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# CHAPTER 4

## **Diagnostic performance of laboratory monitoring to predict severe haemolytic disease of the fetus and newborn in non-RhD-alloimmunised pregnancies**

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## ABSTRACT

In many western countries pregnancies complicated by the presence of clinically relevant red cell antibodies, with a chance that the fetus expresses the antigen, are routinely monitored by repeated laboratory testing. As part of this monitoring usually the antibody titre is determined, but in the Netherlands also the biological activity of the antibodies is evaluated with the antibody-dependent cellular cytotoxicity assay (ADCC). Cut-off levels for titre and ADCC result above which there is an increased risk for severe haemolytic disease of the fetus and newborn (HDFN), defined as necessitating fetal or neonatal treatment with (exchange) blood transfusions, have been evaluated for anti-D, but not for non-D alloantibodies. In this study we evaluated the diagnostic performance of assessment of the antibody titre and the ADCC test value for the identification of cases with a high risk for severe HDFN. We used data obtained as part of a nation-wide evaluation study of 260 cases, all pregnancies complicated by the presence of a clinically significant red cell antibody and an antigen-positive child of which the outcome was known. In this group, 16 children suffered from severe non-D-mediated HDFN.

We concluded for non-D alloantibodies that a titre of  $\geq 16$  (sensitivity of 0.875 (95%-CI: 0.617-0.985); specificity of 0.779 (95%-CI: 0.721-0.829)) or an ADCC test result of  $\geq 30\%$  (sensitivity 0.875 (95%-CI 0.617-0.985); specificity of 0.939 (95%-CI 0.901-0.965)) indicates an increased risk for severe HDFN. The positive predictive value for this cut-off is 21% for the titre and 48% for the ADCC test result, respectively; the negative predictive value of each test is 99%. Furthermore, optimal case-finding, in terms of sensitivity and costs, can be achieved by assessment of first the antibody titre, and addition of the ADCC assay if the titre is below 16.

In conclusion, with this set of tests laboratory monitoring can be used to conduct an expectant management with routine care in most non-D-alloimmunised pregnant women, and to timely detect those alloimmunised women with an increased risk for necessity of fetal or neonatal treatment.

## INTRODUCTION

In most western countries pregnant women are screened early in pregnancy for the presence of red cell alloantibodies.<sup>1-3</sup> If clinically significant red cell antibodies are detected and there is a chance that the fetus expresses the involved red cell antigen, further laboratory testing by assessment of the antibody titre is performed to predict the clinical significance of the antibodies in terms of potency to induce haemolytic disease of the fetus and newborn (HDFN).<sup>1-3</sup> If laboratory monitoring suggests an increased risk for severe HDFN, clinical monitoring is indicated. In the past, fetal haemolysis was evaluated by the invasive procedure of amniocentesis for determination of the Liley index. Currently, fetal anaemia can be diagnosed with a high sensitivity and specificity by non-invasive ultrasonography and Doppler middle cerebral artery blood flow velocity measurements.<sup>4-6</sup>

Most cases of severe HDFN with a need for fetal or neonatal treatment with (exchange) blood transfusions are caused by anti-D antibodies.<sup>1-3;7;8</sup> In general, anti-c, -C, -e, -K, -Fy<sup>a</sup> and -Jk<sup>a</sup> are regarded as red cell alloantibody specificities with the greatest potency to cause HDFN, but for almost all other alloantibody specificities there is only casuistic evidence of a severe course of HDFN.<sup>7-10</sup> We recently reported a nation-wide evaluation study, covering 306,000 screened pregnant women, and concluded that anti-c and anti-K, and in a rare case other Rhesus antibodies, cause severe HDFN. No severe HDFN was observed in the presence of other antibodies.<sup>11</sup>

The policy of laboratory monitoring in alloimmunised pregnant women varies between countries. In some countries all pregnant women with clinically significant red cell alloantibodies are retested regularly (mostly 4-weeks intervals) until a threshold or cut-off value ('critical titre') is exceeded, which implies referral to specialized care.<sup>2;3</sup> In other countries, only pregnant women with anti-D, -c and/or -K antibodies are regularly monitored and pregnant women with other antibody specificities are only retested at 28-34 weeks of gestation, to cover the infrequent high-risk cases in this group.<sup>1</sup>

