13 women who gave consent were invited for the questionnaire. Eight themes were identified: 'Knowledge of possible anti-D donorship', 'Experiences with Rhesus disease', 'Reasons to become a blood donor', 'Organ donorship', 'Knowledge of Rhesus disease', 'Experiences with blood donation', 'Boosting', 'Ways to recruit anti-D donors' and 'Practical considerations'. These topics were all covered in the questionnaire.

Quantitative results – responses

We invited all anti-D donors in the Sanquin donor database meeting the inclusion criteria (340/501) reachable by email and all 410 reachable RhD-immunized women between 43 and 65 years of age from the LUMC database to fill in the questionnaire. The overall response rate – excluding ex-donors – was 41.6% (312/750); 32.7% (134/410) in non-donors and 52.4% (178/340) in donors. In the group of responders

57% were anti-D-donors, 43% non-donors. The number of ex-donors not included in the analysis was 41 (10% of total response).

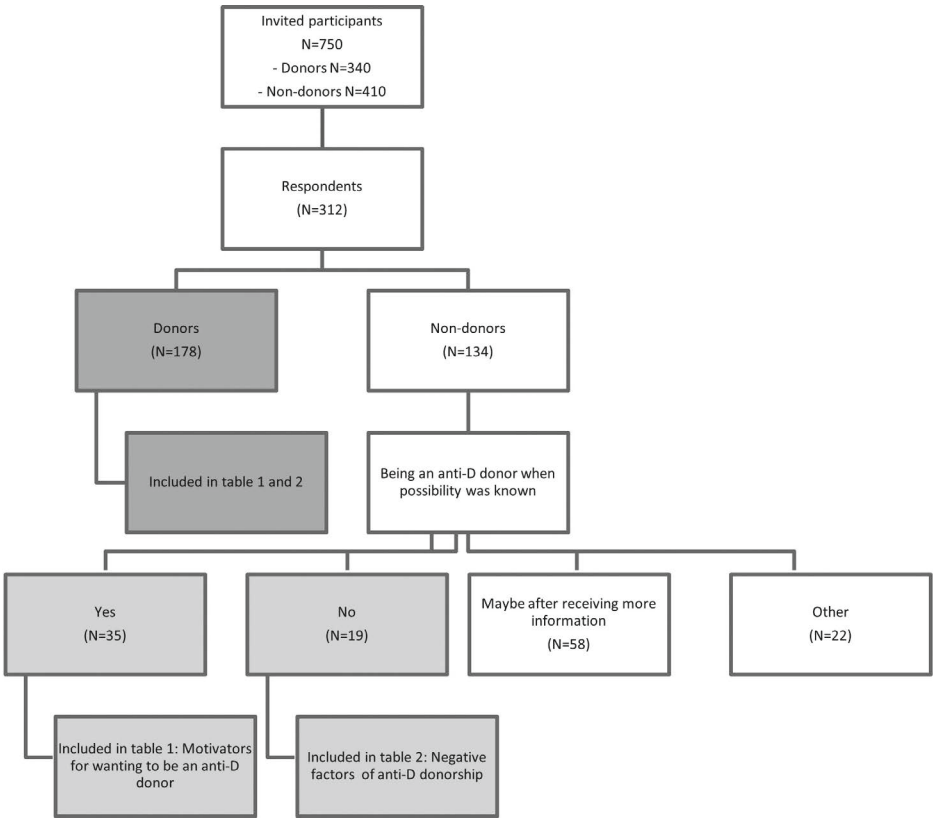

Figure 1. *Flowchart of included participants table 1 and 2*

Attitudes towards anti-D donorship

Almost all responders had ever heard about anti-D injections (98.3%, 347/353) and 94% (332/353) could explain more or less the purpose of anti-D injections. The majority of the 134 non-donors indicated that they would have become a donor if they had known about the possibility (69.4%, 93/134) (Figure 1). Of them, 43.3% pointed out that they wanted to receive more information first. To the question 'in the future I will certainly sign up as anti-D donor', asked to non-donors only, 47% (63/133) gave a neutral response and 35% (47/133) agreed or totally agreed. Table 1 shows the results on motivators for being or becoming an anti-D donor of donors and non-donors with the intention to become a donor (n=35). Anti-D donors gave the reason 'anti-D donors are needed' twice as often as non-donors ($p < 0.001$). Non-

donors responded slightly more often 'they want to do something in return' (p=0.09) or 'want to prevent others having a sick child or losing a child'(p=0.14). Those two reasons (31.2% and 33.9% respectively) were also the most important values for non-donors with the intention to become a donor.

Frequently mentioned negative factors of anti-D donorship by anti-D donors were time investment (63/174) and travel time investment (37/174) (Table 2). Half of them could not think of any negative factors. Respondents who certainly did not want to become an anti-D donor (n=19) named as their reason 'time investment' (42%) and 'negative experiences with blood drawing in the past' (31%). 'Being confronted with memories referring to HDFN' was not mentioned as a major negative factor in either group (anti-D donors 6% and 16% non-donors).

Table 1. *Motivators for being or becoming an anti-D donor*

Motivator	Anti-D donor (N = 178) N (%)	Non-donor* (N = 35) N (%)
'I want to do something in return'	84 (47.8)	22 (62.9)
'It does not cost me much trouble and it delivers much'	108 (60.6)	21 (60.0)
'I want to prevent others having a sick child or losing a child'	82 (45.6)	21 (60.0)
'Anti-D donors are needed'	150 (83.9)	16 (45.7)

**Non-donors with the intention to become a donor.*

Multiple answers were possible.

Table 2. *Negative factors of anti-D donorship*

Negative factor	Anti-D donors (N = 174) N (%)	Non-donors* (N = 19) N (%)
Time	63 (36)	8 (42)
Travel time	37 (21)	4 (21)
Travel cost	7 (4)	4 (21)
Health	6 (3)	2 (13)
Confrontations with memories of HDFN	10 (6)	3 (16)
Negative experience of blood donation	7 (4)	6 (32)
Fear of needles	0 (0)	4 (21)
Negative factor	87 (50)	0 (0)
Other	16 (9)	3 (2)

Table 2. *Negative factors of anti-D donorship (continued)*

Negative factor	Anti-D donors (N = 174) N (%)	Non-donors* (N = 19) N (%)
Purpose of immunization	2 (1)	0 (0)
No fee	0 (0)	1 (6)
Problems with blood drawing	10 (6)	2 (13)
Opening hours	4 (2)	0 (0)

**Non-donors unwilling to become a donor.*

Multiple answers were possible.

Recruitment of anti-D donors

Among the current anti-D donors 44% became a donor on their own initiative, and 51% via a blood bank flyer or a recruitment campaign. A small group (14%) was made aware of the possibility to donate by a health care provider. Frequently mentioned preferred recruitment strategies were 'personally by the obstetric care provider' (69%), 'personally by the LUMC, the reference center for Rhesus disease' (67%) and 'through social media' (49%). The right timing frequently mentioned was 6 weeks (31%) or 6 months after delivery (33%); 80% of the responders mentioned that they would like to have received a personal letter from the LUMC to make them aware of the possibility of anti-D donorship.

Univariable regression analysis

The general demographics, pro-social values and obstetric medical history, and their contributions in the univariable analysis are described in Table 3. There were no significant differences between anti-D donors and non-donors in religion, educational level and employment. Anti-D donors were slightly older than non-donors (not statistically significant). Overall, non-donors had experienced more severe HDFN in their obstetric history ($p < 0.001$). Anti-D donors were more often a registered organ donor and participated more frequently in volunteer work.

To assess the reliability of the altruism and empathy scales used Cronbach's alpha was determined ($\alpha = 0.73$ and $\alpha = 0.65$ respectively). Only the altruism scale showed good reliability and was significantly different between anti-D donors and non-donors. The trust scale consisted of only two items; Cronbach's alpha was therefore not determined.

Table 3. *Demographics, pro-social values and severity of HDFN and their univariable contributions in predicting anti-D donorship*

Variable	Anti-D donors N = 178 (57%)	Non-donors N = 134 (43%)	P-value§
Age mean (SD)	51.7 (± 9.6)	50.4 (± 4.5)	0.16
Family composition n (%)			0.001
Husband/wife and children	122 (68.5)	106 (79.1)	
Husband/wife	31 (17.4)	7 (5.2)	
Alone	14 (7.9)	4 (3.0)	
Single parent with children	11 (6.2)	17 (12.7)	
Religion n (%)			0.48
Roman Catholic	43 (24.2)	35 (26.1)	
Protestant	47 (26.4)	35 (26.1)	
Muslim	0 (0)	2 (1.5)	
None	81 (45.5)	55 (41.0)	
Christian other	7 (3.9)	6 (4.5)	
Education n (%)			0.65
None/lower education	27 (15.1)	15 (11.1)	
Secondary education	64 (35.9)	59 (44.0)	
Higher education	72 (40.4)	50 (37.3)	
University	14 (7.9)	9 (6.8)	
Employed n (%)	136 (76.4)	105 (78,4)	0.66
Registered organ donor n (%)	122 (68.5)	50 (37.3)	< 0.001
Volunteer work n (%)	88 (49.4)	51 (38.0)	0.05
Pro-social values median (P25–P75)			
Trust scale*	7 (6–8)	7 (6–8)	0.84
Empathy scale†	16 (14–16)	16 (14–17)	0.18
Altruism scale‡	19 (17–20)	20 (18–21)	0.05

Table 3. *Demographics, pro-social values and severity of HDFN and their univariable contributions in predicting anti-D donorship (continued)*

Variable	Anti-D donors N = 178 (57%)	Non-donors N = 134 (43%)	P-value§
Severity hemolytic disease of foetus and newborn n (%)			< 0.001
No disease	53 (29.8)	10 (7.5)	
Fetal demise	14 (7.9)	24 (17.9)	
Prenatal transfusion	27 (15.2)	56 (41.8)	
Postnatal transfusion	64 (36)	27 (20.1)	
Phototherapy only	20 (11.2)	17 (12.7)	

*Dependent variable is anti-D-donors and independent variable is non-donors. *Cronbach's α = 0.49; †Cronbach's α = 0.65; ‡ Cronbach's α=0.73.§ Pearson's chi-square test, Student's t-test or Mann–Whitney U-test.*

Predictors associated with anti-D donorship

All variables with a p-value < 0.20 in the univariable regression were included in the multivariable logistic regression (Table 4). Volunteer work and the empathy scale were not significantly associated with anti-D donorship in the multivariable analysis and were subsequently excluded from the final prediction model. The model was adjusted for age.

Family composition affected donorship; in particular, single women and partnered women without resident children were more likely to be an anti-D donor. Not being registered as an organ donor and 'leaving the choice for organ donation to relatives' were also negatively associated with anti-D donorship. Women who had experienced fetal or neonatal disease, especially women who had experienced severe disease such as fetal demise or prenatal transfusion, were less likely to be an anti-D donor. A higher score on the altruism scale was positively associated with anti-D donorship.

Table 4. *Multivariable logistic regression: predicting the likelihood of anti-D donorship*

Variable	Crude OR (95% CI)	Adjusted* OR (95% CI)	Multivariate p-value
Demographics			
Family composition:			
Husband/wife and children	Ref	Ref	Ref
Husband/wife	6.28 (2.29–17.17)	7.88 (2.68–23.11)	0.03
Alone	4.60 (1.09–19.28)	5.79 (1.32–25.31)	0.02
Single parent with children	0.83 (0.32–2.12)	0.84 (0.32–2.17)	0.71
Pro-social parameters and behavior			
Altruism scale	1.12 (1.01–1.24)	1.12 (1.01–1.23)	0.04
Registered organ donor			
Yes	Ref	Ref	Ref
No	0.25 (0.14–0.47)	0.25 (0.14–0.46)	< 0.001
Choice to relatives	0.46 (0.21–1.02)	0.46 (0.21–1.01)	0.05
I don't know	1.13 (0.05–22.21)	0.91 (0.05–18.4)	0.95
Severity hemolytic disease of foetus and newborn			
No disease	Ref	Ref	Ref
Fetal demise	0.08 (0.03–0.22)	0.08 (0.03–0.27)	< 0.001
Prenatal transfusion	0.09 (0.04–0.22)	0.09 (0.04–0.22)	< 0.001
Postnatal transfusion	0.45 (0.18–1.10)	0.44 (0.18–1.08)	0.07
Phototherapy only	0.26 (0.24–0.67)	0.23 (0.08–0.64)	0.005

CI = confidence interval; OR = odds ratio.
**Multivariable analysis adjusted for age.*
Goodness-of-fit tests showed no evidence of lack of fit (Hosmer and Lemeshow p = 0.65); explained variance 24% (Nagelkerke R2).

Discussion

We tried to gain a better understanding of motivators and barriers of RhD-immunized women to become and remain anti-D donors and to identify the most promising way to approach this specific group of (potential) donors. The results showed that almost 70% of non-donors might have become a donor if they had been informed of the possibility, while almost half of them first wanted to get more information before deciding on becoming an anti-D donor. This finding implies that a lack of knowledge about the possibility of becoming an anti-D donor is a major barrier for becoming one. This was confirmed by the explanation frequently heard in the focus group interviews that the potential donors thought that they could not be a whole blood donor because of the presence of RBC antibodies. Negative factors found were time investment and travel time investment, but half of the donors mentioned no negative factors of being an anti-D donor. 'Being confronted with memories referring to HDFN' was not mentioned as a major disadvantage of anti-D donorship in either focus group. Motivators of non-donors to become an anti-D donor were 'want to do something in return' (31.2%) and 'want to prevent others having a sick child or losing a child' (33.9%).

This study shows that (potential) anti-D donors differ from whole blood and plasma donors in gender, almost exclusively women, while in whole blood donors the gender ratio is more balanced and regular plasma donors are predominantly male.(124) Secondly, in this study, demographic variables as educational level, age and marital status were also associated with the intention to donate.(125, 133)

To indicate pro-social behavior, we used 'altruism', 'organ donorship' and 'volunteer work'. Similar to whole blood donors those indicators showed higher odds of being an anti-D donor.(123) Although the confrontation with memories of HDFN was not mentioned as being a negative factor or barrier in focus group discussions, the experience of severe HDFN was associated with higher odds of not being an anti-D donor in the multivariate model. This might be partly explained by an overrepresentation of women with severe HDFN in the non-donor group. A possible further explanation might be that the severity of the disease restrains the obstetric care worker from discussing the possibility of anti-D donorship with the patient.

For this particular group of potential donors, tailored recruitment strategies should be designed. The obstetric care provider can play a major role in creating awareness of anti-D donorship in women with RhD antibodies. Although responders to this

questionnaire mentioned they would like to have been contacted personally by the obstetric care provider 6 weeks to 6 months after giving birth, privacy and ethical considerations might be a barrier for the professional. Further research on this topic, in particular the view of obstetric care workers, will provide more insight. Possibly, a joint protocol might be created between the different parties involved to make it easier for obstetricians to retrieve consent of RhD-immunized women and to enable the blood bank to contact the woman after a certain time to provide her with information about anti-D donorship.

Strength and limitations

To the best of our knowledge, this is the first study on motivators and barriers of women with RhD antibodies to be or to become anti-D donors. The overall response rate of this study was 42%, comparable with other donor studies.(124, 134) In both anti-D donors and non-donors, we achieved a sufficient response; the response in the anti-D donor group was higher. Possibly, selective response exists among non-donors, since women with a positive attitude to anti-D donorship will be more inclined to respond to both the questionnaire and the focus group discussions than non-donors with a more negative attitude. This might have resulted in overestimation of the proportion of women with a willingness to become anti-D donors. However, we think our results provide a good overview of motivators and barriers to becoming an anti-D donor.

A major strength of our study is that we designed our questionnaire based on two focus group discussions in which we identified themes related to anti-D donorship. Moreover, we used validated scales to measure pro-social values and behavior, which were also used in the DIS.(117) In doing so, we believe that our questionnaire covered all themes. A limitation of the questionnaire was that we asked donors and non-donors who would certainly not want to become an anti-D donor (n = 19) only about negative factors of anti-D donorship. Therefore, information about negative factors of non-donors with the intention to be a donor is still lacking. Non-donors from the focus groups, like the anti-D donors, also indicated that too much time and travel investment might be negative factors for becoming an anti-D donor.

Because we identified non-donors via the LUMC, the reference center for the monitoring and treatment of alloimmunized pregnant women, we might have included a group of non-donors who experienced more severe HDFN than RhD-immunized Dutch women who were not referred to the LUMC. This might partly explain the contradiction in our results that the experience of severe HDFN was associated with not being an anti-D donor, while donors as well as non-donors did not consider being confronted with memories of HDFN as a major disadvantage of anti-D donorship.

Conclusion

The main barrier for women with RhD antibodies to be an anti-D donor is lack of knowledge about anti-D donorship. The profile of (potential) anti-D donors is different from whole blood and plasma donors, mainly because they are women and are eligible to become donors through immunization during pregnancy. Important motivators for being or becoming an anti-D donor mentioned often are ‘want to do something in return’ and ‘want to prevent others having a sick child or losing a child’. Predictive factors positively associated with anti-D donorship are family composition and altruism. Negatively associated predictive factors are ‘not being registered as an organ donor’ and ‘severity of the experienced HDFN’. A blood bank and obstetric care providers should find a way to work together to better inform, recruit and retain women to anti-D donorship.

Chapter 5

Predicting anti-Kell mediated hemolytic disease of the fetus and newborn, diagnostic accuracy of laboratory management.

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Abstract

Background: There is controversy on critical cut-off values of laboratory testing to select pregnancies at increased risk for anti-Kell (K) mediated HDFN (hemolytic disease of the fetus and newborn). Without early detection and treatment, Anti-K mediated HDFN may result in progressive fetal anemia, fetal hydrops, asphyxia and perinatal death.

Objective: We aimed to determine the value of repeated anti-K titer determination and biological activity measurement using the antibody-dependent cellular cytotoxicity (ADCC) test determination in the management of pregnancies at risk for anti-K mediated HDFN.

Study design: Retrospective cohort study of pregnancies with anti-K and a K-positive fetus, identified from January 1999 until April 2015. Laboratory test results and clinical outcome were collected from the Dutch nationwide screening program and the national reference center for fetal therapy in the Netherlands, the Leiden University Medical Center. Diagnostic accuracy (ROC-curves, sensitivity, specificity, positive and negative predictive values) of anti-K titers and ADCC test. The relationship between the titer and ADCC measurements and the two foregoing measurements were computed with a Pearson product-moment correlation coefficient.

Results: In a 16 year unselected cohort, representing screening results of 3.2 million pregnancies resulting in life births in the Netherlands, we identified 1,026 K-immunized pregnancies. 93 pregnant women had anti-K and a K-positive child, without other red cell alloantibodies. Forty-nine children (53%) needed intrauterine or postnatal transfusion therapy. The first anti-K titer showed already a high diagnostic accuracy with an AUC of 91%. The optimal cut-off point for the titer was 4 (sensitivity 100% (91-100; 95% CI), specificity 27% (15-43 95% CI) and positive predictive value 60% (49-71%). The ADCC test was not informative to select high-risk pregnancies. Linear regression showed no significant change during pregnancy, when antibody titer and ADCC test results were compared with every two foregoing measurements ($p<0.0001$).

Conclusion(s): Early determination of the anti-K titer is sufficient to select pregnancies at increased risk for HDFN with need for transfusion therapy. If the K status of the fetus is known to be positive, a titer of 4 or higher can be used to target intensive clinical monitoring.

Keywords: alloimmunization, anti-K, diagnostic accuracy, hemolytic disease of the fetus and newborn (HDFN), intra-uterine blood transfusion, laboratory tests, red blood cell antibodies, screening program.

Introduction

Hemolytic Disease of the Fetus and Newborn (HDFN) is caused by red blood cell (RBC) antibodies developed by the mother and transferred to the foetus.(10, 25) Kell (K) alloantibodies are second to RhD alloantibodies in importance as the cause of severe HDFN.(10, 25) K alloantibodies cause hemolysis of fetal erythrocytes and also inhibit the fetal erythropoiesis.(135-137) Without treatment, HDFN may result in progressive fetal anemia, fetal hydrops, asphyxia and perinatal death. (138) After birth, neonatal hyperbilirubinemia may lead to 'kernicterus', cause of neurodevelopmental impairment with athetoid cerebral palsy, hearing problems and psychomotor handicaps.(3, 5-8, 139, 140) Even though the incidence of fetal alloimmune hydrops has declined in the last decades,(141) this condition is still a well-known risk factor for adverse perinatal and long-term outcomes.(12) Severe anti-K-mediated HDFN may develop early in pregnancy, and often presents with hydrops before 20 weeks gestation.(12, 137, 142) Postnatally, anti-K-mediated HDFN is characterized more frequently by anemia than by hyperbilirubinemia, compared with HDFN caused by anti-D or other type of Rh alloantibodies.6

Red blood cell (RBC) alloimmunization should ideally be detected early in pregnancy upon routine RBC antibody screening. In most centers, to identify pregnancies at risk for severe HDFN, the titer of clinically relevant RBC alloantibodies is determined. (10, 21, 26, 139) If the titer is above a certain threshold, patients are referred to a maternal-fetal medicine center for close surveillance and, if needed, for fetal or neonatal treatment. (21, 26) High-risk pregnancies are monitored with ultrasound and Doppler middle cerebral artery peak systolic velocity (MCA-PSV) measurements, to predict the presence of fetal anemia.(18, 29, 143) Severe fetal anemia can be successfully treated using intrauterine transfusions (IUT). Neonates may require phototherapy or neonatal (exchange) transfusions.(144)

In the Netherlands, fetal K (Kell) genotyping is performed with cell-free fetal DNA isolated from maternal plasma.(22) K-alloimmunized pregnancies with a K-positive fetus are monitored by serial antibody titer measurements and by the Antibody Dependent Cellular Cytotoxicity (ADCC) bioassay, a monocyte based assessment of the destructive capacity of the antibodies.(58, 145, 146) However, there is still controversy on which critical titers and ADCC cut-off levels indicate a high risk for anti- K-mediated HDFN.(21, 26, 61, 147-150)

The aim of this study was to assess the performance of anti-K titer and ADCC measurements in K-alloimmunized pregnancies with a K-positive fetus, to predict severe HDFN requiring transfusion therapy.

Methods

Setting and Prevention program in the Netherlands

In the Netherlands, all pregnant women are typed for ABO, RhD and Rhc blood group antigens and screened for RBC antibodies at the first trimester booking visit. All screen-positive samples are sent to one of two national reference laboratories for confirmation and determination of the antibody specificity. These laboratories are Sanquin Diagnostics, Amsterdam (90% of the pregnant population) and the Special Institute for Blood Group Investigations (BIBO), Groningen (10% of the pregnant population). When clinically relevant RBC antibodies are detected, i.e. antibodies with the potency to destroy fetal RBC's, the father of the fetus is typed for the cognate antigen(s). In case the father is antigen-positive, or his type is not known, non-invasive fetal typing with cell-free fetal DNA isolated from maternal plasma is offered (for *RHD*, *RHC*, *RHc*, *RHE* and *K*), since 2004. (22) If the fetus is antigen-positive, serial testing (starting with every four weeks, from 24 weeks every three weeks, from 36 weeks every two weeks) of maternal antibody titers and the ADCC test is performed. Following current Dutch guidelines, a K-antibody titer ≥ 2 and/or an ADCC-test result ≥30% indicate a substantial risk for K-mediated HDFN, and the fetus will be weekly or every two weeks monitored with MCA Doppler measurements.(26) Laboratory follow-up is stopped if these thresholds are reached. Severe fetal anemia is treated with intrauterine transfusion(s) (IUT's) at the Leiden University Medical Center (LUMC), which is the national Dutch reference center for fetal therapy. The threshold for suspected severe fetal anemia requiring IUT was 1) a MCA-PSV 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 Liley's zone II or zone III (only in the early years of this study). (29, 151)

Laboratory testing

Both reference laboratories assess antibody titers, in phosphate-buffered saline by doubling dilutions, with the indirect antiglobulin test (IAGT), using an anti-IgG reagent and heterozygous K-positive RBCs.(152)

The ADCC test, as described by Engelfriet and Ouwehand, is only performed at Sanquin Diagnostics in Amsterdam.(58) Fetal K typing is also only performed at Sanquin Diagnostics.(22)

Study design

We performed a retrospective cohort study, including all pregnancies diagnosed with anti-K in the Netherlands, between January 1st 1999 and April 1st 2015. All K-immunization cases were identified at the two national reference laboratories. Women with K alloimmunization and antibody titers ≥2 and/or ADCC test results > 30% were usually referred to the LUMC for monitoring or treatment. All these cases could therefore also be identified in the LUMC database. We only included pregnancies with a K-positive fetus.

Outcomes

The primary outcome was the diagnostic accuracy (sensitivity, specificity and predictive values) of antibody titers and ADCC tests to predict severe K-mediated HDFN, which was defined as the need for intrauterine or postnatal transfusion.

Data collection

We collected the results of laboratory monitoring during pregnancy from Sanquin Diagnostics and data concerning clinical monitoring and IUT treatment during pregnancy, from the LUMC databases. Neonatal outcome data on treatment with blood transfusion(s) or phototherapy during the first three months of life were extracted from their medical files, by contacting the obstetric care provider, the pediatrician or the local hospital laboratories.

Analysis

Categorical variables were described as number and percentage and continuous variables by median and interquartile range P25-75%. To establish the optimal cut-off for antibody titer and ADCC test results, Receiver Operating Characteristics (ROC) curves were constructed for both the first and the highest measurement. Subsequently, the sensitivity, specificity and positive and negative predictive values for the prediction of fetal and neonatal hemolytic disease were calculated with 2x2 tables for different cut-off levels. To determine the best interval between consecutive titer and ADCC measurements, in order to adequately predict severe HDFN, a linear regression analysis (Pearson product-moment correlation coefficient) was performed. All analyses were performed with SPSS Statistics (version 23).

Ethical considerations

Clinical data were provided by the health care professionals as part of the quality evaluation of the routine laboratory monitoring of RBC alloantibody-complicated pregnancies The data were stored according to the Dutch established codes of conduct for responsible use of patient material and data, as approved by the Leiden University Medical Center. Ethical approval was not necessary according to the Dutch law on medical scientific research involving human subjects and according to the rules published by the Central Committee on Research involving Human Subjects (http://www.ccmo.nl/nl/niet-wmo-onderzoek).

