



RESEARCH

Diagnostic accuracy of routine antenatal determination of fetal *RHD* status across gestation: population based cohort study



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Abstract

Objectives To assess the accuracy of fetal *RHD* genotyping using cell-free fetal DNA in maternal plasma at different gestational ages.

Design A prospective multicentre cohort study.

Setting Seven maternity units in England.

Participants RhD negative pregnant women who booked for antenatal care before 24 weeks' gestation.

Interventions Women who gave consent for fetal *RHD* genotyping had blood taken at the time of booking for antenatal care and, when possible, at other routine visits such as for Down's syndrome screening between 11 and 21 weeks' gestation, at the anomaly scan at 18-21 weeks, and in the third trimester when blood was taken for the routine antibody check. The results of cord blood analysis, done routinely in RhD negative pregnancies, were also obtained to confirm the fetal *RHD* genotyping.

Main outcome measures The accuracy of fetal *RHD* genotyping compared with RhD status predicted by cord blood serology.

Results Up to four analyses per woman were performed in 2288 women, generating 4913 assessable fetal results. Sensitivity for detection of fetal *RHD* positivity was 96.85% (94.95% to 98.05%), 99.83% (99.06% to 99.97%), 99.67% (98.17% to 99.94%), 99.82% (98.96% to 99.97%), and 100% (99.59% to 100%) at <11, 11-13, 14-17, 18-23, and >23 completed weeks' gestation, respectively. Before 11 weeks' gestation 16/865 (1.85%) babies tested were falsely predicted as *RHD* negative.

Conclusions Mass throughput fetal *RHD* genotyping is sufficiently accurate for the prediction of RhD type if it is performed from 11 weeks' gestation. Testing before this time could result in a small but significant number of babies being incorrectly classified as *RHD* negative. These mothers would not receive anti-RhD immunoglobulin, and there would

be a risk of haemolytic disease of the newborn in subsequent pregnancies.

Introduction

Haemolytic disease of the fetus and newborn is caused by maternal IgG antibodies to red cell surface antigens crossing the placenta and facilitating the immune destruction of fetal red cells or erythroid progenitors. The most commonly implicated antigen is the RhD (RH1) antigen of the rhesus system. Postnatal prophylaxis with anti-RhD immunoglobulin substantially reduces the alloimmunisation of RhD negative women and the prevalence of haemolytic disease of the fetus and newborn.¹ Subsequent introduction of routine antenatal prophylaxis with anti-RhD immunoglobulin in the third trimester for all RhD negative pregnant women is now standard in many countries.² In a predominantly white population, however, about 38% of these women would be carrying an RhD negative fetus and thus receive anti-RhD immunoglobulin, a pooled human plasma product, unnecessarily.³ Prophylaxis after delivery is offered only to RhD negative women who have given birth to an RhD positive baby.

Since the identification of cell-free fetal DNA in the blood of pregnant women,⁴ several laboratories have provided a fetal *RHD* genotyping service for RhD negative women with a measureable concentration of anti-RhD antibody.⁵ Cell-free fetal DNA in maternal plasma is tested for the presence or absence of the *RHD* gene, and results are used to direct management of the pregnancy.⁵

Consequent to the success of fetal *RHD* genotyping in high risk women, the National Institute for Health and Care Excellence

has “endorsed studies into the feasibility of mass testing antenatally for fetal blood group by analysis of cell-free fetal DNA in maternal plasma.”² If fetal *RHD* testing proved accurate enough, there would be no need to test cord red cells serologically for RhD status.

High risk alloimmunised women are tested with a labour intensive low throughput method, which is unsuitable for mass screening.⁶ Two studies have used high throughput methods, including robotic isolation of plasma DNA and real time quantitative polymerase chain reaction (PCR) technology, to routinely determine fetal *RHD* status at 30 weeks in the Netherlands⁷ and at 28 weeks in the United Kingdom,⁸ with 99.4% and 95.7% diagnostic accuracy when compared with cord blood serology. If the results of the UK trials had been applied as a guide to treatment, only 2% of the women would have received anti-RhD immunoglobulin unnecessarily, compared with 38% in the absence of genotyping. Similar accuracy was achieved in validation studies in Germany⁹ and France,¹⁰ but the latter involved re-testing all negative results, which would be uneconomical.¹⁰