In general, for non-Rh alloantibodies a cut-off level of 32 is used (reviewed by Moise et al.<sup>9;10</sup>). For example, Hackney et al. reported a study of anti-c-induced severe HDFN, defined as need for intra uterine transfusion or haemoglobin levels at birth of <11 g/dL, where all cases correctly were predicted with a cut-off level of 32 of the titre.<sup>12</sup> Similarly, Joy et al. concluded for anti-E and McKenna et al. for anti-K, a titre cut-off value of 32.<sup>13;14</sup> However, Leggat et al., and van Wamelen et al., reported that especially if anti-K is present, cases with severe HDFN may be missed.<sup>15;16</sup> Therefore both the British and American guidelines state that anti-K titres <32 may already be critical;<sup>1;2</sup> a titre of 8 has recently been proposed as a threshold for anti-K in a review by Moise et al.<sup>10</sup>

In the Netherlands routinely two laboratory tests are performed as part of the

laboratory monitoring: 1) assessment of the antibody titre and 2) a monocyte-driven antibody-dependent cellular cytotoxicity (ADCC) assay to determine the biological activity of the antibodies.<sup>17</sup> However, before this study, the ADCC assay has only been validated to estimate the risk for occurrence of anti-D-induced severe HDFN.<sup>18</sup> It was found that if ADCC levels are <10%, expectant management with routine obstetric care of the RhD-alloimmunised pregnancy can be considered safe; whereas with ADCC values between 15% and 30% fetal anaemia requiring transfusion is not likely to occur, and an ADCC test result >50% is correlated with increased risk for severe HDFN.<sup>18</sup> ADCC values between 30% and 50% justify referral to specialised centres to monitor these high-risk pregnancies. In case of anti-D antibodies, the advice to the clinician is mainly based on the obtained ADCC test value. In agreement with the literature<sup>19</sup>, Oepkes et al. observed no significant fetal or neonatal disease necessitating (exchange) transfusions if the anti-D titre was  $\leq 32$ .<sup>18;19</sup> For non-RhD alloantibodies the predictive value of the ADCC test has not yet been tested in a large series of samples and thusfar for non-RhD antibodies a cut-off value of  $\geq 20\%$  for anti-K and  $\geq 10\%$  for other non-RhD-specificities was used without empirical evidence. For anti-K, advice for clinical monitoring was solely based on the ADCC test result alone, whereas for anti-c, clinical monitoring was advised if the ADCC test result was  $\geq 10\%$  or the titre was  $\geq 16$ ; for all other non-cDK alloantibodies both the ADCC result should be  $\geq 10\%$  and the titre  $\geq 16$ .

This study was undertaken to establish the accuracy of the diagnostic tests used in the Netherlands to monitor pregnancies complicated by red cell alloantibodies other than anti-D. In the majority of these pregnancies HDFN will not occur. Therefore, the primary aim of laboratory monitoring in case of maternal alloimmunisation is to pre-select the group of women that truly need advanced clinical monitoring to detect fetal anaemia or hyperbilirubinaemia in time. Efficient pre-selection reduces the number of admissions for specialized clinical diagnostics. In this study we established the cut-off values of the titre and ADCC assays for non-D red cell alloantibodies, which enable optimal case-finding. Furthermore, we evaluated the optimal order to perform these two laboratory tests to enable selection of high-risk cases at lowest costs.

## **MATERIALS AND METHODS**

### **Inclusion of cases**

Cases were identified as part of a nation-wide prospective index-cohort study, which was conducted with the aim to evaluate the effectiveness of first trimester red cell antibody screening for early detection of cases at risk for HDFN. All pregnant women with clinically relevant non-RhD RBC antibodies recognized by routine first trimester screening (n=1,002) from September 1<sup>st</sup> 2002 until June 1<sup>st</sup> 2003 and Oct 1<sup>st</sup> 2003 until July 1<sup>st</sup> 2004

(population: n=306,000) were included in this nation-wide study. All women with anti-D antibodies were excluded.<sup>11</sup> Information about the antibody specificity and the antigen typing of the father was collected at the two reference laboratories that routinely perform these analyses as part of the Dutch maternal alloimmunisation prevention programme. Assessment of alloantibody titres and ADCC test results was also performed as part of the routine care of alloimmunised pregnant women. Advice for clinical monitoring was given for anti-K if the ADCC result was  $\geq 20\%$ ; for anti-c if the ADCC result was  $\geq 10\%$  or the titre was  $\geq 16$  and for all other non-cDK alloantibodies if both the ADCC result was  $\geq 10\%$  and the titre  $\geq 16$ .