Results

Study population

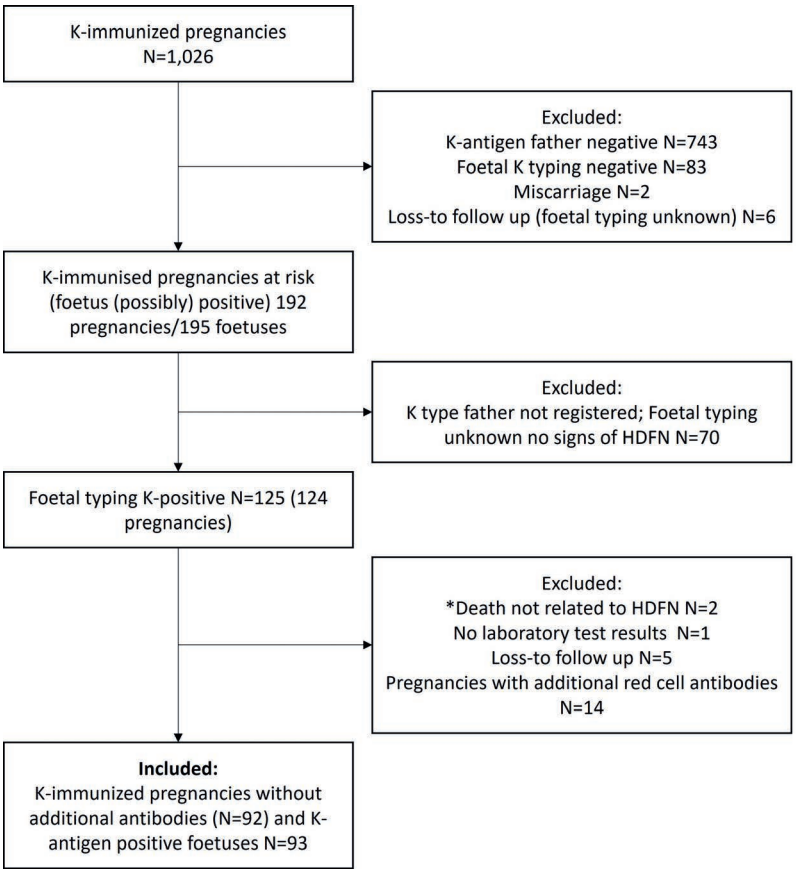
In 16 years, 1,026 K-immunized pregnancies were identified, including three pair of twins (Figure 1). After exclusion of pregnancies with K-negative fathers (n=743) and/or K-negative fetuses/children (n=83), miscarriages (<16 weeks, n=2) and loss-to follow up (n=6), 192 pregnancies with 195 K-positive fetuses remained for analysis. In another 70 pregnancies, the K type of the father and that of the child were unknown (after 2008 all fetal K status were known), but the absence of any sign of HDFN could be confirmed in all cases. After exclusion of these pregnancies, 124 pregnancies with 125 K-positive fetuses remained.

Another seven of these 125 fetuses were excluded from our analysis, because of perinatal death, clearly not related to K-immunization (n=2) or unknown neonatal outcome (n=5). One case with severe HDFN (hydrops) detected late in pregnancy was excluded, because of lack of laboratory data. Furthermore, we excluded 24 cases with additional red cell alloantibodies, which may have contributed to the severity of HDFN. The remaining 93 (92 pregnancies) K-positive fetuses were used in our diagnostic accuracy analysis.

Fifty-six percent (49/93) of children received either intrauterine (48/93; 52%) or neonatal (1/93; 1%) transfusion, whereas the remaining 47% (44/93) had no signs of HDFN or was only treated with phototherapy . There were three cases with two pregnancies, all women had in all pregnancies titers above 4.

There were three cases of perinatal death related to the K-immunization (titers variating from 128-1024). One case was closely monitored every two weeks with ultrasound. At 23 weeks the fetus was unexpectedly found to be hydropic, with an MCA Doppler result indicating fetal anemia. Fetal demise occurred just before the first IUT could be given. One case was a very early onset of fetal anemia with hydrops detected at 15+3 weeks of gestation. The fetus died at 16 weeks; intrauterine transfusion was not performed because of the poor condition and prognosis at this early stage of the pregnancy. In the last case, the fetus was initially predicted to be K-negative with non-invasive fetal K antigen typing, at 12 weeks of gestation. Awaiting the repeated and definitive K-typing result, clinical monitoring was not performed and the fetus died at 18 weeks of gestation. At 19 weeks ‘gestation, non-invasive fetal K typing showed the fetus to be K-positive.

Figure 1: *Flowcharts of inclusion K-immunized pregnancies with a K-antigen positive fetus.*



**Causes of death not related to HDFN were: one fetus had growth restriction in combination with dimorphism and died intra-uterine and one neonate had a congenital neuro-muscular disorder and died 7 days post-partum.*

The median gestational age at the first laboratory testing was 14 weeks (P25-P75: 13-18 weeks). The median gestational age at the last measurement of titer and ADCC test was 26 weeks (P25-75: 18-35 weeks). The median first K-titer was 64 (P25-75: 8-256) and the highest median titer was 128 (P25-75: 16-256). The median first ADCC was 35% (P25-75: 10-57.5%) and the highest median ADCC was also 35% (P25-75: 15-65%). The median number of days from the last test to the first IUT or to delivery was 26 days (P25-P75: 9-77 days). The first IUT was performed in week 24 (P25-75: 22-28 weeks). The median number of laboratory tests performed per pregnancy was 5 (P25-75: 3-8).

Table 1.

First titre Cut-off	Positive tests	Need for transfusion					
		True positive	*Missed HDFN cases	Sens % (95%-CI)	Spec % (95%-CI)	PPV % (95%-CI)	NPV % (95%-CI)
≥2	81	49	0	100 (91-100)	27 (15-43)	60 (49-71)	100 (70-100)
≥4	77	49	0	100 (91-100)	36 (23-52)	64 (52-74)	100 (76-100)
≥8	70	47	2	96 (85-99)	48 (33-63)	67 (55-78)	91 (70-98)
≥16	62	47	2	96 (85-99)	66 (50-79)	76 (63-85)	94 (77-99)
≥32	57	45	4	92 (80-97)	73 (57-85)	79 (66-88)	89 (73-96)
≥64	50	43	6	88 (75-95)	84 (70-93)	86 (73-93)	86 (71-94)
≥128	43	38	11	78 (63-88)	89 (75-96)	88 (74-96)	78 (64-88)

Number of positive tests, sensitivity, specificity and predictive values of K-mediated pregnancies without additional antibodies to predict the need for transfusion by cut-off first titre (N=93).

**Cases with necessity for transfusion therapy that would be missed when cut-off used.*

Diagnostic accuracy of the K-antibody titre and ADCC test

The ROC curves for respectively the first and the highest anti-K titer, correlating with severe HDFN, with need for transfusion therapy (n=93) The Area Under the Curve (AUC) for the first measured K antibody titer to predict the need for transfusion therapy was 0.917, for the highest titer during pregnancy the AUC was 0.906. We defined the optimal cut-off point at a sensitivity of 100% (91-100 95% CI) and combined with the highest specificity of 27% (15-43, 95% CI). Thus, an optimal cut-off for the first and highest titer was assessed at 4 (table 1).

The AUC for the highest ADCC test result was 0.843. If a sensitivity of 100% was taken, the optimal cut off value appeared to be below the first test outcome of ADCC <10% (data not shown). Therefore, additional ADCC testing seems to be not informative for the prediction of severe HDFN (data not shown).

Linear correlation between consecutive measurements of titre and ADCC test

Since the AUCs for the highest titer and the first titer hardly differed, we investigated whether the titer and ADCC test results changed significantly during pregnancy. Linear regression showed no significant change, when antibody titer and ADCC test results were compared with every two foregoing measurements. A Pearson product-moment correlation coefficient was computed to assess the relationship between the titer and ADCC measurements and the two foregoing measurements (for scatterplots see appendix 1 a,b,c,d). Overall, there was a strong correlation between titer and ADCC measurements with the two foregoing measurements. The small, non-clinical relevant difference between the measurements is explained for 94% and 91% (titer) and 87% and 84% (ADCC) by the two foregoing measurements ($p<0.0001$).

Comments

Main Findings

In a 16 year unselected cohort, representing screening results of 3.2 million pregnancies resulting in life births in the Netherlands, we identified 93 pregnancies complicated by the presence of anti-K in the presence of a K-positive fetus. We determined that, if the K status of the fetus is positive, an anti-K titer of 4 identifies all cases with a high risk for severe HDFN, defined as the need for intra-uterine or postnatal transfusions. Test results of both titer and ADCC-test did not change

significantly during pregnancy. The first titer appeared to have the highest power to predict the necessity of transfusion therapy in K-alloimmunized pregnancies.

Strengths and weaknesses

To our knowledge this is the first large registry-based cohort study, including an unselected complete population of K-alloimmunized pregnant women with a K-positive child. Most other studies included a selected group of women with an increased risk for severe HDFN, for example women referred to a regional or national referral center.(147-149)

In our study, 93 out of 1,026 (9%) of the K-immunized pregnancies were considered as at risk for HDFN (fetus K-positive) and included in the analysis. A weakness of our study is that the K status was not known for all fetuses; yet we think no severe cases of HDFN with need for intra-uterine transfusion were missed as they would have been referred to the LUMC.

Clinical implications

Overall, we observed in this unselected population, that over 50% of K-positive fetuses of K-alloimmunized mothers need either intrauterine or postnatal transfusion therapy. At a cut-off of 4 for the first titer the specificity is 27% and the positive predictive value for transfusion therapy is 60%. This relatively high positive predictive value implicates that transfusion therapy is needed in two out of three K-positive children of mothers with a K titer of 4 or above, and may be an argument to accept this relatively low cut-off titer. In order to fine tune the selection of high-risk cases, it would making it worthwhile to add fetal K typing to the diagnostic algorithm. With higher cut-off titers, for example 16, still 96% of severe cases will be followed, raising test specificity to 66% and the positive predictive value to 76%. With this cut-off titers of 16 the number of missed cases of severe HDFN is two.

Our proposed optimal cut-off point of the anti-K antibody titer of 4 is only one dilution step lower than suggested by Moise et al., who proposed a threshold of 8. It is also lower than McKenna et al.[147] who proposed a threshold of 32, based on a smaller cohort with eight cases of severe HDFN, all with titers of >=32. In contrast, Leggat et al., including 16 K-positive fetuses, and our previous study, as reported by Van Wamelen et al. including 41 K-positive fetuses, reported one case each needing intrauterine transfusion therapy at anti-K titers of 2. (148, 149) These studies thus support our finding that also a low anti-K titer can be responsible for a severe course of anti-K-mediated HDFN. However, in our study only 16 pregnancies of 93 cases

had titers below 4, we didn't find severe cases of HDFN in this group. Therefore K-mediated HDFN with need for transfusion therapy in cases with titers below 4 is very rare.

Although titer measurements can vary between laboratories, also with established techniques, in general a comparison be made with one-fold dilution difference between observers and between laboratories. It is advised to use an indirect antiglobulin test without additives.(152) Non-invasive fetal K-typing with cell-free DNA isolated from maternal plasma is not available in all countries. Fetal K-typing can be performed with DNA obtained via amniocentesis. Amniocentesis is an invasive procedure, with risks for the pregnancy and a possible rise in anti-K titers. Therefore, it should be considered, if non-invasive fetal K typing is not possible via a reference laboratory, if close monitoring of anti-K complicated pregnancies with MCA-Doppler can be used for timely detection of the occurrence of fetal anemia.

We observed that the monocyte-based ADCC test was not suitable to accurately select high-risk K-alloimmunized pregnancies. This might be due to the pathogenesis of anti-K-mediated HDFN, in which both the suppression of erythropoiesis and hemolysis of fetal RBC may be of importance.(135, 136, 153) Recently, it was also shown that the glycoprofile of alloantibodies may influence antibody pathogenicity and therefore a putative diagnostic marker.(153) Therefore, it may be that other type of bioassays, testing such antibody characteristics, may improve test specificity. (146, 154, 155, 27)

A first step in the diagnostic algorithm, that we currently use, is non-invasive fetal K typing with cell-free DNA isolated from maternal plasma.(156) Prediction of K-negativity warrants a high sensitivity of this PCR-based testing and the confirmation on sufficient levels of fetal DNA; also in our series early prediction of K-negativity was incorrect once in week 12 of pregnancy.(157) Since cell-free fetal DNA levels raise in the first trimester of pregnancy, for fetal K typing a conclusive result can in general be provided around week 18 of pregnancy.(22)

Conclusion

To select pregnancies with an increased risk for anti-K-mediated HDFN requiring frequent monitoring to detect fetal anemia, determination of the anti-K titer once early in pregnancy is sufficient. The optimal cut-off value is a titer of 4. Following the detection of anti-K, fetal K-typing, preferably using a non-invasive method, is an important step in efficient management. In pregnancies with an anti-K titer of 4 or higher and a positive fetus, 60% of fetuses or neonates requires transfusion therapy. Since the ADCC test is not useful in the prediction of fetal hemolysis in the presence of an anti-K we recommend discontinuing its use for these pregnancies.

Acknowledgements

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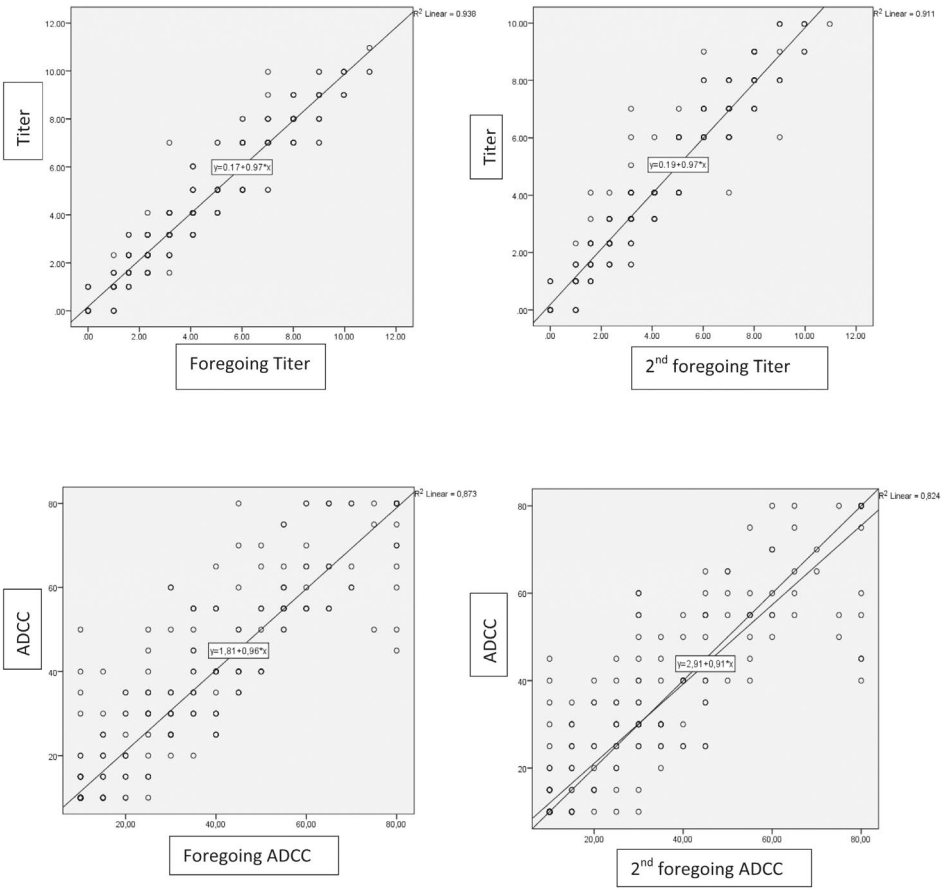

Appendix 1: *Scatterplots of relation between titer and ADCC measurements with the two foregoing measurements.*

Chapter 6

Diagnostic value of laboratory monitoring to predict severe hemolytic disease of the fetus and newborn in non-D and non-K-alloimmunized pregnancies

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Abstract

Background: Pregnant women are routinely screened for red blood cell (RBC) antibodies early in pregnancy. If RBC-alloantibodies are detected, repeated laboratory testing is advised to timely identify pregnancies at high risk for severe hemolytic disease of the fetus and newborn (HDFN). We assessed for RBC alloantibodies, other than anti-D or anti-K, cut-offs for the titer and the antibody dependent cellular cytotoxicity (ADCC) test to select high-risk cases. To advise on test repeat intervals, and to avoid unnecessary testing, we evaluated the chance for exceeding the cut-offs for Rh antibodies other than anti-D, Jk, Fy, and S/s antibodies.

Study design and methods: Diagnostic value of antibody titer and ADCC test was determined with data from a prospective index-cohort study, conducted in 2002-2004. Laboratory test outcomes were from a recent observational cohort (2015-2016).

Results: A titer cut-off of ≥16 showed a sensitivity of 100% (95% CI:73-100%) and a positive predictive value (PPV) of 17% (95% CI:14%-20%). The percentage of pregnancies reaching a titer above the cut-off of ≥16 varied from 0% for anti-Jka / Jk^b (n = 38) to 36% for anti-c (n = 97). The ADCC test showed no cut-off with a 100% sensitivity. However, in cases with a titer ≥16 and an ADCC test ≥30% a PPV of 38% was obtained to detect severe HDFN.

Conclusion: A titer cut-off of ≥16 is adequate to detect all cases at risk for severe HDFN; the ADCC test may add a more accurate risk estimation. Repeated testing is recommended in pregnancies with anti-c. In pregnancies with other Rh antibodies a repeated test in the third trimester is recommended.

Introduction

In most western countries pregnant women are routinely screened early in pregnancy for the presence of red blood cell (RBC) alloantibodies. (25, 26, 64, 158) RBC alloantibodies of the mother can cause hemolytic disease of the fetus and newborn (HDFN). HDFN is characterized by anemia, which can occur early in pregnancy, and by high bilirubin levels after birth. Not all RBC alloantibody specificities cause HDFN. Furthermore, the titer of the RBC alloantibody and its biologic activity are correlated with a mild or more severe course of disease. To timely identify pregnancies at risk for a severe course of HDFN, defined as a need of fetal therapy, preterm delivery, or intensive neonatal treatment, repeated laboratory testing during pregnancy is advised.(25, 26, 64, 158) In this pre-selected group, fetal anemia can be diagnosed with a high sensitivity and specificity by non-invasive ultrasonography, using Doppler middle cerebral artery blood (MCA) flow velocity measurements.(25, 29)

Most cases of severe HDFN are caused by anti-D, less frequently by anti-c and anti-K, and in a rare case by other Rh antibodies.(1-4, 6) Anti-Fya/-Fyb increases the risk for neonatal icterus, needing phototherapy treatment.(6) For almost all other RBC alloantibody specificities there is only casuistic evidence that these cause a severe HDFN disease trajectory.(4, 7, 10)

The policy of laboratory monitoring in alloimmunized pregnant women varies between countries from 4-week intervals to once at 28-34 weeks or only pregnant women with anti-D, –c, and/or -K on a regular basis. When a certain cut-off value (“critical titer”) is exceeded, patients are referred to a maternal-fetal medicine center for close surveillance and, if needed, for fetal or neonatal treatment.(25, 26, 64, 158)

In the Netherlands, in pregnancies with clinically relevant RBC alloantibodies and a fetus (possibly) positive for the cognate antigen, not only serial antibody titer measurements are advised, but also a monocyte-driven antibody-dependent cellular cytotoxicity (ADCC) assay to determine the destructive capacity of the antibodies.4 In case of anti-D, the ADCC is validated to discriminate well between pregnancies with low and high risk for severe HDFN.(7, 19, 139, 159)

For anti-K, we recently reported the results of a nationwide study (1999-2015) concluding that for K-immunized pregnancies a critical titer of four should be used to select pregnancies at high risk for fetal hemolysis, while the ADCC test appeared not to add to the selection of cases at risk for severe HDFN.(160)

In general, for non-D/non-K alloantibodies a cut-off level of 32 is used (reviewed by Moise et al.)(4, 159) and confirmed by a study of Hackney et al., indicating that all cases of severe HDFN with need for IUT or with neonatal hemoglobin levels <10 g/

dL were identified with a titer cut-off of 32. (59) Similarly, Joy et al. concluded for anti-E a titer cut-off value of 32.(161)

In the Netherlands, the titer cut-off value of ≥16 and/or an ADCC test result of ≥30% is used to timely select pregnancies at risk for fetal hemolysis by non-D/non-K alloantibodies. We present the data underlying this policy.(26)

The main goal of laboratory testing is to timely identify all pregnancies possibly at risk for fetal hemolysis, with an almost 100% sensitivity. This might result in a high proportion of unnecessary tests and unnecessary health care costs. For pregnant women, frequent laboratory testing is invasive and can cause unnecessary anxiety; on the other hand, it can also be reassuring.

The aim of the current study was to gain more insight into the optimal frequency of laboratory testing in pregnancies complicated by RBC alloantibodies other than anti-D or anti-K. Therefore, we first report the evidence underpinning the previously determined cut-offs for titer and ADCC test in pregnancies complicated by non-D/non-K RBC alloantibodies, in order to detect severe HDFN requiring transfusion therapy. Second, to assess the added value of repeated laboratory testing for selection of high-risk cases, we investigated the chance of exceeding the determined cut-offs, according to antibody specificity.

Materials and methods

Organization of the prevention program in the Netherlands

In the Netherlands, all pregnant women are typed for ABO, D and c blood group antigens, and screened for RBC antibodies at the first trimester booking visit. All screen-positive samples are sent to one of two national reference laboratories for confirmation and determination of the antibody specificity: Sanquin Diagnostics, Amsterdam (90% of the pregnant population) and the Special Institute for Blood Group Investigations (BIBO), Groningen (10% of the pregnant population). When RBC antibodies are detected with the potency to destroy fetal RBC's, the father of the fetus is typed for the cognate antigen(s). If the father is heterozygous or if his antigen type is unknown, non-invasive fetal typing with cell-free fetal DNA isolated from maternal plasma is offered (for RHD, RHC, RHc, RHE, and K), since 2004.(24, 26) If the fetus is (probably) antigen-positive, serial titration of maternal antibodies and the ADCC test are performed.

The two reference laboratories use an expert opinion–based protocol to determine the interval for repeat testing. The interval depends on the antibody specificity

and the test results: pregnancies with anti-D, anti-K, or anti-c are monitored most frequently, with a 2-week interval during the third trimester. Other Rh antibodies (anti-C/-E/-e) are monitored every 3 weeks during the last trimester, while in case of other antibodies (anti-Fya/-Fyb, -Jka/-Jkb, -S/-s and other) the laboratory testing is repeated only once in Week 30. Since 2009, the Dutch guideline states that for antibodies other than anti-D or anti-K, a titer ≥16 and/or an ADCC test result ≥30% indicates a risk for HDFN; in these cases the fetus will be monitored with MCA Doppler measurements in a specialized center with a frequency depending on the antibody specificity.(58) Laboratory follow-up is usually discontinued if these cut-offs are reached. Severe fetal anemia is treated with IUTs at the Leiden University Medical Center (LUMC), the national Dutch reference center for fetal therapy.

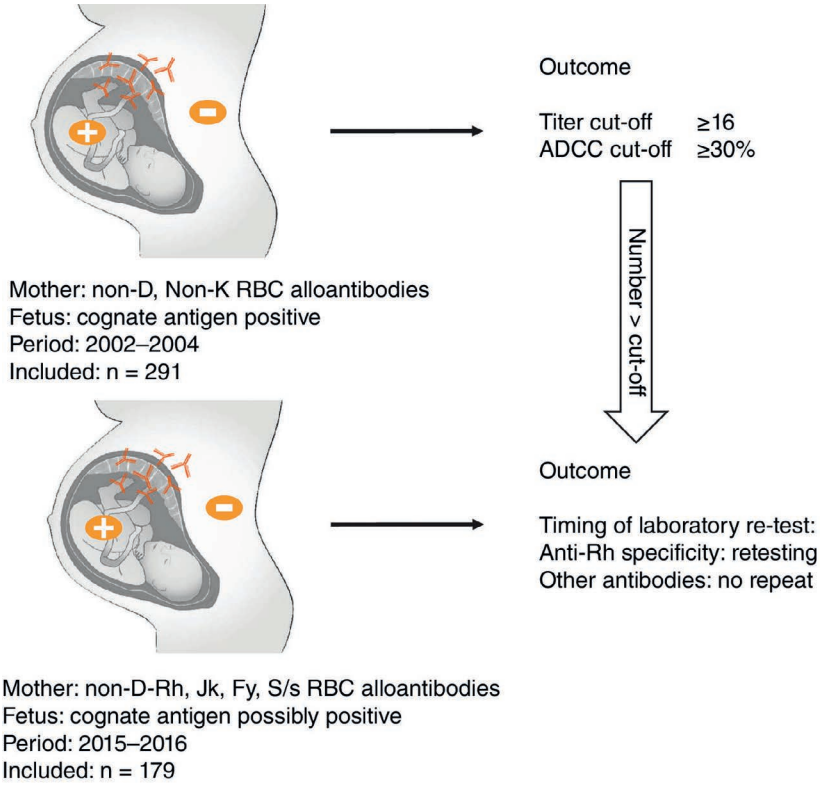
Laboratory testing

Both reference laboratories perform antibody titration in tubes, in phosphate-buffered saline with addition of 2% of a 22% solution of bovine serum albumin, by doubling dilutions, with an incubation time of 30 minutes, with the indirect antiglobulin test (IAGT), using an anti-IgG reagent. For Rh antibodies, (anti-c, -D, and -E), double-dose antigen-positive RBCs were used and for all other antibody specificities (e.g., anti-C, anti-e, -K, -Fya, -Fyb, –Jka, –Jkb, -S, –s) single-dose antigen positive RBCs. Double-dose c, D, and E positive RBCs are also used in the ADCC test. The ADCC test, as described by Engelfriet and Ouwehand, is performed at Sanquin Diagnostics in Amsterdam for all Dutch samples.15 Fetal typing is also only performed at Sanquin Diagnostics.

Study design and study population

An outline of the study is provided in Fig. 1. To assess the diagnostic value of laboratory testing with titer and ADCC test, data were used from a nationwide prospective index cohort study, conducted to evaluate the effectiveness of first trimester RBC antibody screening for early detection of cases at risk for HDFN (OPZI-study). All pregnant women with clinically relevant non-D RBC antibodies, recognized by routine first trimester screening (n = 1,002) from September 1, 2002 until June 1, 2003 and October 1, 2003 until July 1, 2004 (population: n = 306,000) were included. This study is described in more detail, in a previous publication.(10)

Figure 1: *Outline of studies to determine test algorithm for non-D, non-K red blood cell (RBC) alloantibody screening in pregnancy.*



A case was included in the current study if the fetus was positive for one or more antigens against which maternal antibodies were present during pregnancy and if the last test result of laboratory monitoring was performed ≥32nd week of pregnancy or within 21 days before birth, or the last test result indicated a risk for severe HDFN, needing clinical monitoring (titer ≥64 or ADCC test result ≥50%). When antibodies were newly detected in cord blood, for which no laboratory monitoring was performed during pregnancy, the case was excluded.

