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Improving care for red blood cell alloimmunized pregnant women

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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 $\geq 1:16$ and/or ADCC test $\geq 30\%$ 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 1^{sts} 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 $1 / (0\text{-incidence of severe/moderate HDFN/late alloimmunisation in Rhc-negative women, screened in the third trimester})$.

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.

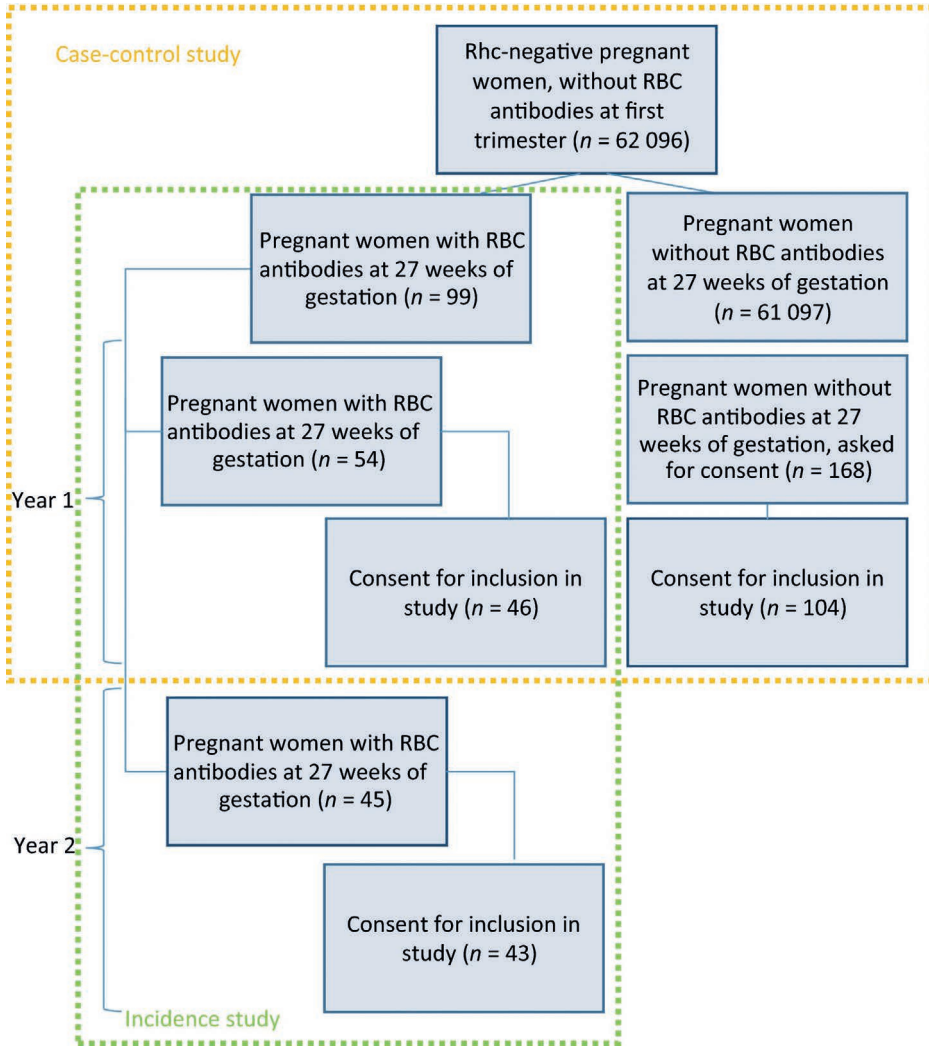


Figure 1: Flowchart of inclusions and exclusions of cases and controls

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: $1 / (0 - \text{incidence in Rhc negative women})$.

Formulae for the calculation of the 95% confidence intervals: $p \pm 1.96 \sqrt{[p(1-p)/n]}$. 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 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 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	2 (2)	7.65 (1.48-39.5)	9.20 (1.16-72.9)		
	6 (13)				

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 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 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 haemorrhage (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.

