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

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Running head: Screening for alloimmunisation in Rhc-negative women

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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 Fetus 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.

Tweetable abstract: 3rd trimester screening for alloimmunisation in Rhc−neg women improves detection and treatment of severe HDFN.

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

Introduction

Haemolytic Disease of the Fetus and Newborn (HDFN) is caused by maternal alloimmunisation against paternally inherited fetal red blood cell (RBC) antigens. HDFN may lead to fetal 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.[1-7] Most severe HDFN cases are caused by RhD-, Rhc- and Kell-antibodies (hereafter called anti-D, anti-c, etcetera).[1-5, 8] Timely detection of maternal alloimmunisation facilitates fetal monitoring, aimed to identify fetuses 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. [9, 10]

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.[1, 11-15]

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. [16, 17] Implementation of screening in Rhc-negative women, comprising 18.7% of pregnancies[18], 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 fetal 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.[8] 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. [8] 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.[19-23] 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.[17] 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 fetal 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 fetal haemolysis. The

father of the fetus is typed for cognate antigen(s) and in case of heterozygosity, non-invasive typing on fetal DNA in maternal plasma is offered (for *RHD*, *RHC*, *RHc*, *RHE* and *K*).[24] If the fetus does not have the cognate antigen(s), further monitoring of the pregnancy is not necessary. If the fetus 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 fetal anaemia is monitored with middle cerebral artery (MCA) Doppler measurements.[25, 26] Severe fetal 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 1st 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 1st 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 fetomaternal (micro-)transfusions (FMT) of antigen-positive RBCs.[12, 20] 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). [27-30] 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.[31]

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 alloimmunised pregnancies. Therefore, the occurrence of severe fetal 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.

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).

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

221 severe case was caused by the combination of anti-c and anti-E, mostly by anti-E (titre 1:256).
222 During this pregnancy, one IUT (pre-transfusion Hb 9.0 g/dL) was performed at 30+3 weeks,
223 followed by induction of labour at 36 weeks. The Hb and Ht levels postpartum were 12.4 (g/dL)
224 and 0.42, respectively. Phototherapy was given during seven days. An exchange transfusion was
225 needed after two operations for pyloric stenosis, carried out after the first week of life. Two
226 months postpartum this child was confirmed to be in a good condition. The other severe case
227 was caused by anti-c only. No intrauterine transfusion was given. Labour was induced at 36
228 weeks + 4 days; Hb and Ht at birth were 13.3 (g/dL) and 0.42, respectively. The lowest Hb was
229 9.8 (g/dL), five top-up transfusions were given, no exchange transfusions were needed.
230 Phototherapy was given in 20 cases (12 anti-c, 5 anti-E and 3 anti-c and anti-E), resulting in an
231 incidence of moderate HDFN of 0.032% of all screened Rhc-negative women (Table 2) and
232 20.20% of screen-positive pregnancies. In cases with known outcome (n=89) the incidence of
233 moderate HDFN was 22.5%(95%-CI:13.8-31.1%).
234 The NNS to detect one case of severe HDFN was 31,048 and to detect one case of moderate
235 HDFN 3,105.
236 Six cases of moderate HDFN occurred in association with laboratory test results below the
237 aforementioned cut-offs.
238 Forty-nine children of the 90 pregnancies with anti-c and/or anti-E, were antigen-positive for the
239 cognate antigens (based on antigen typing of the child (n=26) or homozygosity of the father for
240 the antigens concerned (n=23)), five were antigen-negative and in 36 cases the antigen-typing
241 was unknown. We calculated that 17 children with unknown antigen-typing should have been
242 antigen-positive (Box S1), resulting in a risk for moderate HDFN in antigen-positive
243 fetuses/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 fetal 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 were, 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).

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.[8] 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.[32] 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.[33] 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,[8] a NNS of about 9,000 was expected. An explanation for this decreased incidence might be that timely detection of cases at risk for fetal 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.[34] 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 fetuses. 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 fetal 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 a number of 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%).[31]

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%)[20] 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.[8] 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 fetal 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.[35] 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.[36] Moreover, invasive diagnostic procedures are associated with fetomaternal haemorhage [29], 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).[37]

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[16], 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.[38] We also showed that the psychological burden of antibody screening is small and balanced with the benefits.[39]

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

We thank all the pregnant women and obstetric care providers who participated in the study. 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).

