High-throughput, non-invasive prenatal testing for fetal rhesus D status in RhD-negative women: a systematic review and meta-analysis

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**Abstract**

***Background***

High-throughput non-invasive prenatal testing (NIPT) for fetal Rhesus D (RhD) status could avoid unnecessary treatment with anti-D immunoglobulin for RhD negative women found to be carrying an RhD negative fetus. We aimed to assess the diagnostic accuracy of high-throughput NIPT for fetal RhD status in RhD negative women not known to be sensitised to the RhD antigen, by performing a systematic review and meta-analysis.

***Methods***

Prospective cohort studies of high-throughput NIPT used to determine fetal RhD status were included. The eligible population were pregnant women who were RhD negative and not known to be sensitised to RhD antigen. The index test was high-throughput, NIPT cell-free fetal DNA tests of maternal plasma used to determine fetal RhD status. The reference standard considered was serologic cord blood testing at birth. Databases including MEDLINE, EMBASE and Science Citation Index were searched up to February 2016.

Two reviewers independently screened titles and abstracts and assessed full texts identified as potentially relevant. Risk of bias was assessed using QUADAS-2. The bivariate and hierarchical summary receiver operating characteristic (HSROC) models were fitted to calculate summary estimates of sensitivity, specificity, false-positive and false-negative rates and the associated 95% confidence intervals (CIs).

***Results***

A total of 3921 references records were identified through electronic searches. Eight studies were included in the systematic review. Six studies were judged to be at low risk of bias. The HSROC models demonstrated high diagnostic performance of high-throughput NIPT testing for women tested at or after 11 weeks gestation. In the primary analysis for diagnostic accuracy, women with an inconclusive test result were treated as having tested positive. The false negative rate (incorrectly classed as RhD negative) was 0.34% (95% CI 0.15 to 0.76) and the false positive rate (incorrectly classed as RhD positive) was 3.86% (95% CI 2.54 to 5.82). There was limited evidence for non-white women and multiple pregnancies.

***Conclusions***

High-throughput NIPT is sufficiently accurate to detect fetal RhD status in RhD negative women and would considerably reduce unnecessary treatment with routine anti-D immunoglobulin. The applicability of these findings to non-white women and women with multiple pregnancies is uncertain.

**Keywords**

Fetal rhesus D status; non-invasive prenatal testing; diagnostic accuracy; anti-D immunoglobulin; systematic review

**Introduction**

Pregnant women who have an RhD negative blood type may carry an RhD positive fetus. The presence of fetal RhD-positive cells in the maternal circulation can cause a mother who is RhD negative to produce anti-D antibodies against the RhD antigen. This immune response, termed sensitisation, can happen at any time during the pregnancy, but it is most common in the third trimester and during childbirth. [1]

The process of sensitisation itself has no adverse effects on the mother and does not usually affect the pregnancy during which it occurs. However, in a subsequent pregnancy with an RhD positive fetus in women who have been sensitised to the RhD antigen, the woman’s anti-D antibodies may cross the placenta resulting in haemolytic disease of the fetus and newborn.

This can cause severe fetal anaemia that leads to fetal heart failure, fluid retention and swelling (hydrops), hyperbilirubinaemia, kernicterus and perinatal death. [2]

Prophylaxis with anti-RhD immunoglobulin can substantially reduce the risk of sensitisation in RhD negative women and hence the prevalence of haemolytic disease of the fetus and newborn.[3] The introduction of routine antenatal prophylaxis during the third trimester of pregnancy has led to a reduction in sensitisation, resulting in a decrease in mortality associated with haemolytic disease of the fetus and newborn from 46 in 100,000 births before 1969 to 1.6 in 100,000 births by 1991.[[4](#_ENREF_2)]