As part of the evaluation study, cord blood samples were collected for antigen typing and serological evaluation comprising the direct antiglobulin test (DAT) and analysis of antibody specificity both in a red cell eluate and in the plasma. The national evaluation study was approved by the relevant professional organisations (obstetricians, midwives, general practitioners, paediatricians, clinical laboratories). Representatives of these organisations monitored the study process. Consent for collection of detailed clinical data about the outcome of the pregnancy was given by all women included in this study.

A case (n=260) was included in the current study of the predictive value of the laboratory monitoring if:

- a. the red cell antibodies were directed against a single antigen and the child was typed positive for this antigen, or
- b. a combination of red cell antibody specificities was present, but the child was typed only positive for one of the involved antigens. For these cases, the titre and ADCC test had to be performed with red cells only positive for the 'informative' red cell antigen, AND
- c. at minimally two time points in pregnancy laboratory monitoring was performed with the last test result obtained  $>32^{\text{nd}}$  week of pregnancy. If the fetus was diagnosed with severe HDFN  $<32^{\text{nd}}$  week of pregnancy, test results should have been obtained less than three weeks before diagnosis, or
- d. at the first moment of laboratory testing referral for clinical monitoring was made because test results were above the at that moment used cut-off values.

### **Definition of clinical outcome**

Severe HDFN was defined as occurrence of HDFN-related death (did not occur among the included cases), or the need of intra uterine transfusion, exchange transfusion or transfusion in the first week of life. If only phototherapy was given, HDFN was classified as 'moderate'.

### **Determination of the antibody titre**

The antibody titre was determined by doubling dilution of the serum in saline, with the indirect antiglobulin test using an anti-IgG reagent, according to published recommendations by the American Association of Blood Banks and the British Committee for Standards in Haematology.<sup>1,2,20</sup>

### **Antibody dependent cellular cytotoxicity assay (ADCC)**

The ADCC test was routinely performed as described by Engelfriet al.<sup>7</sup> In short, the test uses peripheral blood mononuclear cells as effector cells and <sup>51</sup>Cr-labeled red cells sensitised with the maternal serum. The biological activity of the maternal alloantibodies is expressed as percentage of <sup>51</sup>Cr release, calculated against a standard obtained with dilutions of a serum containing anti-D antibodies.

### **Determination of test characteristics**

Test characteristics (sensitivity, specificity, and positive and negative predictive values) for the prediction of severe HDFN were calculated with 2x2 tables for a. Titre (range 1 to 16,000) and b. ADCC (range <10% to >80%, with 5% intervals). From these data, receiver operating characteristic curves (ROC) were constructed to determine the test performance and the threshold for optimal discrimination between cases at risk or not at risk for severe HDFN. In theory, the area under the ROC curve ranges from 0.5 till 1.0 with 1.0 meaning a perfect test in terms of 100% discriminating between cases at risk and not at risk. We considered 0.8 or more as a useful test. The optimal cut-off point was constructed by combining a high sensitivity with an acceptable specificity. SPSS version 11.5 was used to construct the ROC curves.

### **Evaluation of optimal design of laboratory monitoring**

Laboratory monitoring was performed in one reference laboratory. The number of tests (titre and/or ADCC assay) performed for each alloimmunised case was counted. The tariff used for a titre was € 55 and for ADCC assay € 136, respectively. In the calculations costs were reflected by units, with costs of a titre=1 unit and performance of ADCC=2.5 units. The sensitivity and specificity to detect severe HDFN and the costs of each possible design were calculated; whereby total costs were divided by the number of correctly identified cases with severe HDFN.

## RESULTS AND DISCUSSION

### Study population

Table 4.1 shows the clinical outcome of the 260 cases that were included in this study. In this table the antibody specificity is listed as an entity if this specificity was found in at least 20 cases. Sixteen cases of severe HDFN, with a need for intra uterine transfusion or (exchange) transfusion were included, in the far majority of cases (n=14) because of anti-c (n=10) or anti-K (n=4) antibodies. The other two cases of severe HDFN were due to anti-E and to anti-e, respectively. Moderate HDFN, only treated with phototherapy, was also mainly caused by anti-c, -E or -K antibodies (Table 4.1). Fy<sup>a</sup> or Jk<sup>a</sup> antibodies nor any of the other red cell antibody specificities (such as anti-C, anti-C<sup>w</sup>, anti-S) caused severe HDFN and induced only in a minority of cases moderate HDFN (Table 4.1). The more detailed clinical outcome of these cases is reported previously.<sup>11</sup>