In Denmark and the Netherlands routine fetal *RHD* genotyping has recently been introduced as a national service at 26 and 28 weeks, respectively.^{11 12} As routine anti-RhD prophylaxis is recommended from 28 weeks, and anti-RhD immunoglobulin is administered earlier in pregnancy after any potential sensitising event, implementation of fetal *RHD* testing earlier in pregnancy would maximise potential benefits and cost reduction. As concentrations of cell-free fetal DNA in maternal blood increase throughout the pregnancy,¹³ it cannot be assumed that tests will be as accurate early in pregnancy as they are at 26-28 weeks’ gestation.

We estimated the diagnostic accuracy and costs of fetal *RHD* genotyping in RhD negative pregnant women from the time of booking until 28 weeks’ gestation to determine the optimal gestation for implementation into routine clinical practice. The economic impact of testing will be reported separately.

Methods

Population

Women booking for antenatal care in 2009-12 at seven maternity units in England were prospectively asked for verbal consent to donate an additional 4 mL of blood at the time of their booking appointment. These appointments were in hospital antenatal clinics or the community according to local clinical practice. Local midwives got verbal consent from women when possible. Blood samples from RhD positive women were discarded, and those from RhD negative women were sent to the International Blood Group Reference Laboratory for fetal *RHD* genotyping, to arrive within 48 hours of blood being drawn. When possible, we also obtained consent to collect additional blood samples opportunistically for fetal *RHD* genotyping from RhD negative women with singleton pregnancies when they attended for Down’s syndrome screening (at 11-20 weeks) and the routine anomaly scan (at 18-23 weeks) (fig 1⇓). While it is recommended that all women book for antenatal care early in pregnancy, before the offer of the combined screening test for Down’s syndrome, which is performed at 11-13 weeks and 6 days, not all do. For the purpose of our study we included only women who booked before 24 weeks’ gestation, agreed to participate in the study, and donated at least one blood sample before 24 weeks’ gestation. Those who booked before 11 weeks might have donated up to four blood samples: at booking; at the time of screening for Down’s syndrome (either the combined test at 11-13 weeks and 6 days

or second trimester screening at 14-20 weeks); at the routine anomaly scan, which, although recommended at 18-21 weeks and 6 days, can be performed as late as 24 weeks in some circumstances; and after 25 weeks at the routine third trimester antenatal visit, when blood is taken to check haemoglobin and antibody concentrations.

Written informed consent was obtained before or at the routine third trimester visit (around 28 weeks), when the women were given the result of molecular testing and an additional blood sample was requested to confirm fetal *RHD* status. When third trimester testing confirmed that the fetus was *RHD* negative, women were allowed to opt out of receiving anti-RhD immunoglobulin if they requested to do so as the reliability of testing at this gestation is proved.^{8 11} All women predicted to be carrying an RhD positive baby, or when the fetal genotyping result was inconclusive, were offered anti-RhD immunoglobulin according to local clinical practice. All women experiencing a sensitising event before 28 weeks’ gestation were also offered anti-RhD immunoglobulin according to local clinical practice.

Fetal genotyping from maternal blood

On receipt of blood samples, plasma was prepared and analysed prospectively by using assay techniques and criteria for the determination of fetal *RHD* status as described previously.⁸

Serological RHD testing on cord bloods

RhD typing on cord blood samples was performed with serological methods routinely used by participating units. Results were ascertained by the research midwives and compared with the genotype predicted.

Data analysis

We included results obtained from RhD negative women before 25 weeks with a singleton pregnancy who gave written consent for fetal *RHD* genotyping and in whom a cord blood RhD group was subsequently obtained. Genotyping results were interpreted as *RHD* positive, *RHD* negative, or inconclusive. Sensitivity for the detection of *RHD* positivity was calculated on the basis that inconclusive results were treated as *RHD* positive. We used generalised linear models with random intercept to quantify trends in sensitivity and specificity with gestational age while taking into account repeat measures from the same woman. All estimates are presented with 95% confidence intervals. We used R (version 2.14.0) library *glmML* to fit the models with 5000 bootstrap samples for estimation of standard errors.