In a second cohort, we evaluated the results of the current policy of laboratory monitoring. For this purpose, we performed a retrospective study, including all women with Rh antibodies, other than D (anti-c/-C, -E/-e), anti-Fya/-Fyb, anti-Jka/-Jkb and/or anti-S/-s, but without the presence of anti-D and/or anti-K, detected at first trimester antibody screening in 2015 and 2016, at risk for HDFN (partner positive for the cognate antigen and/or positive result of non-invasive fetal typing with cell-free DNA). Cases were selected at Sanquin Diagnostic Services.

Outcomes

The primary outcome of the first part of our study was the diagnostic value of laboratory testing, to predict severe HDFN, defined as the need for antenatal or neonatal transfusion therapy during the first week of life, or mild HDFN (only neonatal phototherapy). Intensive phototherapy, starting immediately after birth to prevent (exchange) transfusions in children at high risk for severe HDFN, was not usual care during the study period.(15) In twins with two antigen positive children, the outcome of the most severely affected child was used to categorize disease severity.

We aimed to evaluate the current policy of laboratory monitoring in a second observational cohort, by assessing the chance for exceeding the laboratory cut-offs.

Data collection

For the study on diagnostic value, information about laboratory testing (antibody specificity, antigen typing of father and fetus, titers, and ADCC test results) was collected at the two reference laboratories that routinely perform these analyses. As part of the OPZI-study, the newborn's antigen typing, RBC alloantibody screen, including analysis of an eluate was determined.(10) Clinical outcome data (i.e., number of IUT(s), neonatal blood transfusion(s), phototherapy, gestational age at birth, perinatal death) and neonatal laboratory test results (DAT, antigen typing, hemoglobin level, bilirubin level) were collected from the obstetric care provider. To evaluate the current policy of laboratory monitoring, in the second part of this study, we collected the laboratory testing results from Sanquin Diagnostics (titers, ADCC test, antigen typing of the father, fetal genotyping, formation of additional antibodies) of all samples obtained during pregnancy.(10) No clinical data were collected. We calculated the gestational age at each follow-up laboratory test from the time interval in days between this follow-up test and the routine first trimester blood sampling during pregnancy, assuming that the first trimester screening was performed at 12 weeks (84 days).

Analysis

Categorical variables were described as number and percentage and continuous variables by median and interquartile range P25%-P75%. Associations between categorical variables were tested by Pearson's chi-square test.

Test characteristics (sensitivity, specificity, positive and negative predictive values) for the prediction of severe HDFN (antenatal or neonatal transfusion therapy) were calculated with 2 × 2 tables for different cut-off levels. To establish the optimal cut-off for antibody titer and ADCC test results, receiver operating characteristic curves (ROCs) were constructed. We considered an area under the ROC curve (AUC) of 0.8 or more as a useful predictive test.

All analyses were performed with SPSS version 24.0, except the confidence intervals for measures of diagnostic value. These were calculated with MedCalc version 18.11, available via https://www.medcalc.org/calc/diagnostic_test.php.

Ethical considerations

The index cohort study was approved by the ethical review board of the Academic Medical Center (AMC) Amsterdam. Consent was given by all women included in the study.

In the retrospective study, the laboratory data were anonymized. The data were stored according to the Dutch established codes of conduct for responsible use of patient material and data, as approved by the Leiden University Medical Center. Ethical approval was not necessary according to the Dutch law on medical scientific research involving human subjects and according to the website of the Central Committee on Research involving Human Subjects.

Results

Study population

The index cohort study performed from 2002 to 2004 included 1,002 women with RBC alloantibodies other than anti-D, of which 900 gave consent for collection of cord blood and clinical data. After exclusion of mothers with children with a negative or unknown antigen typing, antigen-positive children of mothers with anti-K, and of mothers with only one laboratory test or a last testing before 32 weeks of the antibody for which the child was antigen-positive, 291 pregnancies remained for

analysis. (Fig. 1 and Fig. S1, available as supporting information in the online version of this paper.)

In the subsequently performed cohort study covering 2015 and 2016, 516 pregnancies (510 women) with RBC alloantibodies directed against antigens in the Rh system other than D, anti-Fya/-Fyb, anti-Jka/-Jkb and/or anti-S/-s, respectively were included. After exclusion of pregnancies with additional anti-D and/or anti-K, and of pregnancies not at risk for HDFN because the partner was antigen negative for all the cognate antigens or the fetus was antigen negative, 279 pregnancies remained for analysis. (Fig. 1 and Fig. S2, available as supporting information in the online version of this paper.)

Diagnostic value of RBC alloantibody titer and ADCC test

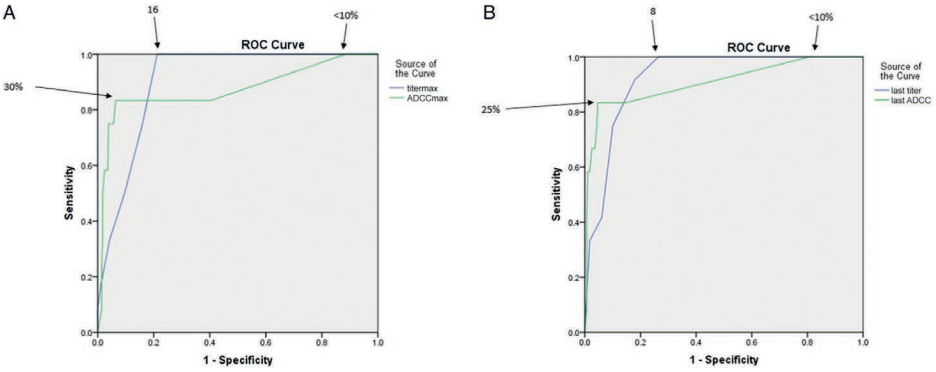
Of the 291 included cases in the index cohort study, 12 children showed severe HDFN, and treatment with an IUT or (exchange) transfusion was needed. This was caused in 10 cases by anti-c or anti-c + anti-E immunization.6 Two other cases were caused by anti-e and by anti-C + anti-Jka, respectively.6 In 49 cases (17%) only phototherapy was given; these cases were mainly caused by anti-c, anti-E, or anti-Fya (Table S1, available as supporting information in the online version of this paper). The ROC to predict the need for antenatal or neonatal transfusion using either the maximum titer, the last titer, or the ADCC test result all showed AUCs above the predefined cut-off of 0.80, with slightly higher values for the last titer and ADCC test result compared to the maximum titer and ADCC. The AUCs to predict the need for transfusion from the first titer or ADCC showed AUCs below the predefined cut-off (Fig. 2A,B and Table 1).

Table 1. *AUC* of maximum and last titer and ADCC test result to predict HDFN disease severity in pregnancies at risk for HDFN*

	Predicted outcome		
	Need antenatal or neonatal transfusionAUC*(95% CI)	Need neonatal phototherapy AUC*(95% CI)	Need antenatal or neonatal transfusion or phototherapy AUC*(95% CI)
Maximum titer	0.90 (0.86-0.95)	0.69 (0.62-0.77)	0.76 (0.69-0.83)
Last titer	0.93 (0.88-0.97)	0.66 (0.58-0.75)	0.74 (0.67-0.81)
Maximum ADCC	0.87 (0.73-1.00)	0.61 (0.52-0.71)	0.68 (0.60-0.77)
Last ADCC	0.91 (0.80-1.00)	0.58 (0.49-0.68)	0.67 (0.58-0.75)

All children were positive for the cognate antigen.
** AUC = Area Under the Curve.*

Figure 2A,B:



A) Receiver Operating Curve to predict the need for transfusion because of HDFN by non-D–/non-K antibodies (n = 291) from the maximum titer and ADCC test result. Legend: All children were positive for the cognate antigen. B) Receiver Operating Curve to predict the need for transfusion because of HDFN by non-D/non-K antibodies (n = 291) from the last titer and ADCC test result. Legend: All children were positive for the cognate antigen.

The AUCs to detect the need for phototherapy and the need for transfusion or phototherapy were all below the predefined cut-off (Table 1, Figs. S3 and S4, available as supporting information in the online version of this paper).

Sensitivity, specificity, and predictive values

To detect HDFN with a need of transfusion therapy, a cut-off for the maximum titer of ≥16 resulted in a sensitivity of 100% with a specificity of 79% and a positive predictive value (PPV) of 17%. In the 10 cases with anti-c, a titer of 16 showed a sensitivity of 100% with a specificity of 69% and a PPV of 27% to detect severe HDFN, needing transfusion therapy (Table 2). For the other RBC alloantibody specificities the PPV was only 6% (Table 2). In the severe HDFN case caused by anti-e, the maximum titer and ADCC test were 32, respectively 70%; in the severe case caused by anti-C + anti-Jka these were 16, respectively <10%.

Table 2. *Number of positive and negative tests, sensitivity, specificity, and predictive values to predict the need for antenatal or neonatal transfusion therapy, by cut-off maximum titer in pregnancies with RBC alloantibodies* and a child positive for the cognate antigen(s)*

n = 291			Need for antenatal or neonatal transfusion therapy (n = 12)				
Cut-off	Test result		True positives	Sensitivity	Specificity	PPV†	NPV†
	+n	−n		% (95% CI)	% (95% CI)	% (95% CI)	% (95% CI)
≥16							
all cases	72	219	12	100 (73.5-100)	78.5 (73.2-83.2)	16.7 (13.8-20.0)	100
anti-c	37	60	10	100 (69.2-100)	69.0 (58.1-78.5)	27.0 (21.3-33.6)	100
other Rh‡	22	89	2	100 (15.8-100)	81.7 (73.1-88.4)	9.1 (6.3-12.9)	100
≥32							
all cases	54	237	9	75.0 (42.8-94.5)	83.9 (79.0-88.0)	16.7 (11.6-23.4)	98.7 (96.7-99.5)
anti-c	27	70	8	80.0 (44.4-97.5)	78.2 (68.0-86.3)	29.6 (20.3-41.1)	97.1 (90.7-99.2)
other Rh‡	17	94	1	50.00 (1.3-98.7)	85.3 (77.3-91.4)	5.9 (1.4-21.2)	98.9 (95.8-99.7)

** RBC alloantibodies = red blood cell alloantibodies; all antibodies, excluding anti-D and anti-K.*

† PPV = positive predictive value; NPV = negative predictive value.

‡ other Rh = all Rh antibodies, excluding anti-D and anti-c.

The ADCC test showed a higher specificity and higher PPVs, but did not reach a 100% sensitivity (Table **S3**, available as supporting information in the online version of this paper). Two cases with the need for transfusion, both with a maximum titer of 16, were missed by using the ADCC test result only. Also no cut-off for the titer or ADCC test with a 100% sensitivity could be determined to detect the need for phototherapy only ór for transfusion and phototherapy combined.

Added value of ADCC test

The added value of the ADCC test to detect severe HDFN was investigated in the 72 cases with a titer ≥16. The ROC-curve showed an AUC of 0.774 (95% CI 0.597-0.950). The PPV of the ADCC test in this group increased from 17.5% to 60% with an ADCC test result of respectively ≥10% and ≥ 60%. In the presence of anti-c the PPV increased from 29% to 83%, in the presence of other antibodies from 4% to 25% (Table **3**).

Table 3. *Number of positive and negative tests, sensitivity, specificity, and predictive values to predict the need for antenatal or neonatal transfusion therapy, by cut-off maximum ADCC test in pregnancies with RBC alloantibodies* and a titer ≥16 and a child positive for the cognate antigen.*

N = 72			Need for antenatal or neonatal transfusion therapy (n = 12)				
Cut-off	**Test result**			**Sensitivity**	**Specificity**	**PPV†**	**NPV†**
	+n	**-n**	**True positives**	**% (95% CI)**	**% (95% CI)**	**% (95% CI)**	**% (95% CI)**
≥10%							
All cases	57	15	10	83.3 (51.6-97.9)	21.7 (12.1-34.2)	17.5 (13.8-22.1)	86.7 (62.7-96.2)
anti-c	31	6	9	90.0 (55.5-99.8)	18.5 (6.3-38.1)	29.0 (23.7-35.0)	83.3 (39.9-97.4)
Rh other‡	15	7	1	50.00 (1.3-98.7)	30.0 (11.9-54.3)	6.7 (1.7-22.7)	85.7 (56.3-96.6)
≥30%							
All cases	26	46	10	83.3 (51.6-97.9)	73.3 (60.3-83.9)	38.5 (27.7-50.5)	95.7 (86.0-98.7)
anti-c	19	18	9	90.0 (55.5-99.8)	63.0 (42.4-80.6)	47.4 (34.6-60.5)	94.4 (72.1-99.1)
Rh other‡	2	20	1	50.0 (1.3-98.7)	95.0 (75.1-99.9)	50.0 (8.6-91.4)	95.0 (82.6-98.7)
≥60%							
All cases	10	62	6	50.0 (21.1-78.9)	93.3 (83.8-98.2)	60.0 (33.2-81.9)	90.3 (84.1-94.3)
anti-c	6	31	5	50.0 (18.7-81.3)	96.3 (81.0-99.9)	83.3 (39.9-97.4)	83.9 (73.6-90.7)
Rh other‡	2	20	1	50.0 (1.3-98.7)	95.0 (75.1-99.9)	50.0 (8.6-91.4)	95.0 (82.6-98.7)

** RBC alloantibodies = red blood cell alloantibodies; all antibodies, excluding anti-D and anti-K.*

† PPV = positive predictive value; NPV = negative predictive value.

‡ other Rh = all Rh antibodies, excluding anti-D and anti-c.

Chance of exceeding the cut-offs

In the observational cohort comprising the years 2002 to 2004, the median number of laboratory tests was 6 (range 1-12) for all RBC alloantibody specificities. In this cohort, including only antigen-positive children, 25% of cases reached a titer of ≥16: anti-c 38%, anti-E 23%, other Rh antibodies 12.5%, anti-Fy^{a}/-Fyb 25%, other antibodies 8.5%. Here it should be noted that anti-c and anti-E are both tested with double-dose antigen positive cells.

From the cohort tested in 2015 and 2016 no follow-up samples were received in 23 out of 279 pregnancies (8%), despite a titer <16 and an ADCC test result <30% and the advice to repeat laboratory testing. This may be because of a miscarriage or because the father was typed antigen negative in the referring center. In the remaining 256 pregnancies, the median number of tests was four (range 1-13), varying from three tests if anti-Fy^{a}/-Fy^{b}, anti-Jk^{a}/-Jk^{b}, or anti-S/-s were present to 6.5 if Rh alloantibodies (other than anti-D) were present.

In 11 cases (4%), the first test results were already above the set cut-offs: anti-c n = 7; anti-c + anti-E n = 1, anti-E n = 3. In another 35 cases the test cut offs for the titer were exceeded during follow up. The risk for exceeding the cut-off differed significantly between antibody specificities, with the highest risk (36%) for anti-c. In none of the pregnancies with anti-C, anti-e, anti-Jk^{a}, and anti-Jk^{b}, the cut-offs were exceeded. The most frequent additional antibody, developed during pregnancy, was anti-c, in addition to anti-E (Table 4).

Table 4. *Number of cases with a titer ≥16 and development of additional RBC alloantibodies**

Antibody specificity N=256	n	n	>Cut-off n (%)	n	Additional antibodies, developed during pregnancy (n = 15)
Anti-c	73		26 (36)		
anti-c		51		21	1 × anti-Fy^a
anti-c + anti-E		18		4	1 × C^w
anti-c + anti-E + anti-Jk^a		1		0	
anti-c + anti-Jk^a		2		0	
anti-c + anti-Kp^a + anti-Wr^a		1		1	
Anti-E	78		15 (19)		
anti-E		77		15	1 × anti-D, 6 × anti-c
anti-E + anti-Jk^a		1		0	
Anti-C/anti-e	14		0		
anti-C		5		0	1 × anti-Jk^a
anti-e		6		0	1 × anti-C
anti-C + anti-e/anti-Ce		2		0	
anti-e + anti-Fy^b		1		0	
Anti-Fy^a/anti-Fy^b	24		3 (12)		
anti-Fy^a		19		3	1 × anti-C
anti-Fy^b		3		0	
anti-Fy^a + anti-S		1		0	
anti-Fy^a + anti-f		1		0	1 × anti-Jk^b
Anti-Jk^a/anti-Jk^b	38		0		
anti-Jk^a		36			1 × anti-f
anti-Jk^a + anti-S		1			
anti-Jk^a + anti-C^w		1			
Anti-S/anti-s	29		2 (7)		
anti-S		27		1	
anti-s		2		1	1 × anti-E
Total	256		46 (18		9 (4)
p value†			<0.001		

** Included are all pregnancies with anti-c/-C,-E/-e, Duffy antibodies, Kidd antibodies, and/or S/s antibodies, but without D and/or K antibodies, detected at first trimester antibody screening in 2015 and 2016 in the Netherlands, at risk for HDFN (partner positive for the cognate antigen and/or positive result of non-invasive fetal typing with cell-free DNA). † p value of testing differences between groups of antibodies (anti-c, -E, -C/-e, -Fya,b, Jka,b, -S/s) in the risk for exceeding the cut-off <0.001 (Pearson's chi square = 29.798, 5 df).*

Discussion

In a nationwide prospective cohort study, including pregnant women with RBC alloantibodies with a specificity other than anti-D or anti-K and an antigen-positive fetus, we found that the maximum titer was, compared to the highest titer and the ADCC test, the best test to differentiate between pregnancies at low and high risk for severe HDFN. A cut-off of ≥16 showed a 100% sensitivity to predict the need for an intrauterine or neonatal blood transfusion, and a specificity of 67%. Because of the low a priori risk for severe HDFN, the PPV was only 17% using this cut-off; 27% in pregnancies complicated by anti-c, and 9% in the presence of other Rh antibodies.

If repeated titer measurements were performed in alloimmunized pregnancies with a possibly antigen-positive fetus, the risk for exceeding the established cut-off was 18%, varying from 0% for anti-Jk^a/-Jk^b to 36% for anti-c.

A major strength of our study of diagnostic value is that we used a prospectively collected cohort of all pregnancies with maternal RBC alloantibodies detected at a routine first trimester screening. The adherence to the free of charge RBC alloantibody screening program in pregnancy is >99% and the majority of women is screened before Week 13 of pregnancy.(62) For this study, all laboratory data and >90% of clinical outcome data were available. Therefore, the sensitivity of the laboratory tests to predict severe HDFN could be determined very accurately. A limitation might be that this dataset was collected more than 15 years ago. However, we think it still is valid for prediction of treatment with an intra-uterine transfusion, since the treatment guideline did not change. Guidelines to start treatment with exchange transfusion did change, resulting in a higher number of severely ill children treated with intensive phototherapy and fewer with exchange transfusions.(15)

Another limitation of our study might be that in our laboratory—different from other laboratories—for anti-c, -D, and -E, double dose antigen positive RBCs are used for antibody titration. Theoretically, this might result in higher cut-offs for anti-c and -E than for other antibodies, but we did not find such a difference. We also did not find a higher cut-off for anti-c/-E than other studies, using single dose antigen positive RBCs. The cut-off of ≥16 obtained in our study is comparable with the cut-off of 32 from other studies.(5, 59, 159, 161) Titer measurements can vary between laboratories, also with established techniques, in general a comparison can be made with a one-fold dilution difference between technicians.

A limitation of our study concerning the chance of exceeding the cut-offs might be that in 8% of pregnancies with Rh antibodies, other than anti-D, only one sample was sent to the reference laboratory, despite a titer below the cut-off and advised follow-up testing. The reason the advice was not followed is unknown; this may

either because of a miscarriage or preterm birth, or antigen-negativity of the father for the involved antigen, as determined in the primary laboratory. However, this will not substantially change the chance of exceeding the cut-offs.

We made the choice for a cut-off with a 100% sensitivity, but with a PPV of only 17%. If this cut-off is exceeded, the woman should be counseled about her risk for severe HDFN. This risk depends on the antibody specificity, with by far the highest risk in association with anti-c and a small risk in case of other anti-Rh specificities.**6** Although severe HDFN will not occur in the majority of these pregnancies, we think clinical monitoring is justified. This non-invasive monitoring detects the cases needing antenatal and/or postnatal treatment and, on the other hand, reassures the majority of women with a pregnancy without severe HDFN.

The ADCC test had no added value above the titer to perform the initial discrimination between high and low risk for severe HDFN. Cellular assays, such as the ADCC test, are technically demanding and costly, and most laboratories do not perform these assays. However, the contribution of these assays to a more precise risk estimation, can be of added value in pregnancies identified as high risk, such as pregnancies complicated by anti-D or anti-c.(19, 146) It may be that a test analyzing IgG-glycosylation patterns can be a cost efficient surrogate test for the biological activity of RBC alloantibodies.(162)

The aim of antenatal RBC alloantibody testing is to provide obstetric care providers with clinically useful information in the most cost-effective manner.(162) Our previously reported data indicated that RBC alloantibodies with specificities other than anti-D, other Rh specificities or anti-K, rarely induce fetal and neonatal disease. (10) Therefore, in these cases, a single test, producing a low titer, may be sufficient to create sufficient awareness of the care providers that the RBC alloantibody may induce neonatal icterus. For anti-K we recently recommended to use a single anti-K titer measurement and non-invasive fetal K typing for selection of high-risk cases. (160) For anti-D, a titer and a cellular assay, combined with non-invasive fetal D typing, can be used to select high risk cases.

The number of cases with titers above the cut-off was highest for anti-c (36% of cases), but also in case of anti-E (20%) and anti-Fy (12%) these high titers are found. Thus, in about one-third of pregnancies complicated by anti-c, titer measurement predicts a risk for severe HDFN, which will actually occur in about 25 percent of those pregnancies. This makes repeated testing in pregnancy useful. For other types of Rh alloantibodies repeated testing is less useful, since the risk for severe disease is lower, but if the antibody titer is <16 early in pregnancy, we do advise to repeat the laboratory testing, early in the last trimester of pregnancy. This seems sufficient to

differentiate between children at risk for hyperbilirubinemia shortly after birth, and children not at risk.

In case of other RBC alloantibodies, it is important that laboratories involved in antenatal testing, actively inform the care provider to create a sufficient level of awareness to monitor the newborn for the increased risk for hyperbilirubinemia, especially in case of anti-Fy.

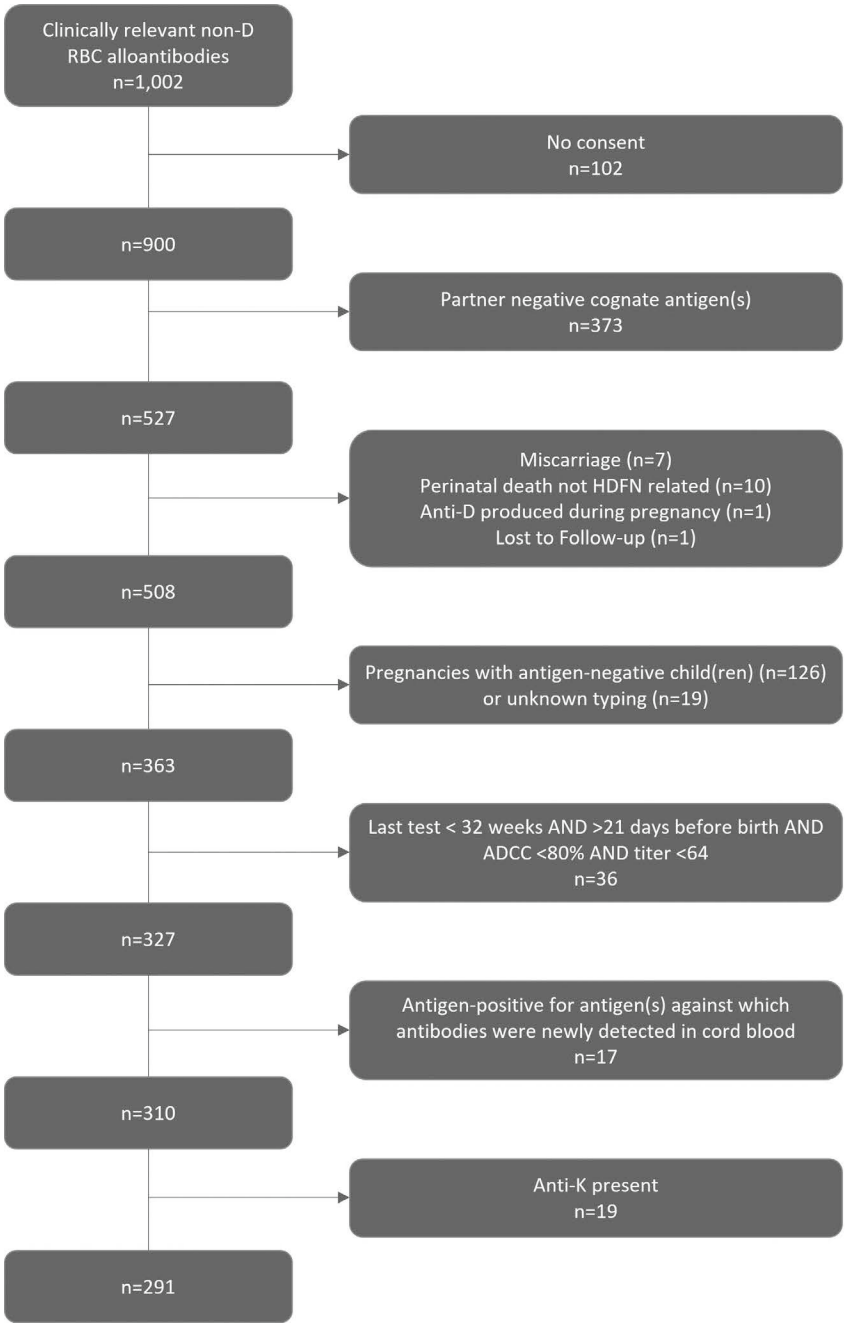
Conclusion

A cut-off of ≥16 for the maximum antibody titer detects all fetuses and children at risk for severe HDFN caused by non-D/non-K RBC alloantibodies. In cases at risk, the ADCC test can be used for a more precise risk estimation. In each pregnancy, one should balance the risk for severe HDFN and the costs, as well as the emotional burden, of repeated laboratory testing.