Currently, the National Institute for Health and Clinical Excellence (NICE) guideline on antenatal care recommends that women should be offered testing for blood group and rhesus D status in early pregnancy.[[5](#_ENREF_3)] In those identified as RhD negative and without preformed antibodies, administration of anti-D immunoglobulin is recommended both as prophylaxis and following potential sensitising events to prevent the sensitisation from occurring. Routine antenatal prophylaxis with anti-D immunoglobulin can be given as two doses at weeks 28 and 34 of pregnancy or as a single dose between 28 and 30 weeks.[5]

Non-invasive prenatal testing (NIPT) of fetal RhD status uses a real time quantitative polymerase chain reaction (PCR) method to detect cell-free fetal DNA - small fragments of extracellular DNA shed from the placenta circulating freely in the maternal plasma. High-throughput NIPT performs the test using an automated platform capable of performing a large number of tests simultaneously, and is therefore suitable for large-scale population screening of pregnant women. High-throughput NIPT for fetal RhD status may enable anti-D immunoglobulin to be withheld from RhD negative women who are predicted to be carrying an RhD negative fetus. Pregnant women found to be carrying an RhD negative fetus could avoid unnecessary treatment with anti-D immunoglobulin (which is a human polyclonal plasma-derived product), along with the potential risk associated with administration of blood products. In addition, these women may not need the provision of anti-D immunoglobulin following potentially sensitising events, and there may no longer be a need for serologic cord testing at birth. High-throughput NIPT is already used in this way in some European countries.[[6](#_ENREF_4) [7](#_ENREF_5)]

However, the diagnostic accuracy of high-throughput NIPT for fetal Rhesus D status in RhD-negative women not known to be sensitised to the RhD antigen is uncertain. The National Institute of Health Research Health Technology Assessment programme commissioned a systematic review and economic evaluation to assess the diagnostic accuracy and cost-effectiveness of high-throughput NIPT for the detection of fetal Rhesus D status in RhD-negative women. This systematic review formed part of a larger report on high-throughput NIPT which also considered broader issues in its clinical value and implementation and a full economic analysis.[8] This work was used to inform the recent NICE guideline regarding the recommendation of high-throughput NIPT for fetal Rhesus D status.

**Methods**

The complete methodology of the original wider review is reported elsewhere. [8] This section summarises the methodology of the diagnostic accuracy review.

***Data sources and searches***

We conducted a systematic review following the PRISMA statement [[9](#_ENREF_7)] and registered the review on PROSPERO. The following databases were searched for relevant studies from inception to November 2015: MEDLINE, MEDLINE In-Process, CINAHL, Cochrane Central Register of Controlled Trials (CENTRAL), Cochrane Database of Systematic Reviews (CDSR), Database of Abstracts of Reviews of Effects (DARE), EMBASE, Health Technology Assessment (HTA) database, Maternity and Infant Care, PubMed and the Science Citation Index.In addition, the following resources were searched for on-going, unpublished or grey literature: ClinicalTrials.gov, Conference Proceedings Citation Index: Science, EU Clinical Trials Register, PROSPERO and the WHO International Clinical Trials Registry Platform portal. An updated search was performed in February 2016.

Both published and unpublished literature were identified from systematic searches of electronic sources, consultation with experts in the field, and reference checking of relevant systematic reviews and included studies. Search strategies were developed by an information specialist (MH).The search strategy can be found in Appendix 1.

***Study selection***

Prospective cohort studies of high-throughput NIPT used to determine fetal RhD status were eligible for inclusion. We considered as high-throughput, any NIPT tests which were conducted using an automated robotic platform (including automated DNA extraction and liquid handling) and were able to process large numbers of samples rapidly for large scale screening purposes. Studies where this test was used for diagnosis (rather than screening) of sensitised women were excluded.

The inclusion criteria for population, index test, reference standard and outcomes are listed below:

* The eligible population were pregnant women who were RhD negative and not known to be sensitised to RhD antigen.
* The index test was high-throughput, NIPT cell-free fetal DNA tests of maternal plasma used to determine fetal RhD status.
* The reference standard considered was serologic cord blood testing at birth.
* The eligible studies had to report diagnostic accuracy data such as absolute numbers of true positive, false positive, true negative, and false negative test results.