Disclosure of interests

There are no competing interests to declare. The ICMJE disclosure forms are available as online supporting information.

Contribution of authorship

YM Slootweg designed the study, carried out data collection, extraction, analysis and interpretation of data and drafted the article and is responsible for the integrity of the work as a whole. JM Koelewijn advised on study design, carried out data collection, extraction and interpretation of data, revised the article critically for intellectual content and approved the final draft for publication. M. de Haas advised on study design, carried out interpretation of the data, revised the article critically for intellectual content, and approved the final draft for publication. JG van der Bom, IL van Kamp and D Oepkes assisted with interpretation of the data, revised the article critically for intellectual content and approved the final draft for publication.

Ethics approval

In the Netherlands this study design does not require formal approval of the Medical Ethical Committee.

Funding

This study was conducted in a partnership of Sanquin Diagnostics Amsterdam and Leiden University Medical Centre. This study was not funded by external sources

Reference List

1. Daniels GL. Blood group antibodies in haemolytic disease of the fetus and newborn; in: Hadley A, Soothill P (eds): Alloimmune disorders of pregnancy. Anaemia, thrombocytopenia and neutropenia in the fetus and newborn. 2002.
2. Moise KJ. Fetal anemia due to non-Rhesus-D red-cell alloimmunization. Seminars in fetal and neonatal medicine; Perinatal Haematology; 2008. p. 207-14.
3. Moise KJ. Management of rhesus alloimmunization in pregnancy. Obstetrics & Gynecology. 2008;112(1):164-76.
4. Franklin I. Prevention of rhesus haemolytic disease of the fetus and newborn. Lancet. 2009;373(9669).
5. Klein HG AD. Haemolytic disease of the fetus and the newborn. In: Klein HG, Anstee DJ, editors. Mollison's blood transfusion in clinical Medicine. 2012:499-548.
6. Watchko J, Tiribelli C. Bilirubin-induced neurologic damage--mechanisms and management approaches. N Engl J Med. 2013;369(21):2021-30.
7. Bhutani VK ZA, Blencowe H, Khanna R, Sgro M, Ebbesen F et al. Neonatal hyperbilirubinemia and Rhesus disease of the newborn: incidence and impairment estimates for 2010 at regional and global levels. Pediatr Res. 2013;74(1):86-100.
8. Koelewijn JM, Vrijkotte TGM, 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):941-52.
9. Lindenburg IT, Smits-Wintjes VE, van Klink JM, Verduin E, van Kamp IL, Walther FJ, et al. Long-term neurodevelopmental outcome after intrauterine transfusion for hemolytic disease of the fetus/newborn: the LOTUS study. Am J Obstet Gynecol. 2012;206(2):141-8.
10. Lindenburg IT, van Klink JM, Smits-Wintjens VE, Oepkes D, Lopriore E. Long-term neurodevelopmental and cardiovascular outcome after intrauterine transfusions for fetal anaemia: a review. Prenat Diagn. 2013;33(9):815-22.
11. American Congress of Obstetricians and Gynecologists. Management of alloimmunization during pregnancy. Obstetrics & Gynecology. 2006;108(2):457-64.
12. Koelewijn JM. Detection and prevention of pregnancy immunisation, The OPZI study. Academic thesis. 2009(Chapter 1):9-31.
13. Canadian Blood Services. Clinical Guide to Transfusion. Canadian Blood Services, editor. 2007.
14. Gooch A PJ, Wray J, Qureshi H. Guideline for blood grouping and antibody testing in pregnancy. Transfus Med. 2007;17(4):252-62.