Repeated testing for anti-c will identify cases with severe HDFN. This is also to be expected for pregnancies complicated by other Rh antibodies. The advice for careful observation because of a risk for neonatal disease should be actively reported by laboratories.

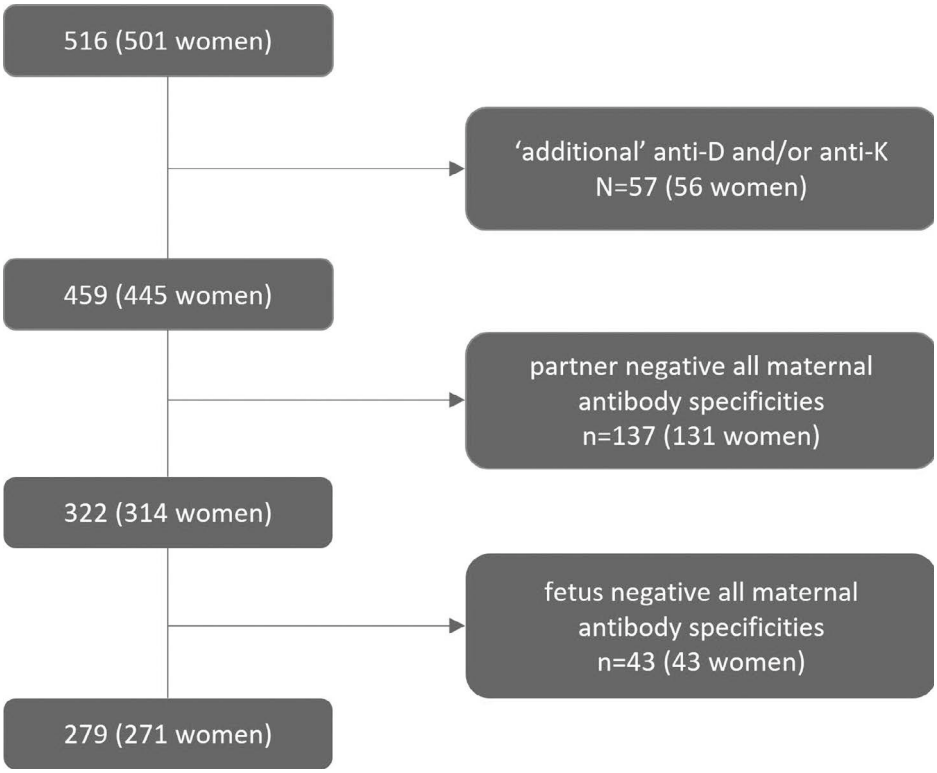
Acknowledgements

We thank all women who participated in the OPZI-study, and we thank obstetric care providers, pediatricians, and collaborators of Sanquin Diagnostic services for collecting clinical and laboratory data.

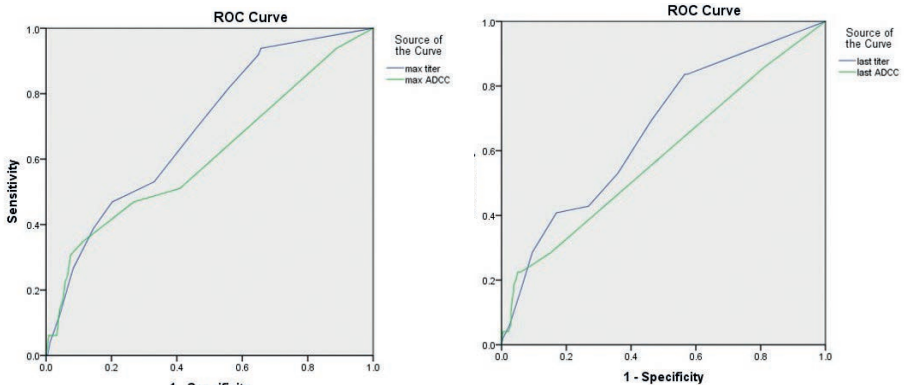


Supplemental Figure 1. *Flowchart of pregnant women with clinically relevant non-D antibodies, detected at first trimester antibody screening in nationwide study in the Netherlands, Sept 1st 2002 until June 1st 2003 & Oct 1st 2003 until July 1st 2004*

Supplemental Figure 2. *Flowchart of pregnancies, at risk for HDFN because of anti-c/-E/-e/-C/Fy^a/-Fy^b/-S/-s/-Jk^a/-Jk^b, detected at Sanquin Diagnostics after positive first trimester antibody screening in 2015-2016*

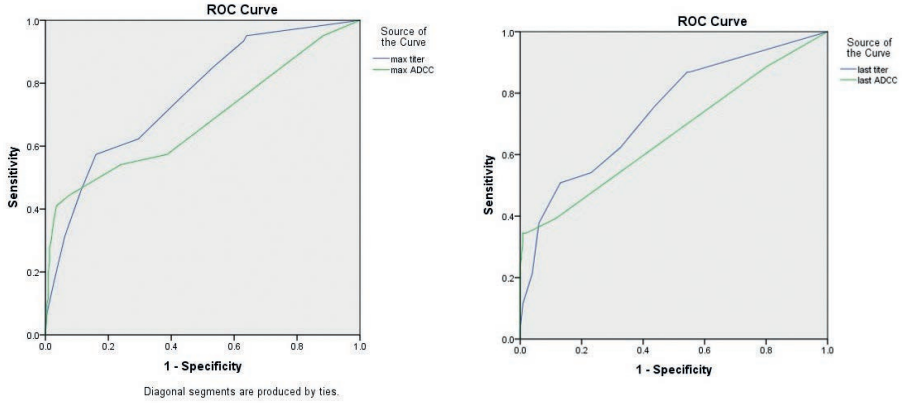


Supplemental Figures 3a,b. *Receiver Operating Curves to predict the need for phototherapy because of HDFN by non-D-/non-K antibodies (n=291) from the maximum and from the last titer and ADCC test result, nationwide study in the Netherlands from Sept 1st, 2002 – June 1st, 2003 & Oct 1st 2003 – July 1st 2004*



Legend: Child positive for the cognate antigen

Supplemental Figures 4a,b. *Receiver Operating Curves to predict the need for antenatal/neonatal transfusion or phototherapy because of HDFN by non-D-/non-K antibodies (n=291) from the maximum and from the last titer and ADCC test result, nationwide study in the Netherlands from Sept 1st, 2002 – June 1st, 2003 & Oct 1st, 2003 – July 1st, 2004*



Legend: Child positive for the cognate antigen

Supplemental table 1. *Prevalence of HDFN in pregnancies at risk for HDFN (child positive for the cognate antigen(s)) because of maternal non-D/non-K RBC alloantibodies, detected at first trimester screening, nationwide study in the Netherlands from Sept 1st, 2002 – June 1st, 2003 & Oct 1st, 2003 – July 1st, 2004*

Specificity RBC antibodies	Antenatal or neonatal transfusion n (%)		Only phototherapy n (%)		No HDFN therapy n (%)		Total	
Anti-c	10 (10.3)	8	24 (24.7)	17	63 (64.9)	50	97	75
. anti-c		2		6		5		13
. anti-c + -E						2		2
. anti-c + -Fy^a				1		4		5
. anti-c + -Jk^a						2		2
. anti-c + -S								
Anti-E	0		13 (16.5)	13	66 (83.5)	66	79	79
. anti-E								
Other Rh	2 (6.3)	1	4 (12.5)	1	26 (81.3)	12	32	13
. anti-C						9		10
. anti-e		1		1		1		2
. anti-C + -e						1		2
. anti-C + Jk^a				2		3		5
. anti-C(w)								
Anti-Fy^a	0		6 (16.7)	6	30 (83.3)	26	36	32
. anti-Fy^a						3		3
. anti-Fy^a + -S						1		1
. anti-Fy^b +-Jk^a								
Other	0		2 (4.3)	1	45 (95.7)	25	47	26
. anti-Jk^a						1		1
. anti-Jk^b				1		10		11
. anti-S						2		2
. anti-s						7		7
Other specificities*								
	12 (4.1)		49 (16.8)		230 (79.0)		291	

**Anti-M (IgG), anti-f, anti-Lu^b, anti-P*

These outcome data were published before in Koelewijn JM, Vrijkotte TG, van der Schoot CE, Bonsel GJ, de Haas M. Effect of screening for red cell antibodies, other than anti-D, to detect hemolytic disease of the fetus and newborn: a population study in the Netherlands. Transfusion 2008;48(5):941-952.

Supplemental table 2. *Number of positive tests, sensitivity, specificity and predictive values to predict the need for antenatal or neonatal transfusion therapy, by cut-off last titer in pregnancies with non-DK RBC antibodies and a child positive for the cognate antigen(s), detected at first trimester screening, nationwide study in the Netherlands, Sept 1st, 2002 – June 1st, 2003 & Oct 1st, 2003 – July 1st, 2004*

n=291		Need for antenatal or neonatal transfusion therapy (n=12)				
Cut-off	Positive tests n	True positive n	Sensitivity % (95% CI)	Specificity % (95% CI)	PPV* % (95% CI)	NPV* % (95% CI)
≥1:8						
all antibodies	86	12	100 (73.5-100)	73.5 (67.9-78.6)	14.0 (11.8-16.5)	100
. anti-c	42	10	100 (69.2-100)	63.2 (52.2-73.3)	23.8 (19.2-29.2)	100
. other Rh	30	2	100 (15.8-100)	74.3 (65.1-82.2)	6.7 (4.9-9.0)	100
≥1:16						
all antibodies	61	11	91.7 (61.5-99.8)	82.08 (77.1-86.4)	18.03 (14.0-23.0)	99.6 (97.2-99.9)
. anti-c	32	9	90.0 (55.5-99.8)	73.6 (63.0-82.5)	28.1 (20.7-37.0)	98.5 (90.9-99.8)
. other Rh	20	2	100 (15.8-100)	83.5 (75.2-89.9)	10.0 (6.8-14.5)	100
≥1:32						
all antibodies	37	9	75.0 (42.8-94.5)	89.96 (85.8-93.2)	24.3 (16.6-34.2)	98.8 (96.9-99.6)
. anti-c	21	8	80.0 (44.4-97.5)	85.1 (75.8-91.8)	38.1 (25.5-52.6)	97.4 (91.4-99.2)
. other Rh	11	1	50.0 (1.3-98.7)	90.8 (83.8-95.5)	9.1 (2.2-31.1)	99.0 (96.1-99.8)

**PPV=Positive Predictive value; NPV=Negative Predictive Value*

Supplemental table 3. *Number of positive tests, sensitivity, specificity and predictive values to predict the need for antenatal or neonatal transfusion therapy, by cut-off maximum ADCC test in pregnancies with non-DK RBC antibodies and a child positive for the cognate antigen(s), detected at first trimester screening, nationwide study in the Netherlands, Sept 1st, 2002 – June 1st, 2003 & Oct 1st, 2003 – July 1st, 2004*

n=291		Need for antenatal or neonatal transfusion therapy (n=12)				
Cut-off	Positive tests n	True positive n	Sensitivity % (95% CI)	Specificity % (95% CI)	PPV* % (95% CI)	NPV* % (95% CI)
≥10%						
All antibodies	124	10	83.3 (51.6-97.9)	59.1 (53.1-65.0)	8.1 (6.2-10.5)	98.8 (95.8-99.7)
. anti-c	57	9	90.0 (55.5-99.8)	44.8 (34.2-55.9)	15.8 (12.4-19.9)	97.5 (85.7-99.6)
. other Rh	36	1	50.0 (1.3-98.7)	67.9 (58.3-76.5)	2.8 (0.7-10.5)	98.7 (94.8-99.7)
≥30%						
All antibodies	28	10	83.3 (51.59-97.91)	93.5 (90.0-96.1)	35.7 (24.95-48.14)	99.2 (97.4-99.8)
. anti-c	20	9	90.0 (55.5-99.8)	87.4 (78.5-93.5)	45.0 (31.2-59.6)	98.7 (92.2-99.8)
. other Rh	2	1	50.0 (1.3-98.7)	99.1 (95.0-100.0)	50.0 (8.4-91.6)	99.1 (96.4-99.8)
≥60%						
All antibodies	11	6	50.0 (21.1-78.9)	98.2 (95.9-99.4)	54.5 (29.9-77.2)	97.9 (96.3-98.8)
. anti-c	9	5	50.0 (18.7-81.3)	97.7 (91.9-99.7)	71.4 (35.7-91.8)	94.4 (90.1-97.0)
. other Rh	2	1	50.0 (1.3-98.7)	99.1 (95.0-100.0)	50.0 (8.4-91.6)	99.1 (96.4-99.8)

** PPV = Positive Predictive Value; NPV = Negative Predictive Value*

Chapter 7

Knowledge, attitude and practices of obstetric careproviders towards maternal red-blood-cell immunization during pregnancy

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Abstract

Background and objectives: A successful routine RBC alloantibody screening programme should not lead to unnecessary emotional burden during pregnancy due to inadequate counselling on the risk of severe Haemolytic Disease of the Foetus and the Newborn (HDFN). Rareness of this disease may result in insufficient knowledge and subsequent inadequate information transfer to women, diagnosed with RBC antibodies. We investigated the current knowledge, views and experiences of Dutch obstetric care providers regarding RBC alloimmunisation during pregnancy.

Materials and methods: We performed a quantitative cross-sectional study, using a structured digital questionnaire to measure knowledge, attitude and practices (KAP) regarding maternal RBC alloimmunisation among Dutch obstetric care providers in 2016.

Results: About 10% of obstetric care providers completed the questionnaire. A sufficient level of knowledge was found in 7% of all participants (N=329). Knowledge about RhD immunisation and prophylaxis was sufficient in 60% of the responders. Knowledge gaps were found concerning the relevance of non-RhD RBC antibodies, the indications for giving extra RhD-prophylaxis and the interpretation of laboratory test results. Health care providers estimated their own level of knowledge “sufficient” (primary/secondary care) to “good” (tertiary care) and all participants considered their professional role important within the screening programme.

Conclusion: Dutch obstetric care providers showed a lack of knowledge regarding maternal RBC immunisation. Awareness of the lack of knowledge is necessary to help obstetric care providers to be careful in giving information and even to decide to contact the expert centre before counselling the patient.

Introduction

Haemolytic disease of the foetus and new-born (HDFN) is still a known cause of pregnancy complications. HDFN is caused by red blood cell (RBC) antibodies developed by the mother and transferred to the foetus. (7, 10, 25) Untreated HDFN may result in progressive foetal anaemia, hydrops, neonatal icterus and even death. (8, 9) Antibodies causing severe HDFN are mostly of the anti-Rh(D) type, and less frequent of the anti-Kell (anti-K1) or anti-Rh(c) type. Severe HDFN is rarely caused by other Rh-antibodies, and only very rarely by non-Rh antibodies (Duffy, Kidd, or S).(4, 10)

Preventive measures such as prenatal and postnatal RhD-immunoglobulin prophylaxis, matched blood transfusions for Rh- and K antigens to women of fertile age (<45 years) and routine prenatal screening for RBC antibodies, together with improvements in monitoring and therapeutic possibilities, have substantially reduced the risk on maternal alloimmunisation and improved outcome of HDFN over the past decades.(10, 32, 44, 91, 163).

Obstetric care providers nowadays only see a few immunised pregnant women during their career, due to the success of the maternal red blood cell alloimmunisation prevention programme. This might result in insufficient knowledge, inadequate information transfer and substandard care to women who are diagnosed with RBC antibodies. In the Netherlands, approximately 180,000 pregnant women are year are entering the screening program. Thanks to a well-organized obstetrical network with multiple safety nets during the process, the coverage of the national prevention programme is almost 100% (57). The reference laboratories (Sanquin Diagnostics and BIBO Groningen) and the national expert centre for the management of alloimmunisation in pregnancy (Leiden University Medical Center, LUMC) are at any time available for advising and consultation on the rare occasion of RBC alloimmunisation.

Pregnancies complicated by the presence of maternal RBC antibodies are monitored by laboratory measurements, consisting of maternal serum testing for antibody levels (quantification of titre) and, in the Netherlands, the antibody dependent cell-mediated cytotoxicity (ADCC) test.(19, 160) If laboratory findings indicate that a pregnancy is at risk for development of HDFN, frequent monitoring is started with ultrasound and Doppler middle cerebral artery (MCA) peak systolic velocity (PSV) measurements, to reliably predict foetal anaemia.(18, 29) If severe foetal anaemia develops, treatment with intrauterine transfusions (IUT) is started and/or preterm delivery is induced, usually followed by neonatal phototherapy and/or (exchange) transfusions.(28, 30)

A Dutch questionnaire survey in 2004, including 233 pregnant women with and without RBC alloimmunisation, showed that women were moderately satisfied with the quantity and comprehensibility of information provided by their obstetric care provider.(63) Fifty to 70% of the women, particularly those with RBC antibodies, indicated that they needed more information, preferable orally, about the consequences of the RBC alloantibodies for their child. Supportive written information (e.g., folders / hand-outs) was lacking, both prenatally and postnatally.

A more recent survey from the UK, performed in the London area, including 270 RhD-negative women, showed that their knowledge about the consequences of screening for RhD-antibodies was limited; 30% of respondents needed more information, via folders or diagrams and through midwives.(23) The authors concluded that midwives needed training on this topic. Wee et al. performed a study on knowledge and practices of RhD-prophylaxis among gynaecologists, residents and obstetric care workers in Singapore. Only 49% appeared to have an adequate level of knowledge on this topic.(164)

In the Netherlands, after adapting the national screening programme in 2011, training and e-learning were developed and offered. However, it is yet unclear what these refresher courses have brought. More insight in the current knowledge of Dutch obstetric care providers on this topic is needed, to identify gaps in knowledge, and to develop strategies to meet these gaps.

The aim of this research was to investigate the current knowledge, views and experiences of Dutch obstetric care providers regarding RBC alloimmunisation during pregnancy.

Methods

Aim/objectives

The aim of the present study was to measure knowledge, attitude and practices (KAP) regarding maternal RBC alloimmunisation among Dutch obstetric care providers. More specifically, the objectives of this KAP study were:

1) to investigate the knowledge of Dutch obstetric care providers about the prevention (strategies) and detection of RBC alloantibodies and identification and treatment of HDFN.

2) to explore the attitude of Dutch obstetric care providers towards the maternal RBC alloimmunisation prevention programme.

3) to examine the practices of Dutch obstetric care providers in participating in the care for pregnant women with RBC alloimmunisation and (risk for) HDFN.

Design

We designed a quantitative cross-sectional study design, using a structured digital questionnaire. The questionnaire was conducted in 2016.

Research population

Participants were midwives, obstetricians and general practitioners specialised in obstetrics. In the Netherlands, obstetric care providers are working in three echelons. The first echelon, primary care, is provided by midwives and general practitioners, working independently in home practices. The second echelon, secondary care, is the regional hospital and the third echelon, tertiary care, is the university hospital (with neonatal intensive care unit availability); in these latter two echelons the obstetric care is provided by midwives and gynaecologists. Participants were invited through a personal mail or mass mail.

Questionnaire

The questionnaire was developed by a medical student (CW), being supervised by a PhD student/midwife (YS) and a PhD/midwife (JK). To reduce the influence of the knowledge questions on the attitude and practice questions, we first posed

the attitude and practical questions. No validated questionnaire was available. We were advised by an expert on questionnaires of the department of Medical Decision Making of the LUMC and by an expert of the education and training Directorate of the LUMC. Additionally, we compared questionnaires with.(165-167) Knowledge of the care providers was examined using vignettes, whereby the respondents had to apply their available knowledge.(168) An expert panel (including obstetricians specialized in foetal therapy, midwives and a laboratory specialist) reviewed the items on content and face validity. Finally, we used a checklist designed by the Dutch Interfaculty Center for Teacher Training, Educational Development and Training (ICLON) (Leiden University).

Measurements

Professional background. Questions about professional background, such as: "In which echelon are you working (primary, secondary, tertiary care)?", year of graduation, work experience (years), prior experienced a pregnancy complicated with maternal RBC alloimmunisation (yes/no), prior experienced a foetus or newborn with haemolytic disease (yes/no), number of deliveries of practice/hospital**,** latest e-learning (2011, provided by the Dutch National Institute for Public Health and the Environment) done (yes/no), latest training on this topic (year). The variable "year of graduation" was categorised as: ≤1998, 1999-2011 and >2011. These time sets were based on the introduction the routine first trimester screening in 1998, the introduction of the foetal RhD-typing and third trimester screening of Rhc-negative pregnant women in 2011.

Knowledge. To test the knowledge about maternal RBC alloimmunisation we used vignettes, case descriptions with questions like "What information do you give your patient?" "What is the right policy in this case?" etc. There were 7 vignettes, the domains were: Screening and prevention of RhD-immunisation (2 questions), Rhc-immunisation (2 questions), K-immunisation (2 questions), risk factors for RhD-immunisation and indications for extra RhD-immunoglobulin prophylaxis (4 questions primary caregivers, 5 questions secondary and tertiary caregivers), laboratory testing for monitoring alloimmunised pregnant women (4 questions), monitoring and treatment of pregnancy with an increased risk of HDFN (only secondary and tertiary care, 2 questions), follow-up of neonate with or without increased risk for hyperbilirubinaemia (2 questions). In total, there were 16 questions to be answered by the primary caregivers and 19 questions for the secondary and tertiary caregivers.

The attitude part consisted of 13 items. *The attitude towards professional role* consisted 4 items: the participants indicated the importance of their own role in the whole process of screening, diagnosis and treatment of maternal alloimmunisation

and HDFN. They indicated if they have enough time per patient to well-inform them, if they find it their job to well-inform them and if they feel that this improves the level of care. *The attitude towards competences* consisted 5 items: participants rated their competences in providing information on the several fragments of this topic and their competences to accompany pregnant women with RBC antibodies and/risk of HDFN. *The attitude towards self-assessment of level of knowledge* consisted 4 items: The participants assessed their own level of knowledge and their satisfaction with it. All items were measured at a five-point Likert Scale (1-5, Completely agree-strongly disagree).

The practices part contained 5 items in which the participants valued the necessity, importance and intention to improve their knowledge and to attend a training. Furthermore, the participants were asked to indicate how often they provide information about the purpose and possible outcomes of the screening program, just before the blood test was taken. All items were measured at a five-point Likert Scale (1-5, good-poor or completely agree-strongly disagree or always-never).

Data collection

The questionnaire was made with NetQ version 2014.Q3. The questionnaire was spread in July 2016 and after two reminders, closed for analysis. Data-analysis was done in SPSS version 23 (SPSS, Inc.).

Data-analysis

On the knowledge questions, the maximum score for primary care was 16 points and for the secondary and tertiary care 19 points. Following the study of Wee et al. and after discussion with the expert panel, it was decided that a score of 80% is a sufficient level of knowledge.

Dichotomous outcomes were described as numbers and percentages, normally distributed continuous variables were described as means and standard deviations, and non-normally distributed continuous variables as median and range. Differences between primary, secondary and tertiary care were tested univariably All variables with a p value less than 0.20 were included in a multivariable logistic regression analysis to assess the association between those variables and the level of knowledge. We intended to add variables with a significant ($p<0.05$) association in a regression analysis in a prediction model that predicted level of knowledge of alloimmunisation.

Ethical considerations

Approval of the Medical Advisory Council of the LUMC was not necessary according to the rules published by the Central Committee on Research involving Human Subjects (http://www.ccmo.nl/nl/niet-wmo-onderzoek).The study was approved by the Science Commission of the Department of Obstetrics.

Results

Response

A total of 402 obstetric healthcare providers opened the link to the questionnaire, 359 of which filled in the attitude/practices part completely and 329 completed the questionnaire (Figure 1).

On January 1st, 2016, approximately 3,321 midwives were active, of them 8.2% (272/3321) filled in at least the attitude/practices part of the questionnaire. Of 66 registered general practitioners specialized in obstetrics, 12.1% filled in at least the attitude/practices part of the questionnaire (ref registration CHBB). In 2009, 842 actively practicing gynaecologists were registered, more recent data are not available, of whom 8.2% filled in the questionnaire at least partly (https://nvl004.nivel.nl/nivel-2015/sites/default/files/bestanden/Rapport-de-arbeidsmarkt-voor-gynaecologen-in-Nederland.pdf).

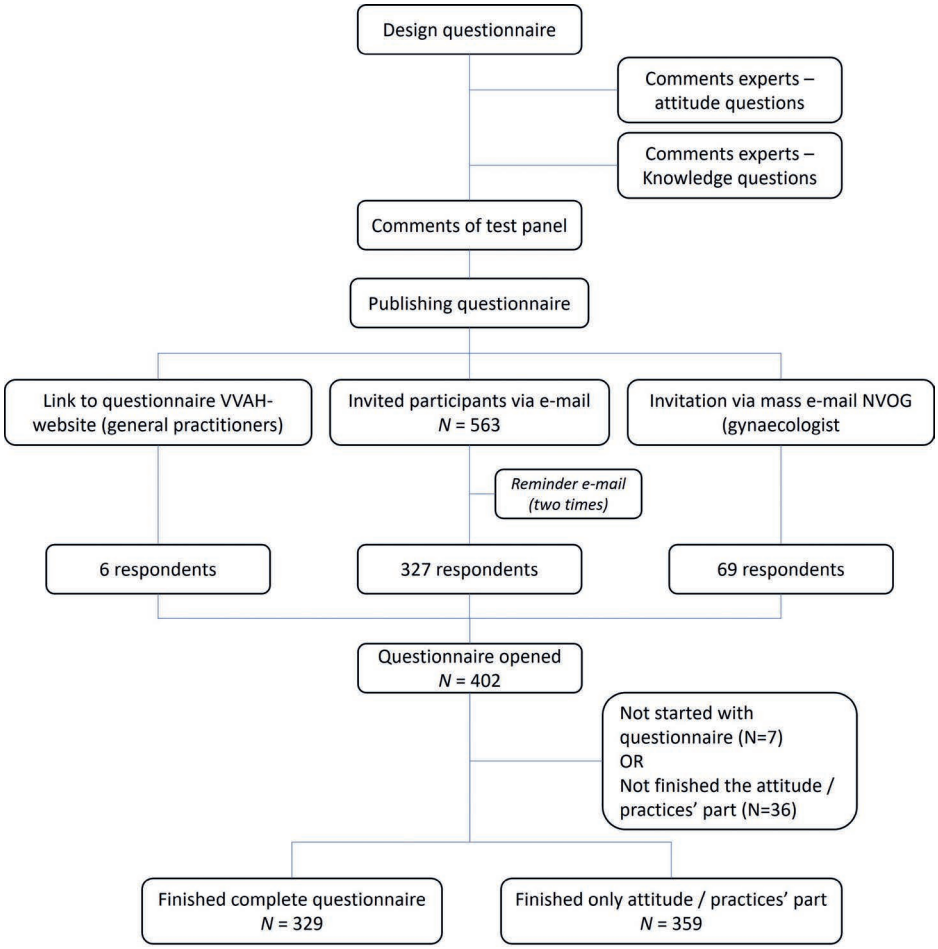

Figure 1: *Flowcharts of study design, distribution of the questionnaire and overview of the responders.*

Background variables

Table 1 shows the background variables of the obstetric healthcare providers who filled in the questionnaire completely (n=329). From all participants, 54% graduated between 1999 to 2011. Most had less than 20 years of work experience. The average number of births supervised annually per clinic/practice was between 250 and 500 in the home practices (primary care), in secondary care, 54% of obstetric care providers attended 1000-2000 births annually and 35% more than 2000 births/year. In tertiary care 59% of the care providers had supervised between 1000 and 2000 births/year. The chance of experiencing a case of maternal alloimmunisation or of HDFN increased from primary to secondary care. Forty-two percent of participants followed a training in RBC alloimmunisation and prevention less than five years ago, 25% between 5-10 years ago or longer than 10 years ago. One fifth of obstetric healthcare providers did not know if or when the last training on this topic was attended. The 2011 e-learning was completed by 32% of all participants.