**TABLE 4.1** CLINICAL OUTCOME OF CASES AT RISK FOR SEVERE HDFN, ACCORDING TO RED CELL ANTIBODY SPECIFICITY

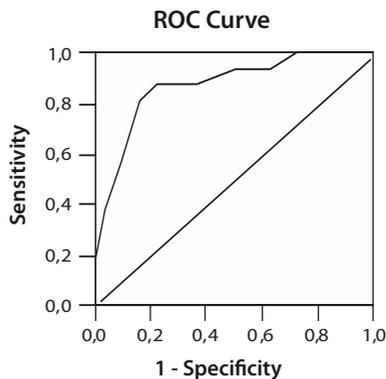
	Severe HDFN: intra uterine or (exchange) transfusion	Moderate HDFN: only phototherapy	No therapy	Total
Anti-c	10	16	46	72
Anti-K	4	3	7	14
Anti-E	1	12	65	78
Anti-Fy <sup>a</sup>	0	5	26	31
Anti-Jk <sup>a</sup>	0	1	22	23
Other specificities*	1	5	36	42
Total	16	42	202	260

\*One case of HDFN because of anti-e was treated with an exchange transfusion.

### Predictive values of the titre and ADCC assay

Figure 4.1a and 4.1b show the ROC curves for respectively the antibody titre and the ADCC test result to detect severe HDFN in all cases irrespective of the antibody specificity. The ROC curve for the accuracy to detect severe HDFN revealed an area under the curve of 0.871 for the titre (Figure 4.1a) and an area under the curve of 0.899 for the ADCC assay (Figure 4.1b). The optimal cut-off (highest sensitivity with acceptable specificity) for the titre was 16, with a sensitivity of 87.5%, and a specificity of 77.9% (Table 4.2a). This is one titration step lower compared to generally used in the literature.<sup>1,2;10</sup> The optimal cut-off for the ADCC assay was found to be 30%, also with a sensitivity of 87.5% but with a higher specificity of 93.9% (Table 4.2b). Also for anti-D has been previously concluded that a cut-off of 30% for the ADCC result justifies referral to a specialized centre, however, in that study a cut-off of 50% was correlated with severe HDFN.<sup>18</sup> With the use of these optimal cut-off values, similar calculations were performed for the group with anti-c antibodies (Table 4.2a and 4.2b). Because only four cases of anti-K-mediated severe HDFN and two

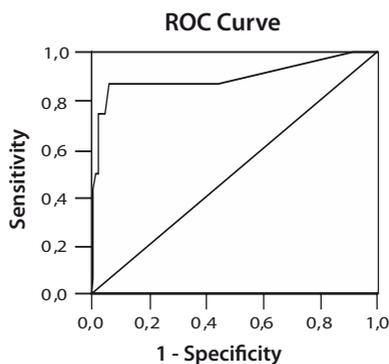
**FIGURE 4.1A** RECEIVER OPERATING CHARACTERISTIC CURVES FOR TITRE TO PREDICT SEVERE NON-RHD-MEDIATED SEVERE HDFN



Titre ? 1:16		HDFN		
		+	-	
test	+	14	54	68
test	-	2	190	192
		16	244	260

Positive predictive value = 21% (14/68)  
 Negative predictive value = 99% (190/192)

**FIGURE 4.1B** RECEIVER OPERATING CHARACTERISTIC CURVES FOR THE ADCC TEST RESULT TO PREDICT SEVERE NON-RHD-MEDIATED SEVERE HDFN



ADCC ? 30%		HDFN		
		+	-	
test	+	14	15	29
test	-	2	229	231
		16	244	260

Positive predictive value = 48% (15/29)  
 Negative predictive value = 99% (229/231)

**TABLE 4.2A** TEST CHARACTERISTICS OF ANTIBODY TITRE FOR THE PREDICTION OF SEVERE HDFN, GIVEN A CUT-OFF VALUE OF 16 FOR NON-D ANTIBODIES, RESPECTIVELY FOR ANTI-C

	Titre	Sensitivity (95%-CI)	Specificity (95%-CI)
<b>All non-D antibodies</b>	16	0.875 (0.617 – 0.985)	0.779 (0.721 – 0.829)
<b>Anti-c</b>	16	0.900 (0.555 – 0.998)	0.661 (0.530 – 0.777)