Results

Women were recruited from 2009 to 2012. Of the samples received at the International Blood Group Reference Laboratory, 172 were excluded from the analysis because of extreme haemolysis (average transit time 8.5 days), and a further 22 were not analysed because they did not contain sufficient blood. Samples from 30 women were excluded because of multiple pregnancy, 185 because no cord blood result was subsequently obtained, and 372 because of lack of written consent. We included results of fetal *RHD* genotyping from 2288 women in the final analysis. We had 4913 fetal genotype results with up to four analyses per woman; 436 had only one measurement, 1132 had two, 667 had three, and 53 had four. Most women were white (78%), 5.8% were Asian, 3.9% were black or mixed race, and 12.3% were unknown. Gestational age at measurement varied from five to 35 weeks (mean 18.67, median 19).

Genotyping results were inconclusive for 393/4913 (8%, 95% confidence interval 7.27% to 8.79%) of the samples (table 1). Analysis of cord blood showed that 2023 samples were from RhD negative babies, of which 18 had been falsely predicted antenatally to be genotypically *RHD* positive and 85 had been inconclusive; 2890 were RhD positive, of which 308 had been inconclusive on fetal *RHD* genotyping and 19 were falsely predicted genotypically to be *RHD* negative (table 1).

The odds of correctly identifying *RHD* positive and *RHD* negative fetuses increased significantly with gestational age, with low levels of false negative results after 11 weeks' gestation. Table 1 shows the summary statistics for <11, 11-13, 14-17, 18-23, and >23 weeks. Figure 2 shows how sensitivity for correctly identifying *RHD* positive fetuses increased on a week by week basis and how before 11 weeks, confidence intervals were wider and sensitivities not good enough to support routine introduction of the test.

Discussion

Previous studies have shown that mass throughput *RHD* genotyping of cell-free fetal DNA is accurate if performed in the third trimester of pregnancy.^{7 8} In this population based cohort study, we evaluated accuracy across a wider range of gestations and have shown that it is highly accurate from 11 weeks' gestation; before this the percentage of babies falsely predicted as RhD negative increases. For routine application of fetal *RHD* genotyping, false negative results—whereby the fetus is predicted to be RhD negative when it is really RhD positive—pose the greatest concern as mothers will not receive prophylactic anti-RhD immunoglobulin and might alloimmunise, which would put babies in subsequent pregnancies at risk of considerable morbidity or mortality. As the sensitivity improves with gestation, this risk can therefore be mitigated by optimal timing of testing, by repeating cell-free fetal DNA testing in women predicted to be carrying an RhD negative baby, or by testing cord blood of babies predicted to be RhD negative. The latter two interventions would, however, impact on the cost effectiveness and applicability of a large scale programme. In our study, the incidence of false *RHD* negative results before 11 weeks' gestation was 16/865 (1.85%, 95% confidence interval 1.14% to 2.98%). This might be because of low concentrations of cell-free fetal DNA in early pregnancy.¹³ After 11 weeks the incidence of false negative results was considerably lower (at 1/956 between 11-13 weeks; 0.1%, 0.02% to 0.59%) and did not decrease between 11 and 24 weeks, in line with recent evidence that concentrations of cell free fetal DNA increase only minimally between 10 and 20 weeks' gestation.¹⁴ The small number of false *RHD* negative results after 11 weeks' gestation would only minimally increase the risk of alloimmunisation in the population, around 0.07/100 000 births (table 2). This figure could well be offset by the potential to improve overall uptake of the immunoprophylaxis programme for RhD negative women carrying an *RHD* positive baby, as counselling and follow-up could be targeted at these women to try to improve anti-RhD immunoglobulin uptake when it is definitively needed.