Table 1: *Background variables of participants divided into three echelons: primary, secondary and tertiary care.*

		Primary care n=252	Secondary care n=60	Tertiary care n=17
		n (%)	n (%)	n (%)
Profession	Midwife	246 (98)	7 (12)	1 (6)
	General practitioner	6 (2)	0	0
	Gynaecologist	0	53 (88)	16 (94)
Graduation year	Until 1998	66 (26)	12 (20.0)	2 (12)
	1999 – 2011	144 (57)	27 (45)	8 (47)
	>2011	42 (17)	21 (35)	7 (41)
Work experience	0-10 year(s)	116 (46)	22 (37)	2 (12)
	11 - 20 years	89 (35)	23 (38)	11 (65)
	21 – 30 years	33 (13)	15 (25)	3 (18)
	31 - 50 years	14 (6)	0	1 (6)
Average number of births attended	<250	130 (52)	0	0
	251 – 500	105 (42)	0	0
	501 – 750	13 (5)	1 (2)	0
	751 – 1000	2 (1)	2 (3)	0
	1001 – 1500	2 (1)	3 (5)	10 (59)
	1501 – 2000	0	20 (33)	3 (18)
	>2000	0	13 (22)	4 (24)

Table 1: *Background variables of participants divided into three echelons: primary, secondary and tertiary care. (continued)*

		Primary care n=252	Secondary care n=60	Tertiary care n=17
Experienced a foetus or new-born with haemolytic disease?	yes	53 (21)	21 (35)	15 (88)
Experienced a pregnancy complicated with RBC antibodies?	yes	171 (68)	44 (73)	17 (100)
Last training about alloimmunised pregnant women	< 5 years ago	102 (41)	56 (93)	12 (71)
	5-10 years ago	45 (18)	24 (40)	2 (12)
	>10 years ago	18 (7)	12 (20)	2 (12)
	Unknown	87 (35)	3 (5)	1 (6)
Latest (2011) followed e-learning provided by the RIVM*?	yes	92 (37)	21 (35)	5 (29)
	No	113 (45)	10 (17)	11 (65)
	Unknown	47 (19)	34 (57)	1 (6)

Knowledge

Table 2 shows the number of correct answers per question of primary, secondary and tertiary caregivers. The questions on the indications for RhD prophylaxis administered in pregnancy were correctly answered by 95% of primary care participants, compared with 15% and 6% respectively of the secondary and tertiary care participants. The question about the indication and quantity of RhD prophylaxis after caesarean section was significantly better answered by secondary and tertiary caregivers. The knowledge about indication for RhD-prophylaxis in case of a spontaneous abortion (72%) as well the indication of RhD-prophylaxis in case of an abortion with curettage was less frequently correctly answered by participants in primary care (43%). The indication for RhD prophylaxis in case of fetal demise was poorly answered; this question was only submitted to secondary and tertiary caregivers. The knowledge score of screening of RhD and Rhc-negative women was over 80%, hence sufficient, in all echelons, but the purpose of the third trimester screening of Rhc-negatives appeared to be often unclear. Less than 20% of all participants gave the correct answers to the question about purpose and policy in case of K-immunisation. In general, questions about laboratory monitoring were moderately to poorly answered. The score for questions about detection of HDFN prenatally or postnatally was in general sufficient. Only tertiary care participants had some difficulties with correctly answering a question about unexpected hyperbilirubinemia.

Table 2: *Correctly answered questions by participants of primary, secondary or tertiary care.*

	Primary care	Secondary care	Tertiary care	p-value*
Question	n=252	n=60	n=17	
Correct	n (%)	n (%)	n (%)	
1a Screening policy RhD-negatives	244 (97)	60 (100)	17 (100)	0.286
1b Antenatal RhD-prophylaxis	231 (96)	9 (15)	1 (6)	<0.001
1c RhD-prophylaxis policy caesarean	16 (6)	35 (58)	11 (65)	<0.001
1d RhD-prophylaxis policy abortion (9 weeks)	229 (91)	53 (88)	14 (82)	0.473
1e RhD-prophylaxis policy abortion (12 weeks)	181 (72)	57 (95)	15 (88)	<0.001
1f RhD-prophylaxis policy abortion + curettage (12 weeks)	109 (43)	56 (93)	16 (94)	<0.001
2a Screening policy Rhc-negatives	252 (100)	60 (100)	17 (100)	-
2b Purpose third trimester screening Rhc-negatives	52 (21)	18 (30)	4 (24)	0.294
3a Screening policy K-immunisation	48 (19)	6 (10)	3 (18)	0.250
3b Follow-up K-immunisation	22 (9)	11 (18)	3 (18)	0.067
4a RhD-prophylaxis policy foetal demise	-	34 (57)	8 (47)	0.483
5a Risk HDFN ADCC-test 10%/ titre 1:8	74 (29)	27 (45)	8 (47)	0.031
5b Policy ADCC-test 10%/ titre 1:8	106 (42)	8 (13)	2 (12)	<0.001
5c Risk HDFN ADCC-test 35%/ titre 1:16	78 (31)	23 (38)	12 (71)	0.003
5d Policy ADCC-test 35%/ titre 1:16	168 (67)	34 (57)	14 (83)	0.113
5e Doppler monitoring to detect foetal anaemia	-	57 (95)	16 (94)	<0.001

Table 2: *Correctly answered questions by participants of primary, secondary or tertiary care. (continued)*

Question	Primary care	Secondary care	Tertiary care	p-value*
5f Frequency of doppler monitoring	-	48 (80)	15 (88)	<0.001
6a follow-up neonate with negative RBC screening	239 (95)	53 (88)	16 (94)	0.179
7a Cause hyperbilirubinaemia neonate and negative third trimester screening	198 (79)	42 (70)	9 (53)	0.031

**comparing primary, secondary and tertiary care (or secondary and tertiary care when restricted question); Pearson's chi-square test, Fisher's Exact with expected value < 5 in 1 or more cells.*

Level of total knowledge of participants

Table 3 shows how many participants from primary, secondary and tertiary care achieved a sufficient score on the test (defined as: 13, respectively 15 correctly answered questions in primary and secondary/tertiary care). Only 7% of all participants achieved a sufficient score. No significant differences between the echelons were measured. None of the background variables showed an association with the total test-result with a p-value < 0.20. When the cut-off was lowered from 80% to 60% or 70%, 35%, respectively 21% of all participants had a sufficient score.

Table 3: *Total test-result of participants shown as sufficient with cut-off at 80% correctly answered questions.*

	Sufficient	
	N	%
Primary care	19	7.5%
Secondary care	3	5.0%
Tertiary care	1	5.9%
p-value *	0.843	

** Comparing primary, secondary and tertiary care; Pearson's chi-square test, Fisher's Exact with expected value < 5 in 1 of more cells.*

Attitude and practices

Table 4 shows the median scores on self-assessed attitude and practices per question per echelon (N=359). For several domains a Cronbach's alpha was calculated, showing that only the domain "Attitude to competences" turned out to be 0.84, while the other domains were between 0.24 and 0.49 (respectively "practices" and "knowledge").

The tertiary health care providers estimated their own level of knowledge significantly higher (median score "good") than primary care and secondary care (median score "sufficient"). The tertiary care participants were more satisfied with their own level of knowledge and found it less necessary to participate in trainings than participants of primary and secondary care. The tertiary care participants considered their role within the screening programme and treatment of RBC immunisation and HDFN less important. The primary care participants considered themselves less capable in the care of pregnant women with RBC alloimmunisation without signs of foetal anaemia. All participants considered it their task to well inform pregnant women about the prevention programme. The opinion about time available to well inform pregnant women about the prevention programme was significant different between echelons, variating from surely enough time (tertiary care) to neutral (secondary care).

Primary care providers felt themselves significantly less competent (median score "partly agree" vs "completely agree") in providing information about the development of RBC antibodies during pregnancy and in explaining the blood test results to women with newly identified RBC antibodies, as well as on possible risk of HDFN. The secondary care providers explained significantly less frequent to the patient (median score "often" vs "always") that the routine first trimester screening includes the ABO blood group, Rhesus-D antigen typing and presence of RBC antibodies.

Table 4: *Overview of median scores on the attitude and practices questions divided in primary, secondary and tertiary care.*

	Primary care (n=270)	Secondary care (n=70)	Tertiary care (n=19)	P-value**
	Median (P25-P75)	Median (P25-P75)	Median (P25-P75)	
Attitude towards professional role				
I'm important within the trajectory of detection and treatment of RBC alloimmunisation and HDFN	1 (1-2)	2 (1-2)	2 (1-2)	<0.001
It is my job to well inform the pregnant women about the goal of the RBC screening	1 (1-1)	1 (1-1)	1 (1-1.75)	0.322
Providing information about the prevention programme alloimmunisation improves the level of care	1 (1-2)	1 (1-2)	1 (1-2)	0.694
The time per pregnant women is sufficient to well inform the pregnant women about the goal of the RBC screening programme	2 (1-4)	3 (2-4)	1.5 (1-3.75)	0.011
Attitude towards competences				
I am competent in explaining the meaning of the titre and ADCC result to pregnant women with RBC antibodies	2 (2-3)	1 (1-2)	1 (1-1.75)	<0.001
I am competent to accompany a pregnant woman with RBC antibodies without any signs of haemolytic disease of the foetus	2 (1-3)	1 (1-1)	1 (1-1)	<0.001
I am competent to provide information about alloimmunisation during pregnancy	2 (1-2)	1 (1-2)	1 (1-1.75)	0.003
I am competent in explaining the blood test result to pregnant women for whom RBC antibodies have been found	2 (1-2)	1 (1-1.5)	1 (1-1)	<0.001
I feel competent to provide information about the possible risk of haemolytic disease due to RBC antibodies during pregnancy	2 (1-2)	1 (1-2)	1 (1-1.75)	<0.001

Table 4: *Overview of median scores on the attitude and practices questions divided in primary, secondary and tertiary care. (continued)*

	Primary care (n=270)	Secondary care (n=70)	Tertiary care (n=19)	P-value**
	Median (P25-P75)	Median (P25-P75)	Median (P25-P75)	
Attitude towards self-assessment of level of knowledge				
My knowledge about alloimmunisation is: ~	3 (3-3)	3 (2-3)	2 (1-3)	<0.001
It is necessary to extent my knowledge about alloimmunisation.	2 (2-3)	2 (2-3)	4 (2.25-5)	0.027
My plan is to extent my knowledge about alloimmunisation.	2 (2-3)	2 (2-3)	4 (3-5)	0.126
I'm satisfied with my level of knowledge	3 (2-3)	3 (1-3)	2 (1-2)	0.044
Practices followed courses, actual information provided and intention or need for training				
I would attend a training/ course on providing information	2 (1-2.25)	2 (1-2.5)	2.5 (2-3)	0.007
I find it important to follow a training/ course about RBC alloimmunisation	2 (1-2)	1 (1-3)	2 (1.25-3)	0.363
Attending the e-learning about prevention and detection of RBC alloimmunisation was useful/relevant # (primary care n=149, secondary care n=17, tertiary care n=8)	1 (1-2)	1 (1-2)	1 (1-1)	0.207
Before the first trimester screening, I explain that the blood test contains the ABO and RhD blood group and RBC antibodies*	1 (1-1)	2 (1-3)	1 (1-2)	<0.001
Before the first trimester screening, I explain the possible test results and the risk of RBC antibodies during pregnancy *	3.5 (3-4)	4 (3-4.5)	3 (2-4.75)	0.329

***Differences between primary, secondary and tertiary care were tested using Kruskal-Wallis test.*
1=Completely agree, 2=partly agree, 3=neutral, 4= partly disagree, 5=strongly disagree
~1=very good, 2 = good, 3 = sufficient, 4= insufficient, 5 = poor
#respondents who didn't follow the e-learning were excluded
**1= always, 2= often, 3= sometimes, 4 = rarely, 5 = never*

Discussion

In this nationwide study with 329 participants, only 7% of obstetric care providers appeared to have sufficient knowledge of all aspects of maternal RBC alloimmunisation, needed to provide sufficient support and counselling during pregnancy. The participants of the tertiary care were more satisfied with their own knowledge on the subject than the participants of primary and secondary care and judged it to be less necessary to follow an additional in-service training on management of maternal RBC alloimmunisation. All echelons considered themselves important within the process of detection and treatment of RBC alloimmunisation and HDFN.

Strength and weaknesses

For each professional group, the response was approximately 10% of the total number of active care providers. The response may have been negatively influenced by the length of the questionnaire and by sending it around in the summer period. In our opinion, this relatively low response is sufficient to at least have an impression of the level of knowledge. However, selective response of care providers who have an affinity with the subject, may have resulted in an overestimation of the level of knowledge and a too optimistic assessment of the attitude.

Previous findings and interpretation

Our estimation was that at least half of the obstetric care providers should have sufficient knowledge about RBC alloimmunisation, defined as answering 80% of all questions correctly.(164) This cut-off value was also used in a study from Singapore, exploring the knowledge about RhD immunisation and prophylaxis. Our study included questions on all aspects of the screening program, whereas the Singapore study focused on prevention of RhD immunisation. Also in our study, 60% of the questions about RhD screening and prophylaxis were correctly answered. The knowledge gaps we found concerned mainly aspects of non-RhD RBC antibodies, the indications for administering extra RhIg and the interpretation of ADCC and antibody titre results. This probably does not mean that mistakes are made in the care for pregnant women with RBC alloimmunisation.(10, 28, 44, 76) A lack of active knowledge may be explained by the fact that, the care provider receives necessary information about the follow-up policy, and if necessary, advice to consult the expert centre at LUMC, via the laboratory report from the reference laboratories. The finding that obstetric care providers are often not aware of their own low level of knowledge is not only remarkable but also worrisome, as self-knowledge and introspection are

essential to warrant an adequate level of care. Presumably, lack of knowledge has consequences for the adequate counselling and understanding of this complex matter by patients. It therefore may explain the moderate satisfaction of pregnant women with the content and comprehensibility of information they receive on this condition, as we previously showed.(63) Poorly provided information after detection of RBC antibodies or during follow up, can influence the emotional pregnancy experience of women. From the evaluation of similar situations, like informing parents about a positive test result for any of the diseases tested during the new-born screening, Moody et al (2017) advised to arrange direct face-to-face contact between the specialist team and the family, continued support and the availability of accessible condition specific information. Various studies about parents' recommendations how to inform them about a positive new-born screening result suggest that it is important to offer realistic reassurance and hope, to address and support parents through the moments of anxiety and to keep the content simple, clear and actionable.(169-172)

In our study the obstetric care providers considered it important to provide information about the national screening programme and found their own professional role important within the process of detection and treatment of RBC alloimmunisation and HDFN. This positive attitude can form the basis to fill the knowledge gaps by a targeted e-learning based training or by up-to-date information on the web. Awareness of giving the patient news that can cause anxiety, already helps to respond more adequately on emotions and socio-psychological aspects of the message, thus diminishing stress and anxiety in the pregnant woman.(173)

Conclusion

Awareness of the lack of knowledge is necessary to help obstetric care providers to be careful in giving information and even to decide to contact the expert centre before counselling the patient.

This will improve adequate counselling with the aim to empower the pregnant woman and her partner to appropriately translate the message of the presence of RBC alloantibodies into risks for their unborn child, to minimise unnecessary anxiety during pregnancy.

Table S1: *Knowledge scores classified per level of obstetrical care*

		Primary care n=252	Secondary care n=60	Tertiary care n=17	p-values*
		n (%)	n (%)	n (%)	
Prevention of maternal alloimmunisation in RhD-negative women Screening policy RhD-negative women Antenatal anti-D administration	Correct	225 (80.9)	7 (11.7)	1 (5.9)	<0.0001
Prevention of maternal alloimmunisation in Rhc-negative women Screening policy Rhc-negative women Purpose screening at 27 weeks in Rhesus-c-negative women	Correct	52 (18.1)	18 (30.0)	4 (23.5)	0.3092
Screening for maternal K-immunisation Follow-up laboratory tests in K-immunised pregnant women	Correct	6 (2,4)	0	1 (5.9)	0.282
Indications Anti-D Anti-D policy caesarean section Anti-D policy spontaneous abortion (9 weeks) Anti-D policy spontaneous abortion (12 weeks) Anti-D policy abortion + curettage (12 weeks)	Correct	85 (34.0)	28 (46.7)	9 (52.9)	0.075
ADCC-test and titre measurements Risk of haemolysis and policy if ADCC-test 10%/ titre 1:8 Risk of haemolysis and policy if ADCC-test 10%/ titre 1:8 Risk of haemolysis and policy if ADCC-test 35%/ titer 1:16 Risk of haemolysis and policy if ADCC-test 35%/ titer 1:16	Correct	32 (12.8)	3 (5.0)	1 (5.9)	0,175

Table S1: *Knowledge scores classified per level of obstetrical care (continued)*

		Primary care n=252	Secondary care n=60	Tertiary care n=17	p-values*
		n (%)	n (%)	n (%)	
Follow-up of the neonate Follow-up neonate with negative antibody screening Causes of neonatal icterus with a negative antibody screening at 27 weeks.	Correct	186 (74.4)	36 (60.0)	9 (52.9)	0.023

**Differences between primary, secondary and tertiary healthcare providers, Pearson's Chi-square and Fisher's Exact with a minimal expected value of <5 in 1 or more cells.*

Table S2: *Univariate analysis of the association of background variables with the level of knowledge.*

	Sufficient n (%)	p-value*
Profession Midwife (n= 254) General practitioner (n= 6) Gynaecologist (n= 69)	 17 (6.7) 0 4 (5.8)	0.783
Year of graduation <1998 (n=80) 1999-2011 (n=179) >2011 (n=70)	 4 (5.0) 13 (7.3) 4 (5.7)	0.547
Years' of obstetrical experience 0-10 years (n=130) 11 - 20 years (n=116) 21 - 30 years (n=49) 31 - 40 years (n=12) 41 - 50 years (n=1)	 10 (7.1) 7 (5.7) 2 (3.9) 2 (14.3) 0	0.684
Experienced a foetus or new-born with haemolytic disease Yes (n=103) No (n=205)	 9 (8.0) 12 (5.5)	0.378
Experienced a pregnancy complicated with red blood cell immunisation Yes (n=227) No (n= 81)	 17 (7.0) 4 (4.7)	0.463
Average number births attended per year < 1000 (n=239) >1000 (n=69)	 17 (6.6) 4 (5.5)	0.484
Last training about alloimmunised pregnant women < 5 years ago (n=130) > 5 years ago (n=75) I don't know (n=103)	 8 (5.8) 7 (8.5) 6 (5.5)	0.652
Latest (2011) followed e-learning provided by the National Institute for Health and environment? yes (n=140) No (n= 94) unknown (n=58)	 8 (5.4) 8 (7.8) 4 (6.5)	0.741

** Pearson's Chi-square and Fisher's Exact with a minimal expected value of <5 in 1 or more cells.*

Chapter 8

When a pregnancy is complicated by red blood cell alloimmunization: the importance of sincere information – a qualitative study of women's experiences

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In preparation

Abstract

Background: In the Netherlands Red blood cell (RBC) alloimmunization occurs in approximately every 300 pregnancies, 80/year caused by Rhesus-D-antibodies of which 25% have severe hemolytic disease of fetus and newborn (HDFN). No research examining women's experiences of this condition has been published.

Objectives: to describe women's experience of a pregnancy complicated with RBC alloimmunization. **Methods:** A descriptive study was conducted using in-depth interviews. A convenience sample of 10 pregnant women with RBC alloimmunization and at risk for HDFN were interviewed during their complicated pregnancy or a few to several years after giving birth. Women were recruited from another cohort study on women with RhD alloimmunization (OPZI 2.0 study). Transcripts of the interviews were analyzed using content analysis to describe their experience.

Findings: The severity of the RBC alloimmunization during pregnancy varied from RBC alloimmunization without risk for HDFN to severe HDFN and perinatal death. Five themes were identified from the descriptions of the experience as related by the participants. They encompassed the experience of the moment they first heard about the RBC alloimmunization, experience of care, knowledge about HDFN by obstetric care workers and patients, impact of pregnancy turning from physiologic to pathologic and the impact on family planning.

Applications: The key word in all the themes was confidence; the trust in the pregnancy and well-being of the fetus and/or newborn has decreased. The experience of care and the way of providing information about the risks and possible treatments can break or increase the trust in the pregnancy and neonatal period.

Introduction

Hemolytic disease of the fetus and newborn (HDFN) is nowadays a rare disease, affecting approximately 290–410 pregnant women per 100,000. It is caused by red blood cell (RBC) alloantibodies, developed by the mother and transferred to the fetus trans placentally. Without treatment, HDFN may result in progressive fetal anemia, fetal hydrops, asphyxia and perinatal death. After birth, neonatal hyperbilirubinemia may lead to 'kernicterus', causing neurodevelopmental impairment with athetoid cerebral palsy, hearing problems and psychomotor handicaps.(3, 4, 6-9, 20)

Several preventive measures, such as administration of anti-D Ig to RhD-negative women with an RhD-positive child and preventive matching of blood transfusions, have substantially reduced the prevalence of RBC alloimmunization in pregnancy, resulting in an even lower risk of severe HDFN. Routine RBC antibody screening in pregnancy has provided the means for timely referral and treatment in secondary or tertiary care centers.

In general, pregnancies at risk for HDFN, as indicated by laboratory investigation, need frequent monitoring with ultrasound and Doppler middle cerebral artery (MCA) peak systolic velocity (PSV) measurements, to reliably predict the development of severe fetal anemia.(18, 29, 143) Fetal anemia is treated with intrauterine transfusions (IUT). Depending on gestational age, it may alternatively be decided to induce (preterm) labor, followed by neonatal phototherapy or (exchange) transfusions if necessary.

Because of the low current prevalence of alloimmunization in pregnancy, obstetric care providers (OCP) rarely encounter alloimmunized women and hardly ever women with a pregnancy at risk for HDFN. OCP's may become unaccustomed to managing these complicated pregnancies and properly counselling the parents on treatment policy and associated risks. In our previous study we found that Dutch OCP's showed a lack of knowledge regarding maternal RBC alloimmunization and were not aware of this lack of knowledge.(174) Furthermore, women showed only moderate satisfaction with the information provided about the screening program concerning RBC antibodies.(63) We did not find other reports specifically describing the experiences of RBC alloimmunized women with a risk of HDFN. During focus group interviews undertaken to gain insight into barriers and motivators of women to becoming anti-D plasma donors after RhD alloimmunization during pregnancy, we observed that women shared other aspects of their experience in relation to the counselling received during the pregnancy in which RBC alloimmunization was detected first and often had difficult memories even years afterward.(175)

However, there are studies available about the experiences of women with a complicated pregnancy due to other causes. A recent qualitative study, interviewing 12 women with a complicated pregnancy, showed that they often felt out of control, fearful and confused.(176) The authors suggest that midwives can play a key role in translating medical jargon and providing emotional guidance and support. Two cross-sectional observational questionnaire studies showed high-risk pregnancies and/or those complicated by a medical disorder to be anxiety provoking and to increase the likelihood of depression, as well as causing stress and distress in the pregnant woman.(177, 178) Côté-Arsenault et al. showed that parents of a neonate with a lethal diagnosis valued receiving intensive psychological guidance,(179) and this contributed to a positive experience of received care.

The present study was designed to gain more insight into the experience of women and their partners regarding the care currently provided during a pregnancy involving RBC alloimmunization, with a risk for HDFN. Our aim was to describe the perceived and desired guidance for a complicated pregnancy and to formulate recommendations for potential improvement of care and at the level of communication.

Methods

Design

We conducted an explorative study with a qualitative descriptive design, following the principles of 'Abbreviated Grounded Theory' (Glaser and Strauss).(180) Semi-structured interviews were conducted based on a topic list.

The study followed an interpretive approach using sensitizing concepts – that is, concepts that might be related to the experience of care. The concepts were not used as interview questions but kept in mind as possible dimensions. The sensitizing concepts were derived from the literature and from our own experience of care for this group of patients.

Data collection

Study sample

Alloimmunized women with a pregnancy at risk for HDFN were invited for interview. Data were collected between 2011 and 2018. If the woman was accompanied by her partner, the partner could also participate in the interview. Women were selected

via purposive sampling with the intention of ensuring a heterogeneous group of participants. We attempted to have variation in the following characteristics: timing of interview either during or after the alloimmunized pregnancy; antenatal care in primary, secondary or tertiary care; Dutch or non-Dutch ethnicity; and disease severity.

Most participants were enrolled in the OPZI 2.0 study, a nationwide cohort study on D immunization in pregnancy. When giving consent for the OPZI 2.0 study, the participants also gave permission to be approached for further research. A minority of women were recruited via their obstetrician at the Leiden University Medical Center (LUMC), the tertiary care center for alloimmunization in pregnancy in the Netherlands. Women were informed that they could decide not to participate or withdraw from participation at any time without explanation. Interviews were conducted until data saturation was achieved and no new information emerged.

Topic list

The topic list was based on the clinical experience of YM and JK and sensitizing concepts such as: received information (knowledge); fear/anxiety surrounding pregnancy complication and coping mechanisms (behavior); expectations of care (norms and values); and suggestions for improvement of care (intention). The topic list was prepared by YS and refined by JK.