**TABLE 4.2B** TEST CHARACTERISTICS OF ADCC FOR THE PREDICTION OF SEVERE HDFN, GIVEN A CUT-OFF VALUE OF 30% FOR ALL NON-D ANTIBODIES, RESPECTIVELY FOR ANTI-C

	ADCC	Sensitivity (95%-CI)	Specificity (95%-CI)
<b>All non-D antibodies</b>	30%	0.875 (0.617 – 0.985)	0.939 (0.901 – 0.965)
<b>Anti-c</b>	30%	0.900 (0.555 – 0.998)	0.903 (0.801 – 0.964)

cases due to anti-E and anti-e respectively were included, it was not possible to further differentiate between the various antibody specificities.

Figure 4.1 shows that if the calculated cut-off values were used, the antibody titre as well as the ADCC test result both were inaccurate in detecting two cases of severe HDFN in two cases. Since in one case both the titre and ADCC test result were below the cut-off level, this concerned three alloimmunised pregnant women. In the first case, with anti-K antibodies, the titre was determined as 4 only once early in pregnancy. At that time the ADCC test result was already >80%, hence, indicative of clinical monitoring and laboratory monitoring was not continued. This child received intra uterine transfusions. For the only case with both a titre and an ADCC test result below the cut-off level, it was concluded by the paediatrician that severe HDFN, treated with a top-up transfusion, was due to anti-c antibodies, however only data on the locally determined positive test result of the DAT were available, and no eluate was made to confirm the diagnosis. No cord blood was received by the reference laboratory as part of the evaluation study. In this case, the observed anaemia in the newborn may have been caused by an OA antagonism between mother and child. This, because the maternal anti-A IgG titre was 64, whereas the anti-c antibodies, determined one week before birth were only weakly reactive (no titre) and showed an ADCC test result <10%. In the third case, severe HDFN due to anti-c was concluded with ADCC test results <10% and a titre of 16, as determined the day before birth. This child received an exchange transfusion, followed by a platelet transfusion at day 2 and a top-up transfusion at day 3 after birth.

As can be concluded from Figure 4.1, in alloimmunised women carrying an antigen-positive child the negative predictive value of a non-D alloantibody titre below 16 or ADCC test result below 30% is 99%. Therefore, test results below the cut-off can be used to continue an expectant management with routine obstetric care for non-D-alloimmunised women. In our group of 260 cases, 54 cases (21%) showed a titre of 16 or higher, without occurrence of severe HDFN, whereas only 15 showed an ADCC test result of 30% or higher without the occurrence of HDFN (6%). The positive predictive value of a threshold for the titre of  $\geq 16$  is 21% (14 out of 68 cases), whereas the positive predictive value of a threshold for the ADCC test result of  $\geq 30\%$  is 48% (14 out of 29 cases). However, in this study only alloimmunised pregnancies with antigen-positive children were included. In the routine situation, cases are followed based on typing of the father, thus, the positive predictive value of the test will be lower because also pregnancies with antigen-negative children from a heterozygously positive father will receive laboratory monitoring. Therefore, determination of the fetal antigen status with non-invasive fetal genotyping should be considered if the threshold for the titre or for the ADCC result is exceeded, or perhaps routinely once the paternal status is known.<sup>21;22</sup> The fetal D, c, C, E or K typing can already be performed before there is a risk for fetal anaemia (before week

16).<sup>21,22</sup> Although currently Doppler ultrasound-based non-invasive methods are used in specialized centres to accurately detect fetal anaemia, laboratory monitoring still serves a role to prevent unnecessary intensified clinical monitoring of alloimmunised women. Therefore, the higher specificity of the ADCC test to predict the risk for severe HDFN can be used to improve the risk assessment in the clinical management of non-D-alloimmunised women.

