Hemolytic disease of the fetus and newborn

Zwiers, C.

CHAPTER 3

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

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ABSTRACT

Objective

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

Methods

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

Results

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

Conclusion

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

INTRODUCTION

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

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

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

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

METHODS

Setting

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

Study design and population

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

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

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

Sample size calculation

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

Data collection and outcome definitions

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

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

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

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

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

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

Ethical considerations

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

Statistical analysis

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

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

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

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

RESULTS

Selection and characteristics of study population

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

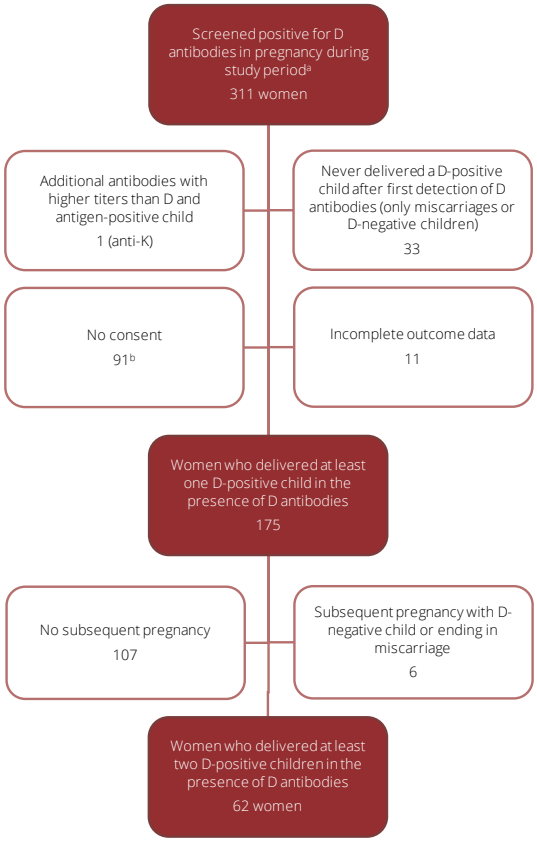


Figure 1. Composition of the study population. [a]Newly immunized women or a new pregnancy after previous immunization. [b]In 21 of these women the antibody was first detected at 27th week screening and no subsequent pregnancy occurred during the study period.

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

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

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

Severity of HDFN in the first immunized and the subsequent pregnancy

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

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

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

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

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

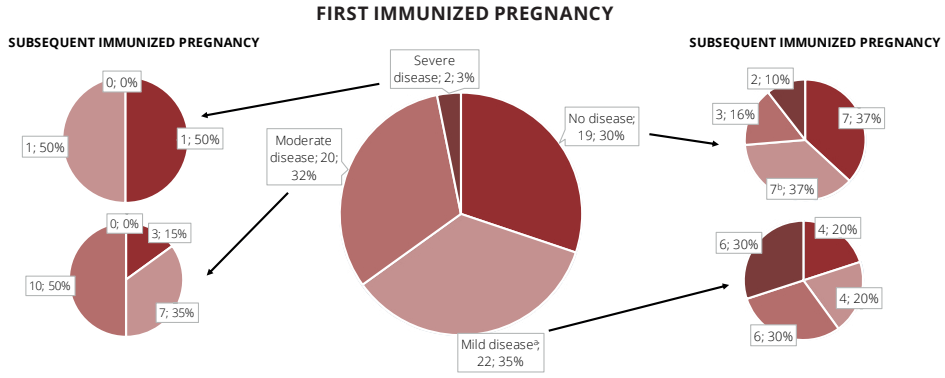


Figure 2. Severity of HDFN in the first and subsequent immunized pregnancy in 61 women (63 vs. 61 D-positive children). Outcome of woman with fetal death to a cause other than HDFN not shown. [a]Intensity of phototherapy missing in one child (2 days of phototherapy), interpreted as mild. [b]Intensity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

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

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

Severity of HDFN according to the time of antibody detection

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

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

Predicting severe disease in the second pregnancy with D antibodies

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

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

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

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

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

Disease severity in third and later pregnancies at risk for HDFN

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

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

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

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

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

DISCUSSION

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

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

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

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

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

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

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

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

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

CONCLUSION

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

SUPPLEMENTAL MATERIAL

Risk of bias

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

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

[a]Logistic regression
[b]Chi-Square

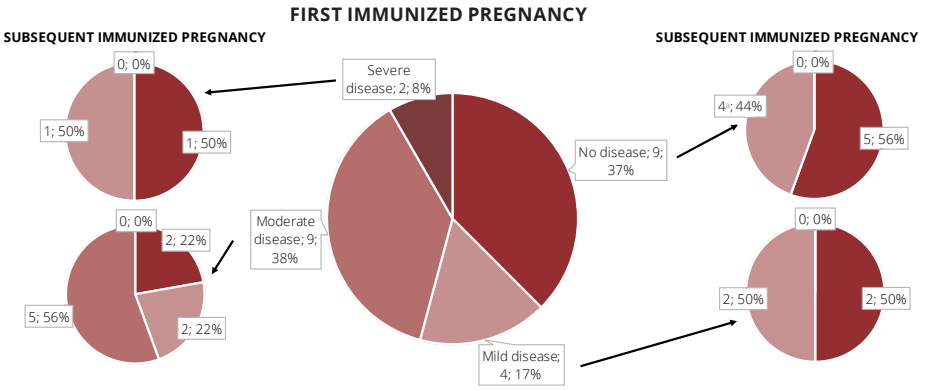
During our study period, 311 women were found eligible for inclusion, as they were pregnant and screened positive for D-antibodies. 55 women were excluded because they had no subsequent pregnancy, only D-negative children or miscarriages after developing D antibodies or because they had other antibodies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

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

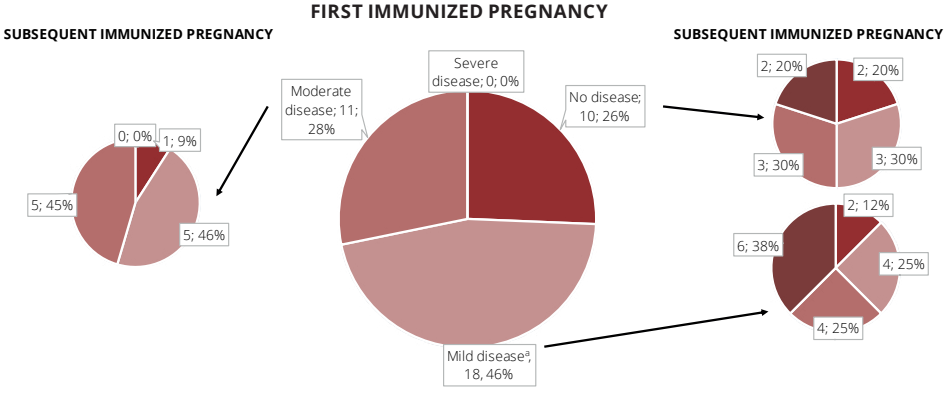
Subsequently, the risk of selection bias was assessed, possibly introduced by selecting women with a subsequent pregnancy only. Data was collected on all pregnancies of women with a positive D antibody screening in pregnancy at the time of our study

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

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



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



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

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

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

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

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

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

Grey boxes reflect the variables excluded at each step.

[a]Log2 transformation to achieve normal distribution.

Disease severity in third and later pregnancies at risk for HDFN

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

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

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