Interviews

The interviews were conducted by YS and two midwifery students, CV and IT, who used the data for their bachelor´s thesis. Semi-structured interviews were conducted either in the participant's home or in a quiet room at the hospital. Each interview lasted approximately 30–45 minutes. The interviews began with general questions about the participant's (obstetric) background. Subsequently, the woman was asked to tell her personal story concerning the alloimmunized pregnancy. Follow-up questions were asked to give the woman the opportunity to clarify relevant aspects of her initial answers. The interviews were recorded on audio tape and were transcribed verbatim by a secretary who had signed a binding agreement to secure confidentiality.

Analysis

Thematic analysis was undertaken, with the aim of formulating relatively broad themes that summaries the content of the data. First, the transcript was read several times, for the purposes of data familiarization. The transcripts were then open, axially and selectively coded; so that the main themes became visible. These main themes were captured in phrases, described as expressed by the participant. Finally, links were made between the different themes and a core theme/category was established. The interview transcripts were analyzed by YS and JK, who both reviewed the findings and came to a consensus. The themes were confirmed by repeatedly returning to the original data and ensuring transparency in data processing; all results can readily be traced to the underpinning data.

Results

Participants

We carried out eight interviews, after which data saturation was reached. Half of the women were accompanied by their partner during the interview. In all interviews the woman mainly spoke and was supported on some points by her partner. The findings and quotations therefore relate to the women participants. Two women participated in the study during their first alloimmunized pregnancy. Both received tertiary care in the LUMC. One of them underwent an intrauterine transfusion (IUT) (Table 1). The other six women were interviewed 9–36 months after giving birth; two of these received care at the LUMC. The other four women never received tertiary care. All participants received primary or secondary care at some point in their first pregnancy at risk for HDFN or during the pregnancy before the one at risk. Seven women had RhD immunization with or without additional antibodies and one woman had K immunization. One of the babies did not require a RBC transfusion or phototherapy for HDFN.

Table 1. *Overview of participants*

Participant number	GPA*	Line of care	Intra-uterine transfusion	Neonatal transfusion	Photo-therapy	Ethnicity	Gestational age	Months post-partum#	Year of interview
1	G4P3	Tertiary (LUMC)	No	-	-	Caucasian	27	NA	2011
2	G2P1	Tertiary (LUMC)	Yes	-	-	Caucasian	34	NA	2011
3	G3P3M2	Tertiary (LUMC)	Yes	Yes	Yes	Arab	NA	9	2018
4	G2P2	Secondary	No	No	No	Mediterranean	NA	36	2017
5	G3P3	Secondary	No	Yes	Yes	Caucasian	NA	28	2016
6	G1P1	Secondary/ tertiary	No	Yes	Yes	Caucasian	NA	16	2016
7	G2P2	Tertiary (LUMC)	No	Yes	Yes	Caucasian	NA	36	2016
8	G3P3	Secondary	No	No	Yes	Caucasian	NA	36	2017

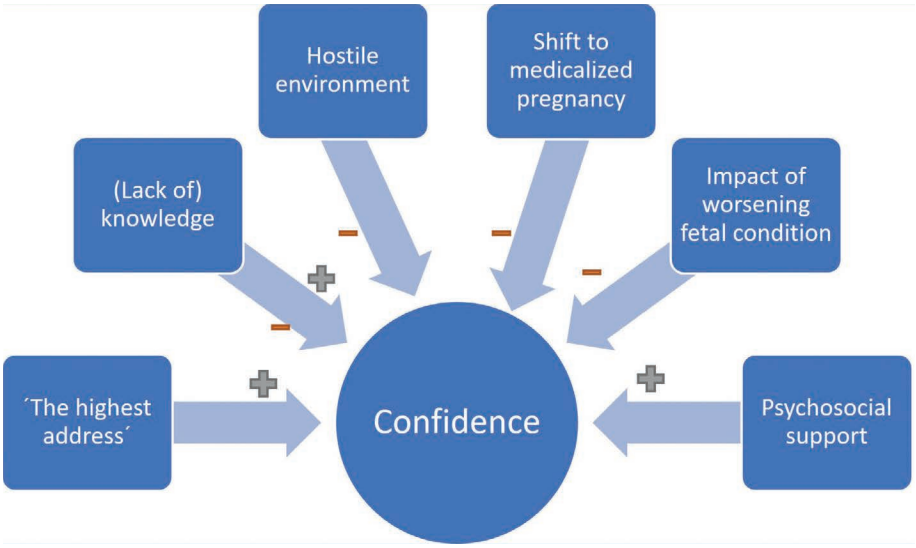
**Gravity and parity and (if applicable) number of children alive (M); #NA = not applicable*

Themes

After analysis of the transcript and codes, six main themes could be formulated. These were: shifting from having a normal pregnancy to one with a potential medical complication; experiencing the impact of worsening of the fetal situation; experiencing the body as a hostile environment for the baby; experiencing needing psychological support; experiencing lack of knowledge; and experiences if the woman reached the LUMC center for fetal therapy, the 'highest address' of knowledge/skills.

The core theme, to which these six themes are related, is 'confidence'.

Figure 1 shows the core theme and main themes and their mutual relationship. Knowledge of RBC alloimmunization and its risks and possible complications plays a role in the woman's confidence in the pregnancy, and when knowledge is lacking or not properly communicated, this creates unease. When the problem suddenly deteriorates (impact of worsening fetal condition) confidence in a successful outcome decline, but as soon as the pregnant woman arrives at the LUMC (the 'highest address') and receives the best available care, confidence in a successful outcome of the pregnancy increases again. Furthermore, the pregnant woman loses confidence in her body when there is erythrocyte immunization, which is further explained under the theme 'hostile environment'. Confidence in a normal course of pregnancy has disappeared and a shift from non-medical to top medical care is experienced as drastic.



(Lack of) knowledge

Difficult subject matter for both OCP and patient

Within the theme 'lack of knowledge', one participant described the issue of RBC alloimmunization as 'a difficult topic'. Most participants found the problem difficult to understand and said that they fully relied on the gynecologists in their decisions. On the other hand, according to the participants, the primary or secondary OCP seemed not always to have sufficient and complete knowledge about the problem. This resulted in providing too little or even incorrect information at crucial moments. In the interview excerpt below, a participant received the result by telephone that RBC antibodies had been detected. Little information was given about what this result meant for the pregnant woman, and it was mostly emphasized that she should not be concerned. When asked what information she received by telephone, the participant said:

"*She said what it was, but she also said to us, don't worry because the pregnancy has been going throughout, so don't search the internet. When you come to the gynecologist you will get all the information.*"

Later, the same participant said: "*In the beginning we were quite calm until a few weeks ago when we got the most recent laboratory test result and this result was 80% [YM: result of ADCC test], then it all went very quickly, and we were very shocked.*"

In this context, it seems as though the pregnant woman has consistently held on to reassuring thoughts in order to deal with the uncertain situation, possibly fueled by reassurances from the OCP.

Another participant indicated that she was always reassured, and the seriousness of the situation downplayed. This led to distress after the birth of the child, when the baby was nevertheless born very ill. She would rather have been able to prepare for this.

One participant, who had had three pregnancies, received the result that she had RBC alloantibodies after the birth of her second child, when it was found that he had severe anemia. She had the results from the gynecologist and was not satisfied with the communication. The way in which she received the results was one reason she changed to obstetric care in another hospital for her next pregnancy. "*The way it was told to me was the reason I would never want to give birth in that hospital again. The guidance was very poor, and I was told: 'Antibodies have been found in your blood and from now on every pregnancy is dangerous, but the good news is you can become a blood donor.' [...] This was a lot to process at that time.*" When asked

what additional information was given at that time, the participant said: *"I felt that they actually didn't understand much of it themselves. [...] Afterwards I learned more myself through the internet."*

Various participants stated that they would have preferred the OCP to be honest about their gap in knowledge and to consult an expert or refer to a reliable source of information, if their own knowledge was inadequate to explain the risks of the situation. This would have been better than giving information that could be misinterpreted.

When the OCP indicated the limits of his or her ability straight away and referred the pregnant woman to secondary or tertiary care for more information, participants were very satisfied with the way they had received the bad news.

If the pregnant woman was not satisfied with the information obtained, or if this information was not provided with the help of an interpreter, for example, she looked for information herself. In some women this initially led to fear. This feeling diminished when they received information from an expert or if someone around them with a medical background could explain this to them a little more.

Hostile environment

The child is in danger because antibodies from the mother destroy the child's RBC's

One of the participants referred to the womb as a "hostile environment". For example, she recalled that after the gynecologist explained the problem of RBC alloimmunization, her partner commented*: "It is a very nice idea that at the moment the umbilical cord is cut, the enemy is gone."* The participant stated that the idea of being an "enemy" and at the same time taking care of the growth and maturation of the child was incomprehensible. She said: *"Sometimes I am suddenly really scared; then I think soon she will die and they cannot get her out right now."*

Because of the uncertainty and the unpredictable course of this condition, the quoted pregnant woman above has less confidence in her body to protect her child sufficiently and allow it to grow and be well. At any time, the child could be requiring intervention. Participants indicated that they experienced anxiety about losing their child. This fear was also felt looking back, realizing what could have gone wrong if timely action had not been taken. One participant said: *"If we had waited a week more, he would have been born dead. Yes, that thought..."*

Shift from a normal to a medically complicated pregnancy

"The pink cloud is gone"

Two participants had had a previous pregnancy in which no complications occurred, and they gave birth at home. These participants indicated that they felt more tense during the subsequent pregnancy. They referred to the pregnancy as no longer "*carefree*", *"living from week to week"*, and involving *"practical hassle"*. One participant indicated that she felt more appreciation during this pregnancy when she felt fetal movements. Participants also indicate that they were dependent on their relatives to care for their other children because of visits to or delivery at the tertiary care center, which might be a long distance away. Three of the participants also had to reassure and prepare their family and relatives for the period after birth. There were also participants who found the idea that the baby could be born ill very difficult. Furthermore, two participants indicated that they were sorry that the delivery would be induced and could not give birth at home in their own environment. Additionally, they were worried about their ability to breastfeed normally. What also emerged clearly were the considerations for a subsequent pregnancy. Almost all participants mentioned that they did not dare to plan a subsequent pregnancy. One participant put it like this*: "What is also quite a big thing is that your next pregnancy, if it comes, will already start with a percentage of antibodies.* [...] *I think it will be very long months."* Another participant said, in relation to a possible next pregnancy: *"But now it is done. I hope this all goes well. I will soon have four children; it will also stop at some point. You shouldn't be defying luck. The body has shown that it clearly has more trouble with pregnancy."*

In contrast, participants were very pleased that they had regular check-ups in secondary or tertiary care, which restored their confidence in a successful outcome for the pregnancy. The feeling that they were taken seriously and getting expert information also contributed to the feeling of confidence, although this balance was very unstable: when a result was communicated by a person with little substantive knowledge, or when the doctor or midwife was not well informed about the patient file, this again caused tension. Confidence in and surrender to the expertise of the OCP was then more difficult for the pregnant woman and her partner.

There was a clear need among the participants to know the different scenarios during that could arise during pregnancy, delivery, and the neonatal period. The participants indicated that better preparation provided peace in a stressful situation.

Impact of worsening fetal condition, rapid referral and intervention

No time to think about it

One participant was referred to the tertiary care center to determine whether intrauterine transfusion was necessary, and this was done immediately the very next day. She said: *"It all went very quickly; we had not taken it [intrauterine transfusion] into account anymore. Especially because we were a little naïve about it until then. We were already in the 31st week, so we hoped that with a bit of luck it would all be fine."* The participant indicated that she was upset and overwhelmed by the speed with which the examinations and intervention took place. She didn't have time to think about this and therefore followed the doctors in what they thought was best. When asked what her role was in the decision to give the intrauterine transfusion, she said: *"My opinion wasn't asked, but that is also irrelevant because it was simply necessary."* Another participant said, in relation to the moment she was referred to the tertiary care center: *"You always keep it in mind, but it still scares you. I thought: 'It is getting serious now. It's serious.' And then I felt quite anxious. [...] I did not know what they could do there [YM: in the LUMC], and then I deepened my knowledge on that myself and I became a bit calmer."* The woman indicated that at that time she felt the need to get more information about the possible treatments at the LUMC. She could find this on the LUMC website, and this gave her more peace of mind.

The potentially sudden need for induction of labor and the course of the disease after the birth of the baby were not always clear to participants in advance of these events. Some participants reported experiencing difficulty bonding with their baby due to the fear of losing the child. Participants who had experienced good guidance from the pediatrician indicated that they had confidence in the doctors and treatment. Empathy and calm explanation were again important here. For two of the participants, the child fell seriously ill after discharge. By trusting their own instinct and daring to ask for help, they ensured that their child received the right care promptly. In one case this meant asking for a second opinion in a crucial situation.

The "highest address"

The tertiary care center, the LUMC, is the last link in the case of a complicated alloimmunized pregnancy

Participants indicated that they received a great deal of information and explanation about the examinations and treatments at the LUMC. The information also matched their level of understanding well. They felt good about the investigations being carried out and had confidence in the doctors. One participant said: *"When I heard during the check-up at the LUMC that the baby was doing well, I was always relieved, on to next week."* Another participant indicated that she did not feel "small" and that there

was room for emotions, which she experienced as positive. She went on to say: "*You have the feeling in terms of knowledge and skills that you are at the highest address here.*" On the other hand, when asked whether there was also room for personal questions, the same participant said: "*In the other hospital we knew all the doctors, so you also have a bond. [...] It would have been nice to have had the possibility of discussing some practical things about the delivery.*"

Despite the predominantly positive ratings for the care at the LUMC, participants indicated that they missed the practical information about induction of labor and the neonatal care immediately after birth. One participant, originally from Syria, missed the use of an interpreter when giving important results and information about therapy.

Psychosocial support

Several participants indicated that they experienced feelings of fear and anxiousness during pregnancy or after the baby was born. They were afraid to lose their child. In addition to obtaining information about the expected course of the condition and need for frequent check-ups, participants also indicated that empathy for the situation, a sense of being taken seriously, and the ability to share emotions and experiences contributed to their confidence in the pregnancy. If these aspects were not experienced in the care they had, feelings of anxiousness were still apparent during the interviews. When these aspects were adequately addressed, the participant could put their story in more perspective.

One participant said: "*but just think what tone you use, what words you use. It is very important to someone who has just given birth and is experiencing an uncertain time.*"

Participants valued the continuity of one OCP, especially when attended by primary or secondary care. The need to be aware of the situation and the course of the disease was felt and appreciated. When the primary care midwife remained involved during the pregnancy, even when the pregnant woman had already been referred to secondary or tertiary care, this was also appreciated. The midwife can translate medical jargon and help to ask the right questions, for example about the expected course of the disease or practical matters such as childbirth in this situation.

Discussion

The concept of 'confidence' plays a central role in the experience of women with a pregnancy complicated by RBC alloimmunization. The issue of confidence covers three domains: confidence in one's own body, during the pregnancy, and in the care

providers. Circumstances can influence the experience of pregnant women positively or negatively. In particular, the provision of sincere, open, correct and complete information, and support decisive moments, can positively influence confidence. It is clear from this study that when information is given by persons with considerable experience in relation to RBC alloimmunization, this provides more confidence to the patient.

This study provides a clear picture of participants' experiences. The pregnancy is no longer experienced as carefree, and the pregnant woman regularly finds herself in an uncertain situation, due to the jeopardized fetal condition. To deal with this uncertainty, women use various coping strategies, such as seeking social support, seeking more insight and information, and trying to have faith in a positive outcome. In relation to treatment by intrauterine transfusion, the pregnant woman must rely completely on the knowledge and skills of the doctors, and she and her partner seem to play only a minor role in the decision-making process. Everything is done for the benefit of the baby, suffering from the maternal alloimmunization.

What also emerged from this study is the influence of RBC alloimmunization on opting for a further pregnancy. The participants indicated that their choice is influenced by the course of this pregnancy. Even women who did not have a sick child at the end understood that this can be totally different in a future pregnancy. They do not want to take that risk, or do not want to go through what they experienced in the current pregnancy.

Strength and limitations

To the best of our knowledge, this is the first study concerning the care experiences of women at risk for HDFN. We interviewed eight women, heterogeneous in gestational age or time after the birth of their last child, severity of HDFN (ranging from none to life-threateningly ill), ethnicity, and attendant level of care. Data saturation was reached when comparable experiences were found in relation to the care provided, especially the expressed need for clear, correct and complete information, and the relationship between the information and participants' confidence in the care provider and course of pregnancy. In our opinion, the results of this study are therefore generalizable to all pregnant women with RBC alloimmunization in the Netherlands. The relatively broad time period during which the interviews were conducted (2011–2018) allows us to show that the experiences of care did not change over time.

The participants who received care at the LUMC were made aware that a midwife based at the LUMC was one of the interviewers (YM), and this may have caused participants to reflect more positively on care received at the LUMC. Nevertheless,

those participants also felt sufficiently free to suggest improvements in care at the LUMC.

Previous findings and interpretation

Poorly provided or incomplete information after detection of RBC alloantibodies, or during follow-up, influenced confidence in a positive pregnancy outcome and caused feelings of anxiety in alloimmunized pregnant women. From evaluation of similar situations, such as informing parents of a positive test result for any of the diseases tested for during newborn screening, Moody et al. advised arranging direct face-to-face contact between the specialist team and the family, continuous support and the availability of accessible condition-specific information.(169) In our previous research we found that the knowledge of OCP's about RBC alloimmunization and HDFN was frequently insufficient, and they were often not aware of these gaps in their knowledge.(174) From the perspective of the women in the current study, a lack of knowledge on the part of the OCP should best be shared with her; otherwise this causes feelings of anxiety and insecurity in the pregnant woman and her partner. As described in the results, when the doctor or midwife is aware of the limits his or her abilities and refers for a second opinion from the specialized team, this shortcoming is quite surmountable. This finding is also applicable in other high-risk pregnancies or rare conditions. A study of pregnant women in which twin-to-twin transfusion syndrome was diagnosed showed that patients received limited information about the consequences following the diagnosis.(181) As a result, they started looking for information themselves. We also found this to be a coping mechanism, and Fischbein et al. indicated that this helps families deal with the unpredictability and emotional adjustment.(181) Nonetheless, in our study women and partners indicated that they wanted more information from an expert at these crucial moments. This may also point to a difficulty in readily finding and accessing high-level knowledge via the internet for pregnant alloimmunized women. The tertiary care center (LUMC) has readily accessible information on its website, and it is worthwhile for both pregnant women and OCP's to refer to this information.

Various studies of parents' recommendations on how to inform them in relation to a newborn screening result that indicates a disease suggest that it is important to offer realistic reassurance and hope, to address and support parents through the moments of anxiety and to keep content simple, clear and actionable.(169-172) The same emerges from this study. The pregnant women and their partners also indicated that they wanted honest information about the risks of RBC alloimmunization, and that knowing what might happen and what to expect is very important. In addition, giving the opportunity to share their emotions, and guidance in dealing with their emotions, is appreciated.

Recommendations

Based on this research, we recommend that when RBC alloantibodies are found, this result is communicated face-to-face and that the risks and possible scenarios in relation to the course of the pregnancy are shared. Consulting an expert on this topic before sharing the risks and scenarios is recommended. It is important that the message is clear and contains realistic reassurance, and that the opportunity is offered for sharing emotions. Continuity in the guidance of the pregnant woman is appreciated, and she should be well prepared for any interventions during pregnancy and for having a child who may be or become ill shortly after birth. Prenatal counselling by a neonatologist should be involved, to prepare women and partners for the anticipated neonatal therapy. Furthermore, a preconception consultation should be offered to give women the opportunity to make an informed choice about a subsequent pregnancy. The themes found in this study can form the basis for a quantitative questionnaire to design further improvements in communication with alloimmunized women and provision of knowledge in the rare event of RBC alloimmunization in pregnancy.

Chapter 9

General discussion

The primary aim of this thesis was to evaluate if the high level of care to pregnant women with red blood cell (RBC) alloantibodies could be improved, starting with the perspective of the obstetric care provider, and by collecting input from pregnant women on their experiences. We designed studies to evaluate the performance of new components of the current policies to prevent RBC immunization and to early identify the risk of severe HDFN during pregnancy. Based on our studies, we strive to make recommendations to further tighten preventive measures, and to gain insight into how the patient and the obstetric care provider can be optimally supported in this process.

Pathogenesis

HDFN is caused by maternal RBC antibodies being transferred to the fetus and is usually provoked by fetomaternal hemorrhage during pregnancy or delivery. HDFN is most frequently caused by RhD alloantibodies, although alloantibodies with other Rh specificities (c, C, E, e) or non-Rh alloantibodies (especially K) may also induce fetal hemolysis. Other type of RBC alloantibodies (Fy, Jk, M, S and s) rarely induce severe disease in the Netherlands.(10) Untreated HDFN may result in progressive fetal anemia, hydrops, neonatal icterus and even perinatal death. Preventive measures have substantially reduced the risk on maternal alloimmunization and improved the outcome of HDFN over the past decades.(14, 182) Both Rh and non-Rh alloimmunization in pregnancies is thus becoming a rare condition. The last report on the performance of the national prevention program (2020), showed that among 172,000 pregnant women there were 480-522 (0,28-0,30%) pregnancies in which RBC alloantibodies were identified, including 235-372 (0,14-0,22%) with clinically relevant RBC antibodies.(62)

Prevalence and prevention of RhD immunization

After the introduction of the antenatal RhIg prophylaxis in 1998, at that time only to pregnant women without a living child, the risk of a new RhD-immunization in the next pregnancy in RhD-negative women who gave birth to an RhD-positive (first) child decreased from 0.67% to 0.31%.(44) This rate is comparable to the observed prevalence in three meta-analyses conducted in the UK by the NICE (National Institute for Clinical Excellence).(183)

The extension of the antenatal RhIg prophylaxis to all RhD-negative pregnant women in 2008 (44) and the targeted RhIg prophylaxis exclusively to women with an RhD positive fetus (2011)(24), did not result in a further reduction of the risk for RhD immunization. The prevalence of newly detected RhD immunizations in 2016 was

0.31% (79/25,170) of all RhD-negative pregnant women in the Netherlands. This can be explained by the average rate of 1.7 children per woman (CBS 2008-2016), implicating that only 21% of women experience more than two pregnancies.(184) With a predicted rate of false-negative fetal *RHD* typing of 0.03%, the occurrence of unforeseen and unexpected severe HDFN was estimated as 1 case every three years. (24) Targeted administration of RhIg based on fetal RHD typing simplifies the process, because RhIg can be administered immediately after childbirth, without additional neonatal typing. However, the effect of the latest adjustments on the prevention of RhD immunization seem of minor importance.

Evaluation of repeated RBC antibody screening in Rhc-negative women

In our nationwide cohort of Rhc-negative women (2011-2013), we found 99 (0.16%) Rhc-negative women with newly detected RBC antibodies at the third trimester screening (at 27 weeks) **(Chapter 3)**. This is in line with reported incidences of late alloimmunization, varying between 0.06 and 0.43%. Remarkably, the incidence of severe HDFN in cases with late alloimmunization appeared to be considerably lower than expected, resulting in a NNS (number need to be screened) to detect one case of severe HDFN of 31,048. From earlier research an NNS of about 9000 was expected. This may be explained by the fact that timely detection of alloimmunized cases at risk for fetal hemolysis, followed by induction of labor at week 37, as advised in the Dutch Guideline on maternal alloimmunization, may have prevented the development of severe HDFN. The downside of this, being a potential negative feature of screening, might be several relatively early and unnecessary inductions of labor, performed purely because of the maternal alloimmunization, despite laboratory test results being below the cut-offs. We observed that a foregoing delivery was a risk factor for Rhc alloimmunization detected late in pregnancy. Furthermore, only three nullipara had late RBC alloimmunization and no HDFN due to RBC alloimmunization occurred. Therefore, it could be evaluated if the RBC alloantibody screening in week 27 could be restricted to para 1 and higher.

During pregnancy there are now valid cut-off values available for laboratory management to predict HDFN prenatally **(Chapter 5 and 6)**. However, these are not valid to predict neonatal disease and the need for neonatal phototherapy and/or exchange transfusions. Whether it is necessary to clinically observe a neonate, in order to monitor bilirubin and hemoglobin levels, if maternal titers were low early in pregnancy, requires further studies.

Future adjustments to the screening and prevention program

Most western countries have maternal RBC alloimmunization screening programs. A wide variation in design of these programs exists between and within countries, ranging from several screenings in all pregnant women to a single screening of RhD-negative women only.(21, 25, 26, 183) In the Netherlands, there is a high uptake of both the screening and the prevention program for RBC alloimmunization.(62) As a result, the current numbers of pregnant women with RBC alloimmunization, followed by HDFN with long-term sequelae, are low (described in **chapters 2, 3 and 4**). Since the disease can be serious in antigen-positive fetuses, it is of great value to further reduce the number of red cell immunizations as much as possible. Based on our findings, the options to prevent RhD immunization mainly lie around childbirth and miscarriage. In pregnancies with complicated deliveries, including cases of major bleeding and surgical interventions, such as cesarean section and surgical (manual) removal of the placenta, determination of FMH volume and adjustment of RhIg dosing is necessary to further reduce the RhD alloimmunization rate.

The mechanism of risk factors that are associated with RhD alloimmunization assumes that a complicated delivery gives an additional risk of a larger FMH. On the other hand, one third of the women who had previously given birth to an RhD positive baby, had none of the risk factors that we reported. Possibly, a larger but subclinical FMH than could be covered by the RhIg prophylaxis occurred, as has been reported earlier.(94) Alternatively, some women would respond more strongly to a relatively low volume of fetal blood entering their circulation. (185) The finding that 27% of the women included in our risk factor study was either nulliparous or had an RhD-negative child in history, supports this hypothesis.