In Denmark¹¹ and the Netherlands¹² routine fetal *RHD* genotyping has been introduced at 25 and 28 weeks' gestation, respectively, and is used to direct routine third trimester antenatal prophylaxis with anti-RhD immunoglobulin. While such strategies can minimise the administration of unnecessary anti-RhD immunoglobulin in a routine immunoprophylaxis programme, they do not take account of anti-RhD immunoglobulin required for sensitising events earlier in

pregnancy. Earlier knowledge of fetal *RHD* genotype would further reduce unnecessary administration of anti-RhD immunoglobulin as well as potentially enhancing any financial savings as prophylactic anti-RhD immunoglobulin and costly estimations of fetomaternal haemorrhage by Kleihauer-Betke test or flow cytometry could be avoided in RhD negative mothers carrying an *RHD* negative baby, savings realised not only for routine prophylaxis at 28 weeks but also for sensitising events occurring at earlier gestations.^{15 16}

Conclusions

Mass throughput fetal *RHD* genotyping is sufficiently accurate to be used from 11 weeks' gestation. To maximise reduction in administration of anti-RhD immunoglobulin and to minimise costs to both women and health services, testing should be performed as soon as possible from 11 weeks' gestation to coincide with routine antenatal visits.

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Contributors: LSC, GD, KF, EM, AW, and PS designed the study; LSC, EM, and GD carried out the literature search; AW created the figures; KF, PS, BM, and KO collected the data; AW analysed the data; LSC, GD, KF, EM, and AW interpreted the data; LSC, EM, GD, and AW wrote the paper, which was revised by all authors. LSC is guarantor.

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Ethical approval: The study was approved by the joint UCL/UCLH committee on the ethics of human research (committee A) of the National Research Ethics Service (07/H0714/128).

Transparency: The lead author affirms that the manuscript is an honest, accurate, and transparent account of the study being reported and that no important aspects of the study have been omitted.

Data sharing: No additional data are available.

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What is already known on this topic

Previous studies have predominantly assessed accuracy of fetal *RHD* genotyping at relatively late gestations (24-30 weeks)

At this stage in pregnancy it is highly accurate and can be used to direct immunoprophylaxis to those RhD negative women carrying an *RHD* positive baby and thereby reduce unnecessary exposure to anti-RhD immunoglobulin

What this study adds

Mass throughput fetal *RHD* genotyping is highly accurate from 11 weeks' gestation, but before this the number of babies falsely predicted to be RhD negative is high

To maximise benefit of routine fetal *RHD* genotyping and to avoid potential sensitisation by withholding anti-RhD immunoglobulin in women falsely predicted to be carrying an RhD negative baby, testing should be done only from 11 weeks' gestation

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Tables

Table 1 | Prediction of fetal RhD status by fetal RHD genotyping of cell-free DNA in maternal plasma compared with results of cord blood typing of RhD

	Gestational age in completed weeks*					Total samples
	<11	11-13	14-17	18-23	>24	
Correct RhD negative	337	341	225	321	696	1920
Correct RhD positive	400	535	272	492	864	2563
False RhD negative	16	1	1	1	0	19
False RhD positive	1	4	1	5	7	18
Inconclusive confirmed RhD negative	19	13	10	19	24	85
Inconclusive confirmed RhD positive	92	62	33	50	71	308
Total	865	956	542	888	1662	4913
Sensitivity RhD positive (95% CI)†	96.85 (94.95 to 98.05)	99.83 (99.06 to 99.97)	99.67 (98.17 to 99.94)	99.82 (9.96 to 99.97)	100.00 (99.59 to 100.00)	99.34 (98.98 to 99.58)
Specificity RhD positive (95% CI)†	94.40 (91.51 to 96.34)	95.25 (92.53 to 97.01)	95.34 (91.85 to 97.38)	93.04 (89.86 to 95.28)	95.74 (94.01 to 96.98)	94.91 (93.86 to 95.78)

*Gestational ages shown are in completed postmenstrual weeks (for example, 11 weeks=11 weeks 0 days to 11 weeks + 6 days).

†Assuming all inconclusive results are treated as RhD positive.