Two researchers independently screened the titles and abstracts of all reports identified by the search strategy and full-text papers were subsequently obtained for assessment. Only reports published in English were sought. There were no restrictions for publication date. Full text copies of all studies deemed to be potentially relevant were obtained and two reviewers independently assessed them for inclusion (HY, AL). Any disagreements were resolved by consensus or by a third reviewer (RW).

***Data extraction***

We selected the most recent or most complete report in cases of multiple reports for a given study or when we could not exclude the possibility of overlapping populations. One reviewer independently extracted details from full text studies including study design, participants, index, comparator and reference standard tests and outcome data. The data extraction was checked by another reviewer. Any disagreements were resolved by consensus (between HY and AL) or with a third reviewer (RW).

We extracted the number of true positives, true negatives, false positives and false negatives for each index test evaluated in each study in order to construct 2 x 2 tables. If reported, we extracted data on the number of undetermined or uninterpretable results. Study authors were contacted if some data were unclear or missing.

***Quality Assessment***

Risk of bias was assessed using a modified version of the quality assessment of diagnostic accuracy studies (QUADAS-2) checklist.[[10](#_ENREF_8)] The QUADAS-2 tool consists of four key domains: 1) patient selection, 2) index test, 3) reference standard, and 4) flow of patients through the study and timing of the index test(s) and reference standard. Each domain was assessed in terms of the risk of bias. The first three domains were also assessed for concerns regarding their applicability in terms of whether (1) the participants and setting; (2) the index test, its conduct or interpretation; and (3) the target condition as defined by the reference standard were applicable to the UK context. One reviewer (AL) independently assessed the quality of all included studies in terms of risk of bias. The quality assessment was checked by another reviewer (HY). Any disagreements were resolved by consensus or by a third party (RW).

***Data synthesis***

For diagnostic accuracy outcomes, estimates of sensitivity, specificity, false-positive and false-negative rates were calculated and presented on forest plots and in receiver-operating characteristic (ROC) space to assess the heterogeneity in test accuracy within and between studies. The hierarchical bivariate model [[11](#_ENREF_9)] was fitted to calculate summary estimates of sensitivity, specificity, false-positive and false-negative rates and the associated 95% confidence intervals (CIs). The hierarchical summary ROC (HSROC) model[1[2](#_ENREF_10)] was fitted to produce summary ROC curves. Both models jointly model sensitivity and specificity and account for the correlation between them. Heterogeneity in sensitivity and specificity was also assessed using the I2 statistic. All analyses were performed using R software.[13][14] Because NIPT testing is highly accurate, we present the results in terms of the false positive rate (FPR) (incorrectly testing positive and being offered unnecessary anti-D prophylaxis) and false negative rate (FNR) (incorrectly testing negative; at risk of sensitisation as women do not receive anti-D prophylaxis), rather than the conventional sensitivity and specificity.

Some NIPT results are inconclusive, and unable to predict the RhD status of the fetus. Current UK practice is to treat such test results as if they predicted an RhD positive fetus, and this approach was used in the primary analysis of diagnostic accuracy. Sensitivity analyses were conducted to explore the robustness of the results by including and excluding such inconclusive test results. A further sensitivity analysis included only UK (Bristol)-based studies, as this review was intended to inform UK practice. Furthermore, as test accuracy may vary according to the gestation age when NIPT is performed we investigated the impact of test timing by plotting diagnostic accuracy against time, and performing meta-regressions against test timing. No analysis for small study effects or publication bias was performed because there were too few studies identified to justify this.

**Results**

The literature searches of bibliographic databases identified 3921 references. After initial screening of titles and abstracts, 227 were considered to be potentially relevant and were ordered for full paper screening. In total, eight studies [6,7,15-18]were included in the diagnostic review of high-throughput NIPT testing. Six studies reported inconclusive results. Figure 1 shows a flow diagram outlining the screening process with reasons for exclusion of full-text papers.