The Dutch Association of Obstetrics and Gynecology (NVOG) advice to administer RhIg to all women with a (missed) abortion past 10 weeks, or when invasive treatment is used after 7 weeks of gestation. We found a higher miscarriage rate in RhD-negative women with anti-D detected early or late in their first ongoing pregnancy with an RhD-positive child, as compared with the general population (35% vs 12.5%). We also found that not in all cases RhIg was administrated, according to current protocol. These findings seem to support the policy to administer RhIg in all cases of miscarriage or abortion, irrespective of gestational age or instrumentation. Observational studies on the effect of RhIg after miscarriage/abortion were mostly performed in the early days after the start of RhIg prophylaxis, and randomized controlled trials are unfortunately lacking.(21, 183, 186) As our study, those early studies showed that anti-D is found late in the first ongoing pregnancy with an RhD-positive child, most likely because immunization already occurred around the miscarriage/abortion, but anti-D is only produced at detectable levels during this first ongoing pregnancy.(69, 95, 96)

In RhD-pregnant women with a previous pregnancy with an RhD-positive child, the significance of potential risk factors for a FMH in that previous pregnancy, such as: external cephalic version, abdominal trauma and antenatal bleeding, but also invasive diagnostics in the current pregnancy, is still controversial in the literature. (72-74) Absence of an association of current pregnancy-related risk factors with D-immunization, suggests that the adherence to current indications for RhIg administration is sufficient in the Netherlands.

Availability of RhIg

For prevention of RhD immunization, we are dependent of plasma from RhD-immunized donors. Until 2020 in the Netherlands, RhIg was part of the product portfolio of the plasma fractionation, by collecting plasma from RhD-immunized donors. Nowadays, all RhIg products used in the Netherlands originates from international operating pharmaceutical companies. Since 'natural RhD-immunized' donors (e.g., women immunized by pregnancy) are becoming more and more rare, mainly donations from actively RhD-immunized donors are used. Although RhD immunization may not implicate a donor's health, the presence of RhD antibodies can delay the process of preparing suitable donor blood, especially if RhD-negative blood is not sufficiently available, such as in Asia.(187) If, 'naturally immunized' women can be motivated to become plasma donor, this reduces such undesirable risks for other volunteers. In addition, voluntary unpaid blood donation is recommended by all international authorities (World Health Organization/Council of Europe/ International Society of Blood Transfusion/European Blood Alliance) (122), because it is the best way to strive for self-sufficiency of all blood products, while maintaining an optimal level of quality and safety for both recipients and donors. (121) Since the process of immunization, repeated boosting and frequent donations ask a lot of the donor, alternatively women already being immunized during pregnancy and being aware of the importance of donorship may serve as highly motivated donors. Our work (**Chapter 4**) showed that a way to tackle this challenge is to intensify the collaboration between obstetric care providers and blood banks. Tailored recruitment strategies could be designed for this group of potential donors, with the obstetric care provider having a major role in creating awareness of potential plasma donorship in women with RhD antibodies. This fits well with one of the CanMed roles of the caregiver, for example health advocate and collaborator. Ideally, an international donor program would be designed to always have sufficient plasma available.

Laboratory management

This thesis shows that although K-immunized pregnancies with a K-positive fetus nowadays occur seldomly (6 per year in the Netherlands), the screening and subsequent management of these high-risk cases are of value, as 50% of affected children need intrauterine (IUT) or postnatal transfusion therapy.

We showed that in K-immunized pregnancies with a K-positive fetus, an anti-K titer of 4 identifies all cases with a high risk for severe HDFN defined as the need for IUT or postnatal transfusion therapy **(chapter 5).** Remarkably, the test results of the titer and ADCC did not change significantly during pregnancy. The first titer appeared therefore to have the highest power to predict the necessity of transfusion therapy in K-alloimmunized pregnancies. Our proposed cut-off value of 4 for the titer is on the safe side and in contrast with those proposed by other authors.(5, 147) These studies included cases of severe HDFN and retrospectively described the titers in those pregnancies. In our study we had the opportunity to describe all pregnancies in the Netherlands between 1999 and 2015 with a K-positive fetus and collect all available titer and ADCC results. Therefore, we can accurately conclude that K-mediated HDFN with need for transfusion therapy in cases with titers <4 is very rare. The ADCC test was not suitable to select high risk K-alloimmunized pregnancies. This could be explained by the pathogenesis of anti-K mediated HDFN, in which both the suppression of erythropoiesis and hemolysis of fetal RBC occur. The ADCC test may generally be more correlated with the level of hemolysis. Cost-effectively, the ADCC will not contribute as the specificity of detecting HDFN is not increasing if ADCC test results are added (**Chapter 5**). Moreover, every pregnancy with a titer above the cut-off value will have to be clinically followed with Doppler ultrasound examination to timely detect fetal anemia.(26) Based on our results, we propose not to continue testing with the ADCC assay in K-alloimmunized women.

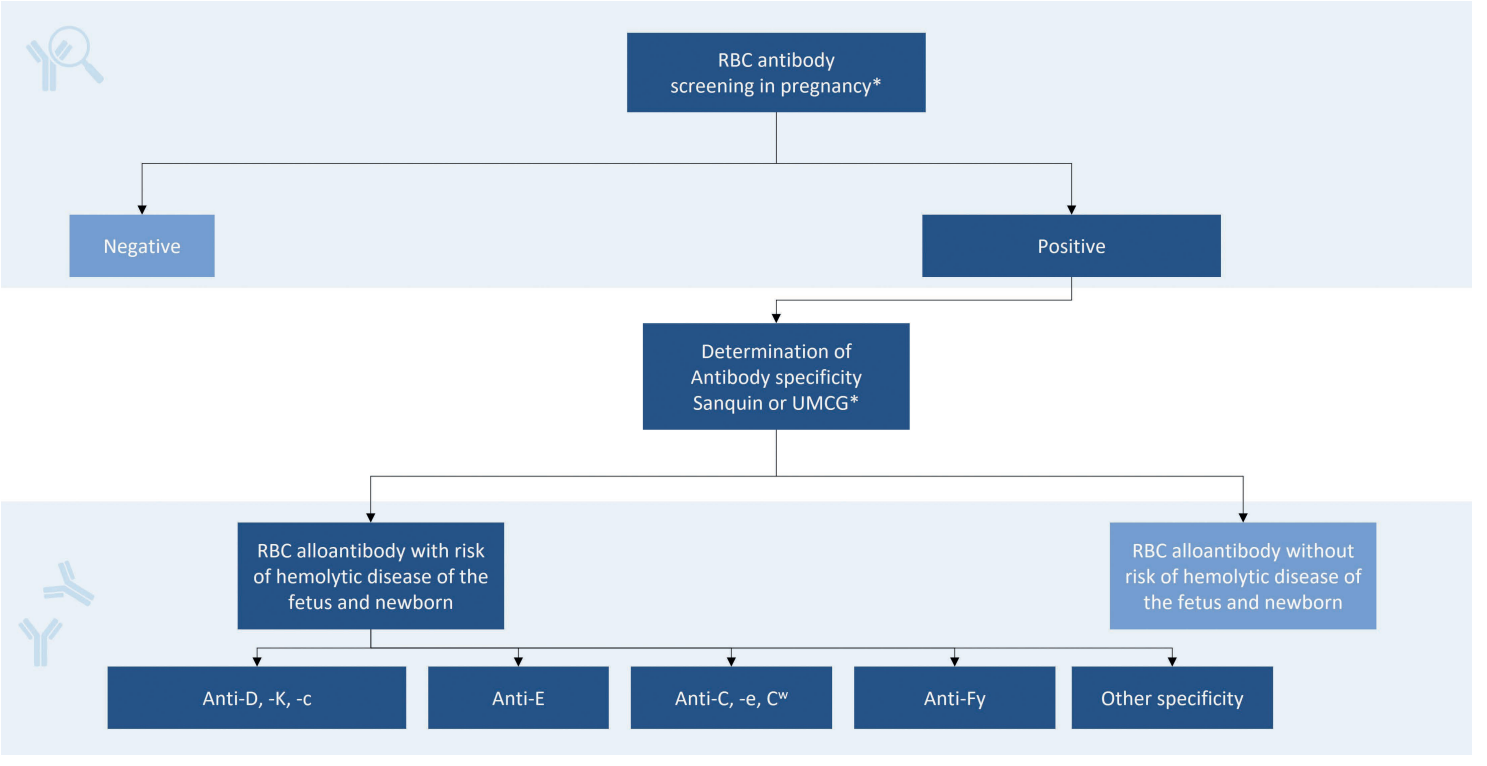
Most cases of severe HDFN are caused by anti-D, less frequently by anti-c and anti-K, and in a rare case by other Rh antibodies.(10) Anti-Fy type of antibodies increase the risk for neonatal icterus, needing phototherapy treatment.(140) For almost all other RBC alloantibody specificities there is casuistic evidence that they may cause severe HDFN disease, underscoring the fact of the very low frequency of those events. In our nationwide prospective cohort study, including pregnant women with RBC alloantibodies with a specificity other than anti-D or anti-K and with an antigen-positive fetus, we found that a maximum titer of ≥16 was the best cut-off to differentiate between pregnancies at low and high risk for severe HDFN. The cut-off of ≥16 obtained in our study is close to the cut-off of 32, derived from other studies. In the follow-up of pregnancies with RBC antibodies with other specificities than Rh, the ADCC test appeared to have no additional value (chapter 6).

Up until now, there is evidence that the glycoprofile of RBC alloantibodies may influence antibody pathogenicity and therefore may be considered a putative diagnostic marker.(153) The OPZI 2.0 cohort, which is described in **chapter 2**, has also been designed to collect a cohort of samples and clinical data to investigate the value of RBC alloantibody glycoprofiles in the prediction of HDFN. Study results are expected in the coming years.

Proposed laboratory management

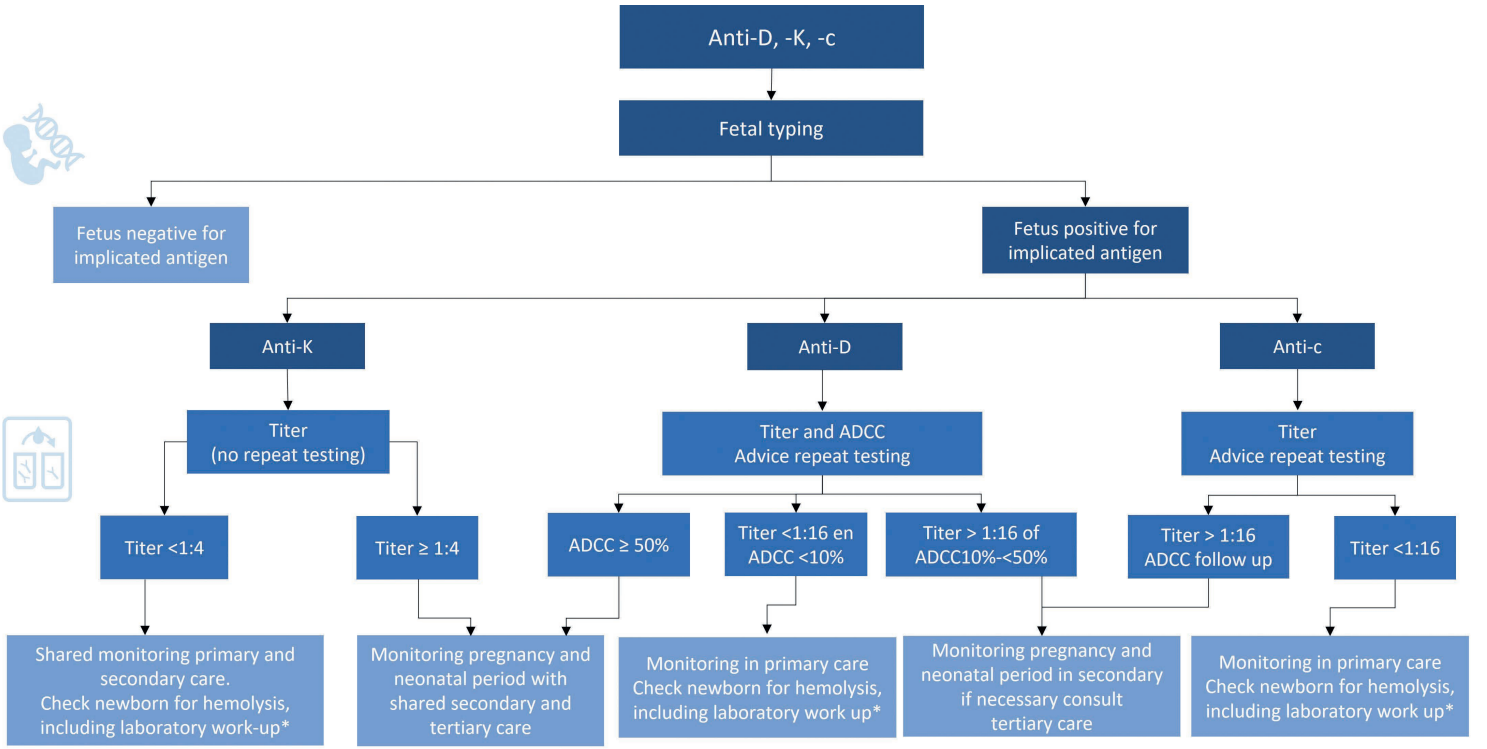
Evaluating the current laboratory management of alloimmunization in pregnancy in the Netherlands, in the context of the results of our studies, we were able to make useful suggestions for adjustments and finetuning of the protocol. In figure 1A,B,C we show how current laboratory management should look like, based on the findings in **chapters 3, 5, and 6**. Our research outcome can be used to update the Guideline Erythrocyte Immunisation and Pregnancy from the Dutch Society of Obstetrics and Gynecology (in Dutch: NVOG), as published in 2009.(26) To further improve the laboratory and clinical management, the care to RBC alloimmunized women would benefit from implementation of a process of continuous monitoring the predictive value of laboratory testing and clinical management in relation to HDFN disease outcome in the newborns. Due to the rarity of the disease, prospective studies to validate traditional and new laboratory tests or to judge necessity of clinical monitoring will be very difficult to perform and take a long time period. Ideally, a process would be developed enabling continuous centralized data collection on RBC alloimmunized pregnancy, making it possible to review laboratory test results and clinical data obtained during pregnancy and after birth.

Figure 1A



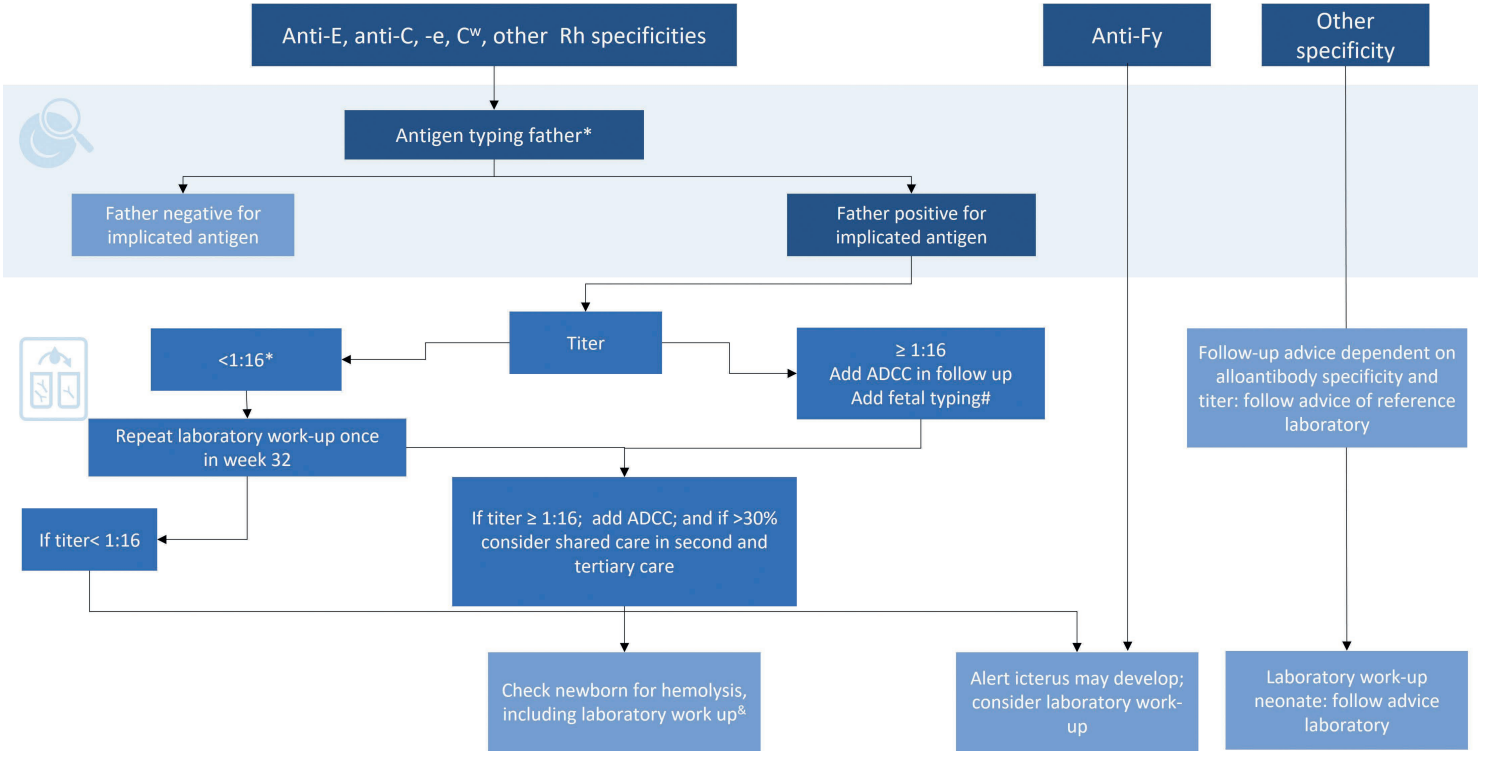
**According to national program: prevention and screening infectious diseases and erythrocyte antibodies in pregnancy (75); #no risk, if of IgM class; or low/absent expression of the antigen on fetal RBCs and no association with HDFN.*

Figure 1B



**For anti-K: if transfusion before 2004 or abroad: consider first K typing of partner: #Laboratory work up: Typing of implicated RBC antigen; Direct Antiglobulin Test, Hb, bilirubin*

Figure 1C



**Fetal typing is possible, but –especially if low are absent titers- antigen typing of the father can be used to reduce costs; #Consider ADCC or fetal antigen typing in follow-up, based on antibody specificity.*

&Laboratory work up: typing of implicated RBC antigen; Direct Antiglobulin Test, Hb, bilirubin

Evaluation prevention program in the framework of the Can MEDS model

Nowadays, due to the success of all preventive measures, obstetric care providers see only few pregnant women with a pregnancy complicated by RBC alloimmunizations This may result in insufficient knowledge, inadequate information transfer and substandard care for women, who are diagnosed with RBC antibodies. In the Netherlands, annually 170-180 000 pregnant women are entering the screening program; the uptake of the RBC alloantibody screening program is very high. Thanks to a well-organized network that has evolved over the years with central coordination of the prevention program by the RIVM, the laboratories and obstetric care providers, the RhD immunization rates are low ((53) and chapter 2) and the timely identification of high-risk pregnancies is successful.(14)

The prevention program contains multiple safety nets during the process of case identification. For example, the obstetric care provider receives all necessary information on RBC alloantibody risks from the reference laboratory, including advice (often also by phone) if a pregnant woman must be referred to a regional hospital or to the fetal therapy center, the LUMC. Presumably, this adds to the prevention of delay or misjudgments of test results, but this also means that the care provider is comforted in such a way, that there is no need to take the effort to be up to date and well-informed concerning current knowledge.

Viewed from the perspective of the Can MEDS framework, several caregiver roles are well represented in the prevention program, such as global leadership in preventing alloimmunization and a high standard of treatment and care. There is a national collaboration of the reference laboratories, the fetal expert center and the National Institute of Public Health and Environment. Centralizing care thus enables a high standard of prevention and treatment. Knowledge obtained from the Dutch program and experience may be shared with international colleagues, to further improve all programs in terms of prevention, diagnostics and clinical monitoring.

Nevertheless, there is also room for improvement regarding the competencies needed to run the screening and prevention program properly. The obstetric care provider can think of education and keeping him- or herself up to date. Since the last adjustments in the screening program in 2011, there was an e-learning accessible for all health care providers, involved in the care of alloimmunization in pregnancy. In 2017, several medical experts did a tour through the Netherlands, to provide healthcare providers with more information about alloimmunization and hemolytic disease of the newborn. However, these education tools are not used by all. In **chapter 8,** we showed that health care providers had little knowledge about items defined in the national guidelines, such as: when an extra dose of RhIg should be administered

or how a titer or ADCC result should be interpreted. These items are until now not clearly enough described in the guidelines, and possibly therefore multi-interpretable. We advise to increase the clarity of recommendations in an updated guideline. In **chapter 7,** we showed that most concern was caused during referral to a reference center and the interpretation of laboratory results. It is therefore important that the national guidelines are amended in such a way, that the healthcare provider has easy access to the necessary information. Attention must also be paid to the information that pregnant women need and must receive and the fact that an expert can always be consulted when there are questions or uncertainties. From the perspective of professionalism, this also means that a care provider knows the limits of own knowledge and skills, especially in the context of rare diseases. In **chapter 8** we report that immunized women indicate to have felt insecure because of too much or incorrect information by their primary obstetric care provider. It is important to create awareness, for instance through national scientific journals, of the impact of incorrect or too much not very concrete or contradictory information by health care providers, in case of a rare disease.

Finally, from the role of health advocacy, there are opportunities to make RhIg more widely available and thus also to reduce the morbidity and mortality of HDFN on a global level. If monoclonal antibody based RhIg is not available, we must rely on enough volunteer anti-D plasma donors, and we might join forces in international context, as is currently done by a working group led by Prof. van der Schoot and initiated by the European Directorate for the Quality of Medicines (EDQM). Sufficient RhIg is also of importance to increase the access to RhIg in low-income countries. This is of major importance for reducing global morbidity and mortality from anti-D mediated HDFN.

Conclusions and future perspectives

In general, we can conclude that the current Dutch screening and prevention program for alloimmunization is on a high standard level. Adjustments can be made to be strict in the policy of recognizing risk factors, determination of estimated FMH volume and adjustment of RhIg dosing, especially in pregnancies with complicated deliveries, including cases of major bleeding and surgical interventions, such as cesarean section and manual (surgical) removal of the placenta. Miscarriage and abortion can be considered as risk factors for alloimmunization, although further research is still necessary to determine the preventive effect of RhIg in all cases. For selection of pregnancies with a high risk of HDFN in K- and non-D immunized pregnancies, the RBC alloantibody titer can be used to make a first selection, preferably after fetal typing of the cognate antigen or after RBC antigen typing of the father. When the titer is equal or above the cut-off value of 4 in K-immunized pregnancies and 16 in other types of RBC immunizations, further clinical follow-up is required. The ADCC test

cannot be effectively used in the first selection of high-risk pregnancies, other than in cases of RhD immunization. Our studies can be used to reduce intensive laboratory management in pregnancies complicated by RBC alloimmunization. Due to the rarity of HDFN, it is of importance to keep the level of knowledge high in expert centers. An up-to-date guideline needs to be available that can be used by all obstetric care providers in primary care. These professionals need to be aware of the limits of their abilities and never refrain from consulting experts in the field for their opinion. This is especially of importance to improve the experience and wellbeing of the immunized pregnant woman and not to confuse and frighten her and her partner unnecessarily.

Chapter 10

Summary

Summary

Pathogenesis and prevention

Hemolytic disease of the fetus and newborn (HDFN) is caused by red blood cell (RBC) alloantibodies, developed by the mother and transferred to the fetus. Most severe cases are caused by RhD, Rhc and K antibodies. Without treatment, HDFN may result in progressive fetal anemia, fetal hydrops, asphyxia and perinatal death. The introduction of this thesis (**Chapter 1**) provides information about the development during the years of the Dutch national program to prevent and timely detect RBC alloimmunization in pregnant women. This program resulted in a decrease in the incidence of RBC alloimmunization and, more importantly, in reduction of perinatal morbidity and mortality. The so-called Prevention and Screening of Infectious Diseases and Erythrocyte Immunization (PSIE) program, currently encompasses respectively prevention of anti-D formation by provision of RhIg prophylaxis (antenatal and postnatal doses); screening for RBC alloantibodies at the booking visit and in RhD-an Rhc-negative women at 27th week of pregnancy. During the years, also the Dutch Transfusion Guideline developed into a policy with matching blood transfusions for Rhc, D, E and K in women below 45 years of age. The timely detection of HDFN has ensured that alloimmune fetal hydrops is rare nowadays. For RhD immunized pregnancies, which remains the most prevalent cause of severe HDFN, selection of high-risk cases and reassurance of low risk by laboratory testing is specific and valid. This was much less well defined for K-immunized pregnancies or if other type of Rh type immunization is present. as described in **Chapter 1**.

As introduced in **Chapter 1**, to identify elements in the RBC alloantibody screening and prevention program that could be improved, the Can Meds framework can be used as a starting point. It can also be used to evaluate the provided care to RBC alloimmunized pregnant women. Therefore, in this thesis, the tools of the Can Meds framework were used to formulate study questions and steps to improve the care.

Incidence and prevalence of RBC alloimmunization

In **Chapter 2** and **3** we evaluated the current incidence of RhD and Rhc immunization in the Netherlands. We evaluated this in two national cohorts. In addition, we also looked at risk factors for RhD immunization and lately detected Rhc immunizations. The prevalence of newly detected RhD immunizations in 2016 was 0.31% (79/25,170) of all RhD-negative pregnant women in the Netherlands. The prevalence of all RhD immunizations (including pregnancies from women who were likely immunized before immigration to the Netherlands) in 2016 was 0.09% of all pregnant women

(158/171,727) and 0.63% of RhD-negative pregnant women in the Netherlands (rates described in **Chapter 2**). In our nationwide cohort (2011-2013) we found 99 Rhc-negative (0.16%) women with newly detected RBC antibodies with the second RBC alloantibody screening at 27 weeks of gestation **(Chapter 3)**. We observed that a previous pregnancy (delivery) was an important risk factor for development of anti-c during the subsequent pregnancy (RR parity (P1: OR, 11.8; 95% CI, 3.00–46.5; P > 1: OR, 7.77; 95% CI, 1.70–35.4). We recommend considering restricting 'week 27 screening' to only those Rhc-negative women with a foregoing pregnancy (55%). Such a policy would improve the number needed to screen (NNS) from 31048 (if screened all) to 17076 (if only ≥P1 would be screened).