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

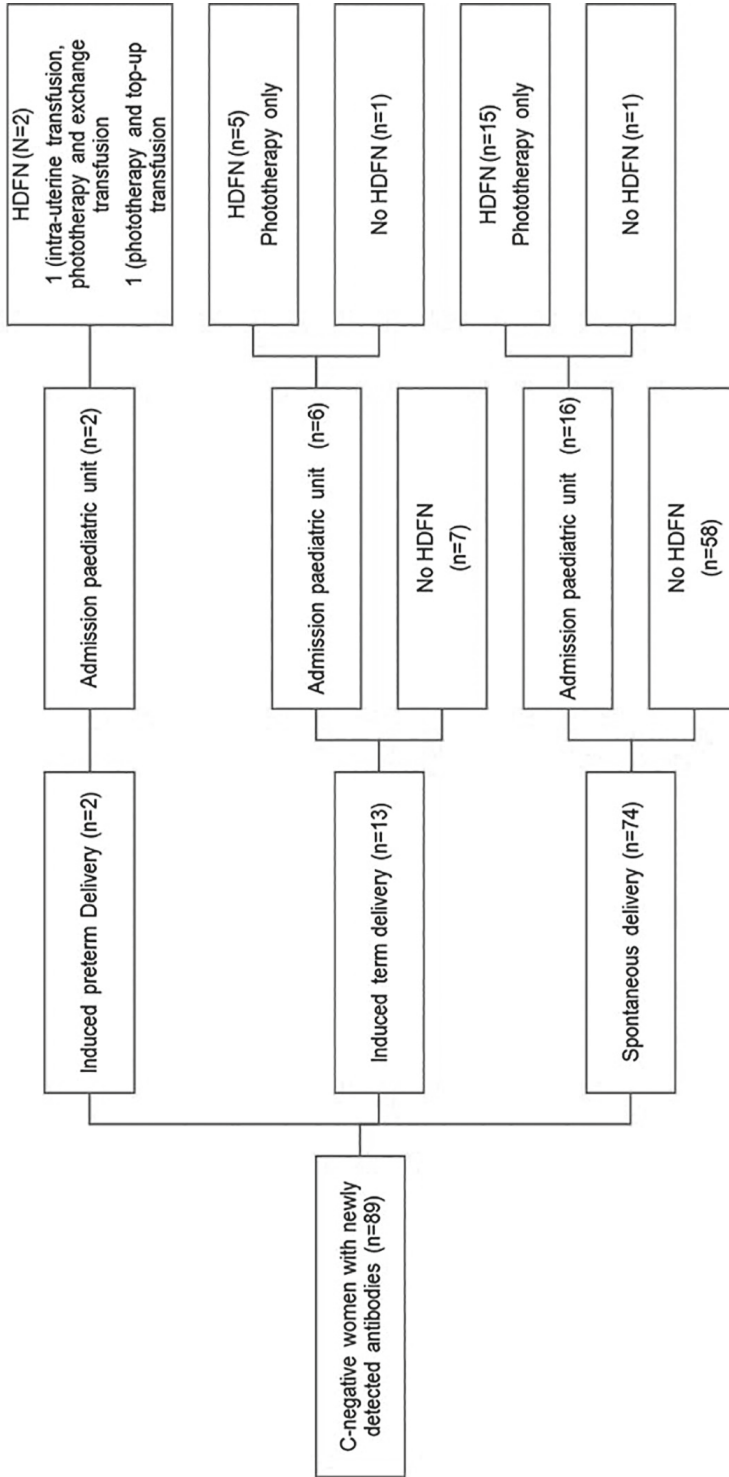


Table S1. Univariate risk factors for the presence of newly formed RBC antibodies at screening in week 27

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	$32.3\% + 49.2 \times 0.5\% = 56.8\%$
Anti-E	13	50%	5	$2.4\% + 26.3 \times 0.5\% = 1.2\%$
Anti-c + anti-E*	5	75%	2	$(100\% - (1 - 56.8\%)) \times (1 - 1.2\%) = 57.3\%$
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)