[Figure 1 here please]

Table 1 presents the summary information of characteristics of the included diagnostic accuracy studies. All the studies were prospective studies and conducted in European countries. Four studies were conducted in England, [15-18] three of which were based at Bristol. [15-17]The sample size of studies ranged from 282 to 18383. Most studies recruited pregnant women with a gestational age of 10 to 28 weeks. Most participants were white, and most pregnancies were singleton. All studies used maternal plasma as their sample source. A robotic DNA extraction instrument was employed in all studies. The studies used a number of robotic platforms including MDx BioRobot, MagNa Pure 96, MagNA Pure LC, and COBAS AmpliPrep. For PCR, all studies targeted at least two exons (generally exons 5 and 7) and at least two controls for *RHD* assay (RhD positive DNA and RhD negative DNA) except for the study by Wikman et al.[20] which targeted exon 4 only and used *GAPDH* DNA as control. The reference standard used in all studies was cord blood serology, except for Akolekar et al.[16] which did not describe its reference standard. Where reported, rates of inconclusive results ranged from 1.0% [20] to 14.3%[18].

[Table 1 here please]

***Risk of bias***

Table 2 presents a summary of the results for the risk of bias assessment. The majority of included studies were judged to be at low risk of bias, but two studies [7 18] were judged to be at high risk of bias. The study by Akolekar et al. [18]reported that the targeted RhD negative women were selected from a database, but it was unclear whether this selection was conducted on a random basis. The study enrolled a large proportion of Africans (19.3%) which may not be representative of the general population of pregnant women in the UK. This may have contributed to the larger than average proportion of inconclusive results (14.3 %). Characteristics of the reference standard were also poorly reported in this study. In the study by Thurik et al. [7]only 80% of participants received a reference standard. The reasons why cord blood serology was not conducted in a significant proportion of the study population were not stated. This study also reported that its prediction algorithm was judged daily and modified as needed, which may have introduced bias in the diagnostic accuracy estimates.

NIPT as an automated procedure was deemed to be of limited risk to human error, and multiple controls were used for *RHD* assays in all except one study. [20]The index test of NIPT was conducted independent of the reference standard and the results of one were considered unlikely to influence the results of the other, therefore the risk of incorporation bias was considered low. It appears that most studies prospectively recruited consecutivesamples from clinical practice. Only three studies stated that their diagnostic threshold was pre-specified during the conduct of the screening programme.[6 15 16]

The results of the studies were considered broadly applicable to the use of high-throughput NIPT for nationwide screening purposes, except for two studies. [18 20] In particular, the NIPT test used in the study by Wikman et al. [21] only targeted exon 4, unlike all other included studies where at least two exons (5, 7 and/or10) were targeted. It is generally accepted that a combination such as of exons 5 and 7 should be targeted to discriminate the pseudogene *RHD*Ψ, particularly present in individuals of African origin.[22]

[Table 2 here please]

***Meta-analysis***

The results of the bivariate meta-analyses are shown in Table 3. These show that NIPT is a highly accurate test. The false negative rate (where women would not be offered anti-D prophylaxis and so be at risk of sensitisation) is very low at 0.34% (95% CI 0.15 to 0.76). When treating women with an inconclusive test result as if they were positive the false positive rate is 3.86% (95% CI 2.54 to 5.82). Excluding inconclusive test results reduces this to 1.26% (95% CI 0.87 to 1.83). Therefore most false-positive results occur in women with inconclusive test results.

There was some evidence of inconsistency across studies. I2 was 75% for the false negative rate and 99% for the false positive rate. It should be noted that these high heterogeneities are, in part, a consequence of the high accuracy of the test and the large size of the studies (and consequent small within-study variance, because I2 increases as the average within-study variance declines). They do not necessarily indicate any clinically meaningful differences between studies. The heterogeneity in false positive rates is likely to be a consequence of differing reporting and handling of inconclusive tests.