15. Gottvall T, Filbey D. Alloimmunization in pregnancy during the years 1992 untill 2005 in the central west region of Sweden. Acta Obstetricia et Gynecologica Scandinavica: Informa Scandinavian; 2008. p. 843-8.
16. Health Counsil of the Netherlands. Pregnancy immunisation by red blood cells. Advise Health Council. The Hague; 2009.
17. RIVM National Institute Public Health and Environment. Prenatal screening infectious diseases and erythrocyte antibodies. the Hague, the Netherlands; 2014.
18. Reid ME, Lomas-Francis C, Olsson ML. The blood group antigen facts book. 3 ed. Oxford: Elsevier; 2012.
19. Lurie S EE, Piper I, Woliovitch I. Is antibody screening in Rh (D)-positive pregnant women necessary? Fetal Neonatal Med. 2003;14(6):404-6.
20. Adeniji AA, Fuller I, Dale T, Lindow SW. Should we continue screening rhesus D positive women for the development of atypical antibodies in late pregnancy? Journal of Maternal-Fetal and Neonatal Medicine: Informa Clin Med; 2007. p. 59-61.
21. Andersen AS, Praetorius L, Jorgensen HL, Lylloff K, Larsen KT. Prognostic value of screening for irregular antibodies late in pregnancy in rhesus positive women. Acta Obstetricia et Gynecologica Scandinavica: Informa Scandinavian; 2002. p. 407-.
22. Dajak S, Stefanovic V, Capkun V. Severe hemolytic disease of fetus and newborn caused by red blood cell antibodies undetected at first-trimester screening (CME). Transfusion. 2011;51(7):1380-8.
23. Bowell PJ AD, Entwistle CC. Blood group antibody screening tests during pregnancy. British journal of obstetrics and gynaecology. 1986;96(10):1038-43.
24. Scheffer PG vdSC, Page-Christiaens GC, de Haas M. Noninvasive fetal blood group genotyping of rhesus D, c, E and of K in alloimmunised pregnant women: evaluation of a 7-year clinical experience. BJOG. 2011;118(11):1340-8.
25. de Haas M, Koelewijn JM, Bilgin K, Vrijkotte TGM, van der Schoot CE, Bonsel GJ. Diagnostic performance of laboratory monitoring to predict severe haemolytic disease of the fetus and newborn in non-RhD-alloimmunised pregnancies. Detection and prevention of pregnancy immunisation, The OPZI-study, Academic Thesis. 2009 2009;Chapter 4:73-85.
26. Oepkes D, Seaward PG, Vandenbussche FP, Windrim R, Kingdom J, Beyene J. Doppler ultrasonography versus amniocentesis to predict fetal anemia. N Engl J Med. 2006;355(2):156-64.
27. Koelewijn JM, Vrijkotte TGM, de Haas M, van der Schoot CE, Bonsel GJ. Risk factors for the presence of non-rhesus D red blood cell antibodies in pregnancy. BJOG: An International Journal of Obstetrics & Gynaecology. 2009;116(5):655-64.
28. Sebring ES, Polesky HF. Fetomaternal hemorrhage: incidence, risk factors, time of occurrence and clinical effects. Transfusion. 1990;30(4):344-57.
29. Sikovanyecz J HE, Pasztor N, Kereszturi A, Szabo J, Pal A. Fetomaternal transfusion after amniocentesis and cordocentesis. Ir J Med Sci. 2011;180(3):697-701.
30. Oxford CM LJ. Trauma in Pregnancy. Clin Obstet Gynecol. 2009;52(4):611-29.
31. Dutch association for perinatal registration. Perinatal Care in the Netherlands 2012. table 1.1.1; 2013.
32. de Vrijer B H-LE, Oosterbaan HP. The incidence of irregular antibodies in pregnancy: a prospective study in the region of 's-Hertogenbosch. Ned Tijdschrift Geneeskd. 1999;143(50):2523-7.

33. Heddle NM, Klama L, Frassetto R, O'Hoski P, Leaman B. A retrospective study to determine the risk of red cell alloimmunization and transfusion during pregnancy. Transfusion.1993;33(3):217-20.
34. NVOG Dutch Association Obstetrics and Gynaecology. Red blood cell immunisation and pregnancy. 2009.
35. Altman D. Practical Statistics for Medical Research. London: Chapman and Hall/CRC 1991. p. 94.
36. CBO Central Guidance Agency. Guideline Blood Transfusion. Utrecht, the Netherlands; 2011.
37. Health Council of the Netherlands. NIPT: Dynamics and Ethics of Prenatal Screening. the Hague: Health Council of the Netherlands. 2013.
38. Koelewijn JM. Economic analysis of the screening programme for red blood cell antibodies, other than RhD, in pregnancy.; 2009. p. 107-30.
39. Koelewijn JM, Vrijkotte TGM, de Haas M, van der Schoot CE, Bonsel GJ. Women's attitude towards prenatal screening for red blood cell antibodies, other than RhD. BMC Pregnancy and Childbirth. 2008;8(1):49.