#### *Optimal design for laboratory monitoring of non-RhD-mediated severe HDFN*

We evaluated the optimal design in terms of sensitivity, specificity and costs of using both or either of the two laboratory tests for high-risk case identification purposes (Table 4.3). We included conditional testing i.e. applying a second test conditional on the result of the first. Costs of an ADCC test are 2.5 times that of determination of a titre, thus, if it is not necessary to have both test results available, costs could be saved. The current setting is depicted as 'design 4'. Table 4.3 shows that if only the titre or the ADCC test is performed, the sensitivity to identify high-risk cases will be 87.5%, which may be regarded as suboptimal compared to the sensitivity of 93.8% obtained with designs 4, 6 and 8 using both tests. With design 6 and 8, the titre (design 6) or ADCC assay (design 8) is performed as first test and the second test, respectively ADCC or titre, is only performed if the result of the first test is below the cut-off level. Thus, the second test is used to detect cases which may have been missed by the first test and aims to increase the sensitivity. These

**TABLE 4.3** SENSITIVITY, SPECIFICITY AND COSTS OF THE EIGHT POSSIBLE DESIGNS OF LABORATORY MONITORING FOR CASES AT RISK OF SEVERE HDFN

		Sensitivity (95%-CI)	Specificity (95%-CI)	Costs (units) per detected case of severe HDFN*
<i>Design 1</i>	Titre Result $\geq 16$	87.5 (61.7 – 98.5)	77.9 (72.1 – 82.9)	67
<i>Design 2</i>	ADCC Result $\geq 30\%$	87.5 (61.7 – 98.5)	93.9 (90.1 – 96.5)	293
<i>Design 3</i>	Titre and ADCC Both results $\geq$ cut-off	81.3 (54.4 – 96.0)	94.7 (91.1 – 97.1)	388
<i>Design 4</i> (Current Design)	Titre and ADCC	93.8 (69.8 – 99.8)	77.0 (71.3 – 82.2)	336
<i>Design 5</i>	Titre, only ADCC if titre $\geq 16$ Both results $>$ cut-off	81.3 (54.4 – 96.0)	94.7 (91.1 – 97.1)	159
<i>Design 6</i>	Titre, only ADCC if titre $< 16$ One of the results $>$ cut-off	93.8 (69.8 – 99.8)	77.0 (71.3 – 82.2)	261
<i>Design 7</i>	ADCC, only titre if ADCC $\geq 30\%$ Both results $\geq$ cut-off	81.3 (54.4 – 96.0)	95.1 (91.6 – 97.4)	321
<i>Design 8</i>	ADCC, only titre if ADCC $< 30\%$ One of the results $\geq$ cut-off	93.8 (69.8 – 99.8)	77.0 (71.3 – 82.2)	331

\*titre : ADCC = 1 unit : 2.5 units

latter two designs reduce the number of performed tests, hence costs, with most benefits resulting from design 6.

#### *Optimal policy for laboratory monitoring*

As reported by us and others, the main alloantibody specificities associated with the occurrence of severe HDFN are anti-D, anti-c, anti-K and to a lesser extent alloantibodies directed against other Rh antigens.<sup>9-11</sup> Therefore, pregnancies complicated by the presence of these red cell alloantibodies justify laboratory monitoring for timely detection of fetal anaemia and risk for postnatal hyperbilirubinaemia. However, since the need for fetal treatment of HDFN in pregnancies complicated by non-D, c or K antibodies is extremely rare, it might not be necessary to continue laboratory monitoring in all cases of these antibodies from early pregnancy onwards. Reports in the literature point to occurrence of HDFN in Fy<sup>a</sup>-alloimmunised pregnant women.<sup>23;24</sup> However, in our series, as published before<sup>11</sup>, of 42 Fy<sup>a</sup>-positive newborns only six needed to treatment with phototherapy and intra uterine transfusions because of anti-Fy<sup>a</sup> are extremely rare in the Netherlands.<sup>11;25</sup> A policy as conducted in the UK, in which all pregnant women with anti-D, -c or -K antibodies are monitored at 4-weeks intervals and 3- to 2-weeks intervals in the last trimester of pregnancy, whereas women with alloantibodies directed against other red cell antigens are retested only once in the last trimester of pregnancy, will meet the aim of laboratory monitoring to timely select cases at risk of severe HDFN.<sup>1</sup> If at the moment of retesting a significant rise in antibody levels ( $\geq 2$  steps in titre) or ADCC test result is noted, laboratory monitoring should be continued or clinical monitoring should be advised.

In conclusion, in this study using data from a nation-wide study, the performance of laboratory monitoring to detect non-invasively severe HDFN was studied in a cohort of 260 cases, including 16 children with severe HDFN. We concluded that a non-D alloantibody titre of 16 or higher or an ADCC test result of 30% or higher indicate high-risk cases. Furthermore, optimal case-finding, in terms of sensitivity and costs, can be conducted by assessment of first the antibody titre, and addition of the ADCC assay if the titre is below 16.

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