Table 2 | Impact of false negative results in terms of alloimmunisation, and risk to subsequent pregnancies

	Gestational age in weeks				
	<11	11-13	14-17	18-23	>24
False RhD negative % (CI)	1.85 (1.14 to 2.98)	0.10 (0.02 to 0.59)	0.18 (0.03 to 1.04)	0.11 (0.02 to 0.64)	0.00 (0 to 0.23)
Risk of alloimmunisation per 100 000 pregnancies if cord blood typing is not performed (CI)*	167 (103 to 268)	9.0 (2 to 53)	16.2 (3 to 94)	9.9 (2 to 58)	0 (0 to 20)
Risk per 100 000 pregnancies of having subsequent severely affected child if cord blood typing is not performed*†	7.4 (4.6 to 11.9)	0.4 (0.08 to 2.4)	0.72 (0.1 to 4.2)	0.44 (0.08 to 2.6)	0 (0 to 0.9)
Risk of alloimmunisation per 100 000 pregnancies if cord typing is performed in pregnancies when cell-free fetal DNA result is <i>RHD</i> negative	19 (11 to 30)	1.0 (0.2 to 6)	1.8 (0.3 to 10)	1.1 (0.2 to 6)	0 (0 to 0.1)
Risk per 100 000 pregnancies of having subsequent severely affected child if cord blood typing is performed in pregnancies when cell free fetal DNA result is <i>RHD</i> negative*	0.82 (0.5 to 1.3)	0.04 (0.009 to 0.26)	0.08 (0.01 to 0.46)	0.05 (0.009 to 0.28)	0 (0 to 0.11)

*Assuming 9% alloimmunised if not given anti-RhD immunoglobulin at delivery (published range 4.3-9%) and 1% alloimmunised if given anti-RhD immunoglobulin at delivery based on cord RhD type.

†Assuming 62% have subsequent baby 71.5% of which are RhD positive, risk of death for affected baby is 5%. Risk of permanent neurological damage in affected baby is 5%.⁸

Figures

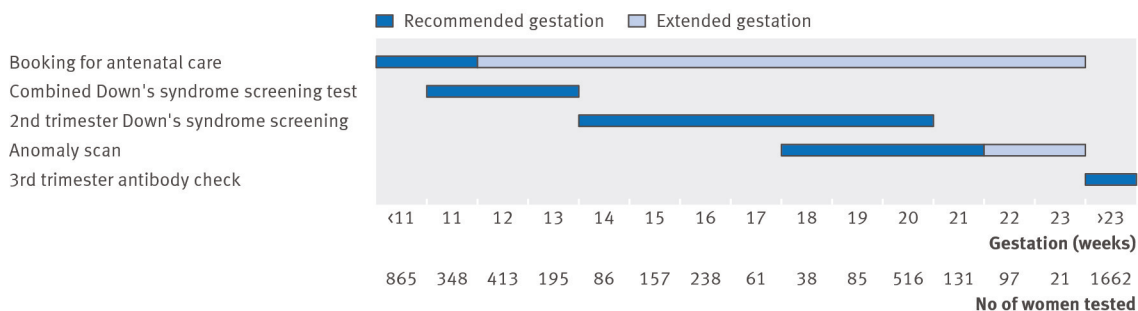


Fig 1 Maternity care pathway followed in England by gestational age together with number of women tested at each completed week of gestation. Dark bars indicate recommended gestations for tests but not all women book early in pregnancy and some attend late for their anomaly scan, so lighter bars show extended range of gestations, when women might have been recruited when attending for these indications

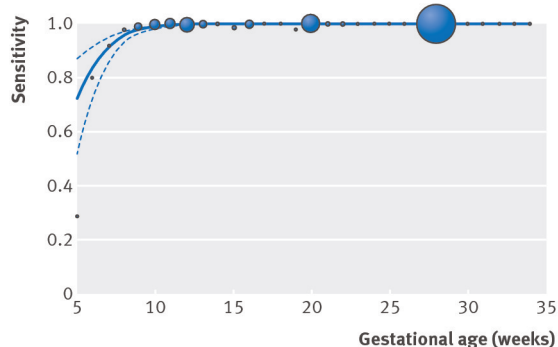


Fig 2 Sensitivity of determining *RHD* positive fetuses according to gestational age (inconclusive results treated as *RHD* positive). Fitted model (solid line) and 95% bootstrap confidence intervals (dashed lines) obtained from generalised linear model incorporating random intercept to take into account repeat measures from same women over pregnancy. Weekly estimates are overlaid with point size proportional to number of women tested during each week