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

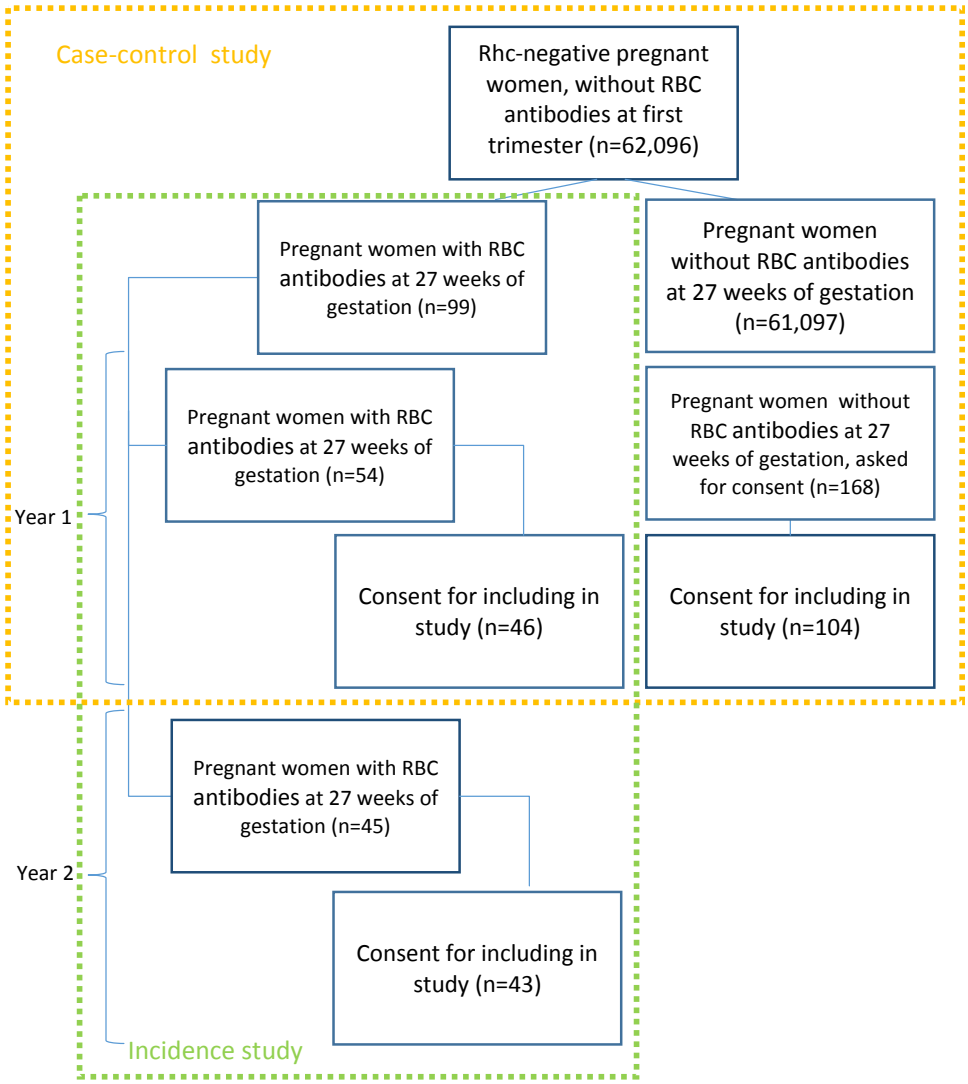

Table 1. Newly detected clinically relevant RBC antibodies in week 27 in Rhc-negative pregnant women

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

*** 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.**

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) of Rhc-negative women	% (95%-CI) of cases with late alloimmunisation	n
Late alloimmunisation	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

* **Assumption calculation NNS: timely detection without screening programme = 0%. NNS calculated as 1/(0-incidence in Rhc-negative women)**

Formula for calculation of the 95%-confidence intervals: p-1.96*ROOT(p*(1-p)/n), resp. p+1.96*ROOT(p*(1-p)/n). p = proportion of alloimmunised women (0.16%) and n = the number of screened women (62,096).

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)

* 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 Chisquare)