Risk factors for RBC immunization

In **Chapter 2** we identified risk factors for fetal maternal hemorrhage (FMH) resulting in RhD immunization despite antenatal and postnatal prophylaxis. In a cohort of 194 women in their first immunized pregnancy, there were 113 women with a foregoing pregnancy above 16 weeks gestation assumed as "exposed" to the RhD antigen. In this group, risk factors for RBC alloimmunization present in a foregoing pregnancy were cesarean section (CS) (OR 1.7, 95% CI 1.1-2.6), perinatal death (OR 3.5, 95% CI 1.1-10.9), gestational age over 42 weeks (OR 6.1, 95% CI 2.2-16.6), postnatal bleeding (>1000mL) (OR 2.0 95% CI 1.1-3.6) or surgical removal of the placenta (SRP) (OR 4.3, 95% CI 2.0-9.3). In the group of nulliparous women and women without an RhD positive child in the previous pregnancy, assumed as "non-exposed" or "possibly" exposed to the RhD antigen, there were no risk factors related to possible events leading to FMH during pregnancy found. Remarkably, we observed a considerable higher miscarriage rate (35%) in this group compared with the average population (12,5%).

Risk factors for late alloimmunization in Rhc-negative women are described in **Chapter 3.** In addition to the data presented in **Chapter 2** for RhD-negative women we found in Rhc-negative women that invasive diagnostic in early pregnancy was a risk factor for Rhc immunization in pregnancy. No other risk factors related to the current pregnancy were identified that might have led to late Rhc immunization detected in week 27 of pregnancy. Independent risk factors for late alloimmunization were blood transfusion in history (OR 10.4; 95% CI, 1.14–94.9), parity (P1: OR, 11.8; 95% CI, 3.00–46.5; P > 1: OR, 7.77; 95% CI, 1.70–35.4) and chorionic villus sampling/ amniocentesis (OR, 9.20; 95% CI, 1.16–72.9) in the current pregnancy.

Donor recruitment

With the effectiveness of the prevention program, the number of RhD-immunized women and thus potential anti-D donors has decreased. In **Chapter 4** it is described that recruiting anti-D donors would benefit from a targeted approach. A lack of knowledge about the possibility to become an anti-D donor was found to be the main barrier to become one. When RhD-immunized women know about this possibility almost 70% of those who were not yet an anti-D donor indicated that they might have become donors if they had been informed. Motivators to become anti-D donors were “want to do something in return” (31%) and “want to prevent others having a sick child or losing a child” (34%). The negative factors we identified were time investment and travel time investment, but 50% of the interviewed anti-D donors mentioned no negative factors of being an anti-D donor.

Timely detection and monitoring

To timely identify pregnancies at risk for a severe course of HDFN, defined as a need of fetal therapy, induced preterm delivery or intensive neonatal treatment, repeated laboratory testing during pregnancy is advised. In this pre-selected group of alloimmunized women, fetal anemia can be diagnosed with a high sensitivity and specificity by non-invasive ultrasonography, using Doppler middle cerebral artery blood (MCA) flow velocity measurements. The tests used for repeated laboratory testing during pregnancy are determination of the RBC antibody titer and antibody activity with the antibody dependent cellular cytotoxicity assay (ADCC). These tests are well validated in RhD-immunized pregnancies, but less well if RBC alloantibodies other than anti-D are present. In **Chapter 5** we evaluated the diagnostic value of the ADCC and titer in case of K-alloimmunized pregnancies with a K-positive fetus. The first measured titer with a value above 4 has the best performance in identifying cases with the need for IUT or postnatal transfusion therapy: sensitivity 100% (95% confidence interval, 91-100); specificity 27% (95% confidence interval, 15-43), and positive predictive value of 60% (49-71%). The ADCC test was not informative to select high-risk pregnancies. Linear regression showed no significant change during pregnancy, when antibody titer and ADCC test results were compared with every 2 foregoing measurements ($P < 0.0001$). Furthermore, we observed in a cohort of 93 pregnancies with a K-positive fetus that >50% of K-positive fetuses need either intrauterine transfusion or postnatal transfusion therapy.

In **Chapter 6** we evaluated test characteristics of RBC alloantibodies with another specificity than anti-D or anti-K. We used a cohort collected recently in 2015-2016 and compared the results with a cohort collected in 2003-2004 of which also HDFN disease severity was collected. The optimal cut-off value for the maximum titer in

pregnancies with an antigen-positive fetus was ≥ 16 with a sensitivity of 100% and a specificity of 67% to predict severe HDFN (need for antenatal or postnatal transfusion). On average in 18% of the pregnancies the cut-off value for the titer was reached; but a higher percentage of 36% was observed if anti-c was present. We calculated the positive predictive value (PPV) of the titer to be only 17%, and if in combination with an ADCC of ≥60% the PPV is 60% (83% if anti-c is present, 25% for other type of antibodies. Based on these results we present in the **General Discussion** a proposal to use extensive laboratory monitoring for high-risk case selection in RhD, Rhc and K alloimmunized pregnancies, and to be more restricted with repeat testing if other type of RBC alloantibodies is present.

The prevention program from health care provider and patient perspective

The low prevalence of RBC alloimmunization and especially the rare occurrence of severe HDFN may result in insufficient knowledge and subsequent inadequate transfer of information to pregnant women, diagnosed with RBC alloantibodies. In **Chapter 7** we measured knowledge, attitude and practices (KAP) regarding maternal RBC alloimmunization among Dutch obstetric care providers. We found that only 7% of 329 participants had sufficient knowledge of all aspects of maternal RBC alloimmunization as judged needed to provide sufficient support and counselling during pregnancy. The knowledge gaps we found concerned respectively aspects of alloimmunization with non-RhD alloantibodies, the interpretation of ADCC and antibody titer results and indications for administering extra doses of RhIg.

Next, we set out a study to investigate how women with a pregnancy complicated by RBC alloantibodies experience the provision of information, the transfer to a specialized hospital and how this all influenced their experience of the pregnancy. In **chapter 8** we describe that woman noticed that the knowledge of the healthcare provider was insufficient to provide satisfactory information about RBC alloimmunization and risks for the fetus. Poorly provided or incomplete information after detection of RBC alloantibodies, or during follow-up, influenced their confidence in a positive pregnancy outcome and caused feelings of anxiety. The study also showed that if the caregiver is aware of the limits of the own knowledge and skills and consults an expert with questions or uncertainties, this shortcoming can be overcome.

In the **General Discussion** our findings in the Dutch context. To put the prevention program in a broader context, we have used the Can Meds framework. Viewed from the perspective of the different roles of a healthcare professional, it can be concluded that the roles of collaborator, leader and medical expert are very much

used and necessary in the care to women with RBC alloimmunization. The rare occurrence of RBC alloimmunization and HDFN makes it difficult to provide sufficient and correct information to RBC alloimmunized pregnant women. Although experts can be consulted, there is also room for improvement to provide a clear guideline and easy access to correct information. Knowledge of the disease can personalize the moments of laboratory and clinical testing. Overall, it was observed that there is still a lot that can be achieved when investing in training of health care providers on theoretical background of the red blood cell alloimmunization prevention program and in the counselling of pregnant women with RBC alloimmunization and at risk of HDFN. The Can Meds roles of communicator, professional and lifelong learning (scholar) represent the challenges of obstetric care providers and investment in those roles would further add to the already high level of care to the small group of women with a pregnancy at risk of HDFN. Finally, the obstetric care provider can certainly also take the role of health advocate, since there are still opportunities to make RhIg more widely available to reduce morbidity and mortality of HDFN on a global level.

Chapter 11

Nederlandse Samenvatting

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Pathogenese en preventie

Hemolytische ziekte van de foetus en pasgeborene (HZFP) wordt veroorzaakt door rode bloedcel (RBC)-alloantistoffen, gevormd door de geïmmuniseerde moeder en via de placenta overgedragen aan de foetus. Ernstige gevallen van HZFP worden veroorzaakt door RhD-, Rhc- en K-antistoffen. Zonder behandeling kan HZFP leiden tot progressieve foetale anemie, hydrops foetalis, asfyxie en perinatale sterfte. De introductie van dit proefschrift (Hoofdstuk 1) geeft informatie over de ontwikkeling van het landelijke programma voor de preventie van en screening op infectieziekten en erytrocytenimmunisatie (PSIE) tijdens de zwangerschap. Dit programma resulteerde in een belangrijke afname van de incidentie van rode bloedcel (RBC)-alloimmunisatie en, belangrijker nog, in een afname van de perinatale morbiditeit en mortaliteit. Het PSIE-programma omvat momenteel de preventie van RhD-immunisatie door verstrekking van RhIg-profylaxe (antenatale en postnatale doses), screening op RBC-alloantistoffen bij alle zwangeren bij de intake van de zwangerschap en opnieuw in de 27e week van de zwangerschap, bij RhD- en Rhc-negatieve vrouwen. Daarnaast is in de Nederlandse richtlijn Bloedtransfusiebeleid opgenomen om vrouwen onder de 45 jaar bloedtransfusies te geven die gematcht zijn voor Rhc, RhD, RhE en K. De tijdige detectie van zwangerschappen met een risico op HZFP, in combinatie met eventuele verwijzing en behandeling, heeft ervoor gezorgd dat foetale alloimmuun hydrops tegenwoordig zeldzaam is. Bij RhD-immunisatie, nog steeds de meest voorkomende oorzaak van ernstige HZFP, zijn de specificiteit en validiteit van laboratoriumtesten om onderscheid te maken tussen hoog- en laagrisico zwangerschappen aangetoond. Deze laboratoriumtesten worden ook gebruikt bij zwangerschappen gecompliceerd door ander type RBC-alloantistoffen, waarbij de waarde van de testuitslag en het herhalen van testen tijdens de zwangerschap niet goed bekend was.

Zoals geïntroduceerd in Hoofdstuk 1, kan het Can Meds-raamwerk als uitgangspunt worden gebruikt om de elementen in het screenings- en preventieprogramma erytrocytenimmunisatie te identificeren die verbetering behoeven. Het kan ook worden gebruikt om de verleende zorg aan zwangere vrouwen met RBC-alloimmunisatie te evalueren. Daarom is in dit proefschrift het Can Meds-raamwerk gebruikt om onderzoeksvragen en stappen te formuleren met het doel de zorg te verbeteren.

Incidentie en prevalentie van RBC alloimmunisatie

In Hoofdstuk 2 en 3 beschrijven wij de incidentie van RhD- en Rhc-immunisatie in Nederland. We evalueerden dit in twee recente landelijke cohorten. Daarnaast

onderzochten wij het voorkomen van risicofactoren voor RhD-immunisatie en laat (27 weken) ontdekte Rhc-immunisaties. Nieuw ontdekte RhD-immunisatie werd in 2016 aangetoond bij 0,31% (79/25.170) van de RhD-negatieve zwangere vrouwen. De prevalentie van RhD-immunisaties (inclusief zwangerschappen van vrouwen die waarschijnlijk vóór immigratie naar Nederland waren geïmmuniseerd) bedroeg in 2016 0,09% van alle zwangere vrouwen (158/171.727) en 0,63% van de RhD-negatieve zwangere vrouwen in Nederland (beschreven percentages in Hoofdstuk 2). In ons landelijke cohort (2011-2013) vonden we 99 Rhc-negatieve (0,16%) vrouwen met nieuw gedetecteerde RBC-antistoffen bij de tweede antistofscreening na 27 weken zwangerschap (Hoofdstuk 3). We zagen dat een eerdere zwangerschap (bevalling) een belangrijke risicofactor was voor het ontwikkelen van anti-c tijdens de volgende zwangerschap (RR-pariteit (P1: OR, 11.8; 95% BI, 3.00-46.5; P > 1: OR, 7.77; 95% BI, 1,70-35.4). Op basis van deze resultaten geven wij in overweging om de 'week 27 screening' te beperken tot alleen die Rhc-negatieve vrouwen met een eerdere zwangerschap; dit betreft (55% van de Rhc-negatieve zwangeren).

Risicofactoren voor RBC immunisatie

In Hoofdstuk 2 identificeerden we risicofactoren voor foetomaternale transfusie (FMT) resulterend in RhD-immunisatie ondanks prenatale en postnatale profylaxe. In een cohort van 194 vrouwen in hun eerste geïmmuniseerde zwangerschap waren er 113 vrouwen met een eerdere zwangerschap van meer dan 16 weken van wie werd aangenomen dat ze "blootgesteld" waren aan het RhD-antigeen. In deze groep waren de volgende risicofactoren voor alloimmunisatie tegen RBC aanwezig in een voorgaande zwangerschap: keizersnede (CS) (OR 1,7, 95% BI 1,1-2,6), perinatale sterfte (OR 3,5, 95% BI 1,1-10,9), zwangerschapsduur langer dan 42 weken (OR 6,1, 95% BI 2,2-16,6), postnatale bloeding (>1000 ml) (OR 2,0 95% BI 1,1-3,6) of operatieve verwijdering van de placenta (MPV) (OR 4,3, 95% BI 2,0-9,3). De groep nullipara vrouwen en vrouwen met alleen RhD-negatieve kinderen in voorgaande zwangerschappen, werd verondersteld "niet-blootgesteld" te zijn aan het RhD antigeen; in de subgroep van deze vrouwen die ook een miskraam in de voorgeschiedenis had werd aangenomen dat zij "mogelijk" eerder blootgesteld waren het RhD-antigeen. Bij deze beide groepen zwangeren, werden geen risicofactoren gevonden die kunnen leiden tot FMT tijdens de zwangerschap. Opmerkelijk is dat we in deze groep van nullipara vrouwen wel een aanzienlijk hoger aantal miskramen (35%) zagen in vergelijking met de gemiddelde populatie (12,5%).

Naast de gegevens gepresenteerd in Hoofdstuk 2 voor RhD-negatieve vrouwen vonden we bij Rhc-negatieve vrouwen (Hoofdstuk 3) dat invasieve prenatale diagnostiek in de vroege zwangerschap een risicofactor was voor Rhc-immunisatie tijdens de zwangerschap. Er werden geen andere risicofactoren met betrekking tot

de huidige zwangerschap geïdentificeerd die zouden kunnen hebben geleid tot laat gedetecteerde Rhc-immunisatie(week 27). Onafhankelijke risicofactoren voor late Rhc-alloimmunisatie waren bloedtransfusie in de voorgeschiedenis (OR 10,4; 95% BI 1,14-94,9), pariteit (P1: OR, 11,8; 95% BI, 3,00–46,5; P> 1: OR, 7,77; 95% BI, 1,70–35,4) en zoals genoemd vlokkentest of vruchtwaterpunctie (OR 9,20; 95% BI, 1,16– 72,9) in de huidige zwangerschap.

Donorwerving

Met de effectiviteit van het preventieprogramma is het aantal RhD-geïmmuniseerde vrouwen en daarmee potentiële anti-D-donors afgenomen. In Hoofdstuk 4 wordt beschreven dat het werven van anti-D-donoren gebaat zou zijn bij een gerichtere aanpak. Gebrek aan kennis over de mogelijkheid om anti-D-donor te worden bleek de belangrijkste barrière om te besluiten dat te doen. Bijna 70% van de RhD-negatieve vrouwen met anti-D die nog geen anti-D-donor waren gaven aan dat ze mogelijk donor zouden zijn geworden als ze geïnformeerd waren. Motivaties om anti-D-donor te worden waren “iets terug willen doen” (31%) en “willen voorkomen dat anderen een ziek kind krijgen of een kind verliezen” (34%). De belemmerende factor die we identificeerden was (reis-)tijdsinvestering, maar 50% van de geïnterviewde anti-D-donors noemde geen negatieve factoren om anti-D-donor te zijn.

Tijdige opsporing en monitoring

Om zwangerschappen met een risico op een ernstig beloop van HZFP, gedefinieerd als de noodzaak tot intra-uteriene transfusie, geïnduceerde vroeggeboorte of intensieve neonatale behandeling, tijdig te identificeren, wordt geadviseerd om tijdens de zwangerschap herhaalde laboratoriumtesten te doen.

De testen die worden gebruikt voor herhaalde laboratoriummonitoring tijdens de zwangerschap zijn bepaling van de RBC-antistoftiter en de antistofactiviteit met de antistof-afhankelijke cellulaire cytotoxiciteitstest (ADCC). Deze testen zijn goed gevalideerd in RhD-geïmmuniseerde zwangerschappen, maar minder goed als er andere antistoffen dan anti-D aanwezig zijn. Als op basis van de testen een verhoogd risico op foetale hemolyse bestaat, kan foetale anemie

met een hoge gevoeligheid en specificiteit worden gediagnosticeerd met behulp van niet-invasieve echografische Doppler stroomsnelheidsmetingen in de ‘middle cerebral artery’ (MCA). In Hoofdstuk 5 onderzochten we de diagnostische waarde van de ADCC-test en antistoftiter in zwangerschappen met maternale K-immunisatie en een K-positieve foetus. De eerste gemeten titer met een waarde groter of

gelijk aan 4 heeft de beste testeigenschappen voor het identificeren van gevallen waarbij IUT of postnatale transfusietherapie nodig is: sensitiviteit 100% (95% betrouwbaarheidsinterval, 91-100); specificiteit 27% (95% betrouwbaarheidsinterval, 15-43), en positief voorspellende waarde van 60% (49-71%). De ADCC-test was niet informatief bij K-immunisatie om risicozwangerschappen te selecteren. Lineaire regressie toonde geen significante verandering tijdens de zwangerschap, wanneer titer en ADCC-testresultaten werden vergeleken met elke twee voorgaande metingen ($P < 0,0001$). De eerst gemeten en eenmalige titerbepaling is dus voldoende om het risico op hemolyse te voorspellen. Daarnaast stelden wij vast in een cohort van 93 zwangerschappen met een K-positieve foetus dat >50% van de baby's een intra-uteriene transfusie en/of postnatale transfusietherapie nodig had.

In Hoofdstuk 6 onderzochten we de test uitkomsten van RBC-alloantistoffen met een andere specificiteit dan anti-D of anti-K die HZFP kunnen veroorzaken en bij zwangeren aangetoond worden. We gebruikten een cohort dat recentelijk in 2015-2016 was verzameld en vergeleken de resultaten met een eerder cohort (2003-2004), waarin ook de ernst van de HZFP bekend was. De optimale afkapwaarde voor de hoogste titer bij zwangerschappen met een antigeen-positieve foetus was ≥ 16, waarbij een sensitiviteit van 100% voor opsporen van ernstige HZFP (noodzaak tot antenatale of postnatale transfusie) als voorwaarde gesteld werd en een specificiteit van 67% vastgesteld werd. Gemiddeld werd deze afkapwaarde voor de titer in 18% van de geïncludeerde zwangerschappen bereikt, het meest frequent in gevallen van anti-c-immunisatie (36%). We berekenden dat de positief voorspellende waarde (PPV) voor HZFP van alleen de titer slechts 17% was, maar dat in combinatie met een ADCC-uitslag van ≥60% de PPV 60% is (83% bij anti-c, 25% voor andere antistofspecificiteiten). Gebaseerd op deze resultaten presenteren we in de Algemene Discussie een voorstel waarin uitgebreide laboratoriummonitoring wordt gebruikt voor de selectie van gevallen met een hoog risico op HZFP, bij RhD-, Rhc- en K-alloimmunisatie. En om het herhaald testen te beperken als er andere specificiteiten RBC-antistoffen aanwezig zijn.

Het preventieprogramma vanuit het perspectief van zorgverlener en patiënt

De huidige lage prevalentie van RBC-alloimmunisatie en met name het zeldzame voorkomen van ernstige HZFP kan leiden tot onvoldoende kennis en daarmee ook tot gebrekkige overdracht van informatie aan zwangere vrouwen met RBC-alloantistoffen. In Hoofdstuk 7 hebben we de kennis, attitude en praktijk met betrekking tot maternale RBC-alloimmunisatie gemeten onder Nederlandse verloskundige zorgverleners. We ontdekten dat slechts 7% van de 329 deelnemers voldoende kennis had van aspecten van RBC-alloimmunisatie bij een zwangere

vrouw, die wij nodig achtten om voldoende ondersteuning en counseling te bieden tijdens de zwangerschap. De kennislacunes die we vonden betroffen aspecten van alloimmunisatie met ander type antistoffen dan anti-D, de interpretatie van ADCC- en antistoftiterresultaten en indicaties voor het toedienen van extra doses RhIg.

Vervolgens hebben we een studie opgezet om te onderzoeken hoe vrouwen met een zwangerschap gecompliceerd door RBC-alloimmunisatie de informatievoorziening en de verwijzing naar een gespecialiseerd ziekenhuis ervaren en hoe dit hun beleving van de zwangerschap beïnvloedde. In Hoofdstuk 8 beschrijven we dat vrouwen aangaven dat zij ervaren hadden dat de kennis van de zorgverlener onvoldoende was om voldoende informatie te geven over RBC-alloimmunisatie en de bijbehorende risico's voor de foetus. Matig verstrekte of onvolledige informatie na detectie van RBC-alloantistoffen, of tijdens follow-up, beïnvloedde hun vertrouwen in een positieve zwangerschapsuitkomst en veroorzaakte gevoelens van angst. Uit het onderzoek bleek ook dat als de zorgverlener zich bewust is van de grenzen van de eigen kennis en vaardigheden en bij vragen of onduidelijkheden een deskundige raadpleegt, deze tekortkoming overkomelijk is.

Om het preventieprogramma in een bredere context te plaatsen, hebben we gebruikgemaakt van het Can Meds-raamwerk. Gezien vanuit het perspectief van de verschillende rollen van een zorgprofessional, kan worden geconcludeerd dat de rollen van 'samenwerking', 'leiderschap' en 'medisch expert' goed ontwikkeld en noodzakelijk zijn in de begeleiding van vrouwen met RBC- alloimmunisatie. Doordat RBC-alloimmunisatie en HZFP inmiddels zeldzaam zijn in de dagelijkse praktijk is het voor de zorgprofessional moeilijk om voldoende en correcte informatie te verstrekken aan zwangere vrouwen met RBC-alloimmunisatie. Hoewel experts gemakkelijk kunnen worden geraadpleegd, is er ook ruimte voor verbetering door een duidelijke richtlijn en eenvoudige toegang tot correcte informatie te bieden. Kennis van de ziekte kan bijdragen tot een beter begrip bij de zwangere en meer individuele context rondom momenten van laboratorium- en klinische testen. Over het algemeen werd geconstateerd dat er nog veel kan worden bereikt door te investeren in de opleiding van zorgverleners over de theoretische achtergrond van het PSIE-programma en in de begeleiding van zwangere vrouwen met RBC-alloimmunisatie en een risico op HZFP. Investeren van verloskundig zorgverleners in de Can Meds-rollen van 'communicator', 'professional' en 'levenslang leren' zouden het toch al hoge niveau van zorg voor deze kleine groep vrouwen met een zwangerschap met een risico op HZFP verder vergroten. Ten slotte kan de verloskundig zorgverlener zeker ook de rol van 'gezondheidsbevorderaar' op zich nemen, aangezien er nog steeds mogelijkheden zijn om RhIg breder beschikbaar te maken om zo de morbiditeit en mortaliteit van HZFP op mondiaal niveau te verminderen.

Appendices

- **Publications**
- **Curriculum Vitae**
- **Dankwoord**
- **Abbreviations**
- **References**

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Curriculum Vitae

Yolentha Slootweg werd geboren op 22 november 1986 in Valkenburg (Z-H) als zesde en jongste kind van drie zoons en drie dochters. Na afronding van de HAVO aan het Visser 't Hooft Lyceum in Leiden ging zij naar de Verloskunde Academie in Amsterdam. In 2008 voltooide zij de opleiding tot verloskundige.

Gedurende een korte periode werkte Yolentha als eerstelijns verloskundige in de omgeving van Leiden. Daarna werkte zij een klein jaar als tweedelijns verloskundige in het Vlietland Ziekenhuis in Schiedam. In december 2009 ging zij werken als klinisch verloskundige in het LUMC.

Intussen wilde Yolentha zich verder gaan verdiepen in het doen van wetenschappelijk onderzoek. Daarom startte zij in 2010 met de Master Midwifery Science aan de Universiteit van Amsterdam en studeerde zij in 2013 af. Voor haar Masterthesis deed Yolentha een evaluatiestudie naar de opbrengst van de tweede screening onder Rhc-negatieve vrouwen, welke in 2011 was toegevoegd aan het nationale programma preventie en screening naar infecties en erytrocytenimmunisatie. Tijdens de module Kwalitatief onderzoek werd de interesse gewekt om de wat meer sociale kant van rode bloedcel immunisatie tijdens de zwangerschap wetenschappelijk te exploreren. Samen met Masja de Haas en Joke Koelewijn van Sanquin, Dick Oepkes en Inge van Kamp van het foetale therapie team van het LUMC werd een promotietraject samengesteld. In 2015 startte zij officieel als promovendus, gedeeltelijk gefinancierd door een beurs van Sanquin Blood Supply. Dit traject was een samenwerking tussen de afdeling Verloskunde van het LUMC en de afdeling translationele immunohematologie van Sanquin.

Tijdens haar gehele promotie bleef Yolentha werken als klinisch verloskundige en vanaf 2019 werd zij leidinggevende van het team van verloskundigen. Yolentha is namens de KNOV lid van de programmacommissie Preventie en screening naar infecties en erytrocytenimmunisatie. Daarnaast is Yolentha actief bestuurslid van het verloskundig samenwerkingsverband Leiden in haar rol als leidinggevend verloskundige.

Yolentha woont in Rijnsburg met haar man Maarten Messemaker en haar zoon Job (2014) en dochter Roos (2017).

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De afdeling Verloskunde van het LUMC en de afdeling Translationele Immunohematologie Sanquin hebben mij de mogelijkheid gegeven om mij op dit promotieonderzoek te richten.

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Abbreviations

ADCC – Antibody-dependent cell-mediated cytotoxity assay
BIBI - Special Institute for Blood Group Investigations
Can MEDS – Canadian Medical Education Directives for Specialists
CS – Caesarean Section
FMH – Fetomaternal hemorrhage
HbF – Fetal hemoglobin
HDFN – Hemolytic disease of the fetus and newborn
HZFP – Hemolytische ziekte van de foetus en pasgeborene
IU – International units
IUT – Intrauterine transfusion
IVIg – Intravenous immunoglobulins
KBT – Kleihauer Betke test
LUMC- Leiden University Medical Center
MCA-PSV – Middle cerebral artery peak systolic velocity
MoAb – Monoclonal antibody
MoM – Multiple of the Median
MRP – Manual Removal of the placenta
NIPT – Non-invasive prenatal test
NNS – Number needed to screen
OCP – Obstetric care provider
OPZI – Opsporing en Preventie van zwangerschapsimmunisatie
PSIE – Prenatal Screening for Infectious diseases and erythrocyte immunization
RBC – Red blood cell
Rh – Rhesus
RHD – Rhesus- D antigeen
RhIg – Anti-D prophylaxis
RIVM – Rijksinstituut voor Volksgezondheid en Milieu
UMCG – University Medical Center Groningen

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