[Table 3 here please]

Studies conducted in Bristol had a lower false negative rate (0.21%, 95% CI 0.09 to 0.48), with a consequently higher false positive rate (5.73%, 95% CI 4.58 to 7.16). This suggests that the Bristol high-throughput NIPT testing approach, in which the MDx Bio Robot machine is used, may be using a different test threshold to other countries, which further minimises false negative findings.

Figure 2 shows the results of the bivariate and the summary HSROC curve for this primary analysis, which is presented in terms of false positive and false negative rates. The black circle is the summary effect estimate from a bivariate model, and the black curve is the HSROC curve. This plot shows that the studies were generally consistent in terms of false negative results, except for two outlying studies. [18 20]The study by Wikman et al. [20] conducted most NIPT tests in the first trimester, earlier than other studies. The studies are less consistent in false positive rates. This is most likely because the studies have different numbers of inconclusive test results, and different methods of handling such results.

[Figure 2 here please]

***Timing of NIPT tests***

Figure 3 shows the false negative rates plotted by gestational age at time of high-throughput NIPT testing. It suggests that false negative rates after the first trimester (i.e. after around 13 weeks’ gestation) were consistent, irrespective of timing, but false negative rates were higher in the first trimester. This pattern is most visible in the Chitty study [15] which reported diagnostic accuracy at a range of test timings. Given the limited amount of data, no formal statistical test could be performed to confirm this conclusion. Appendix Figure 1 shows the false positive rates plotted by gestational age at time of high-throughput NIPT testing. There was no obvious pattern from this figure.

[Figure 3 here please]

We also evaluated the impact of the timing of high-throughput NIPT testing on the number of inconclusive test results. As seen in Appendix Figure 2, there is a suggestion that the percentage of inconclusive results for this test reduces as the gestational age increases. This is most obvious in the study by Chitty et al. [15]

***Impact on UK practice***

We considered the likely impact of implementing NIPT to guide anti-D prophylaxis in the UK by conducting a simulation study, simulating a representation of the UK population using data sourced from the review (see Appendix 2 for input parameters).

Based on the results of the three Bristol-based studies, we assumed that 60.7% of RhD negative women have an RhD positive fetus, and 6.7% of women have an inconclusive NIPT result. Given this, the results of the diagnostic meta-analysis, and parameters described in Appendix 2, using NIPT would result in anti-D prophylaxis being received by 65.9% of RhD negative women. It would reduce the numbers of women receiving unnecessary anti-D from 38.9% to 5.7%. The number of women who miss out on potentially beneficial anti-D would rise from 0.6% to 1.2%, leading to potentially more sensitisations: an extra 3 per 100,000 women if postpartum cord blood testing continues, or 13 per 100,000 if it is withdrawn. Sensitisation rates using universal anti-D administration were estimated to be 280 per 100,000 women, so this increase is small.

It would however mean 0.12% of women with an RhD positive fetus would not be offered anti-D and so would be at risk of sensitisation.

**Discussion**

In this systematic review we identified eight studies that evaluated diagnostic accuracy of high-throughput NIPT. Six of these studies were judged to be at low risk of bias. Meta-analyses showed very high diagnostic accuracy of high-throughput NIPT testing.

Diagnostic accuracy of high-throughput NIPT varied by gestational age. The data suggest that high-throughput NIPT testing is insufficiently accurate in first trimester, but is consistently accurate at any time thereafter. This might be due to low concentration of cell-free fetal DNA in early pregnancy[23] but an increased concentration of cell-free fetal DNA after the end of the first trimester.[24]

Given the very high diagnostic accuracy performance of high-throughput NIPT testing, implementing high-throughput NIPT for fetal RhD screening in all RhD negative women nationwide could be feasible. The results suggest it would substantially reduce the need for antenatal anti-D prophylaxis, while only marginally increasing the risk of sensitisation due to false negative test results. NIPT testing could be conducted, with low false-positive rates, at any time from the 2nd trimester onwards, perhaps to coincide with routine antenatal blood tests. Any nationwide NIPT screening programme will require careful logistical management to ensure that blood samples are transported to laboratories and tested quickly, and that results are reliably returned to general practitioners and midwives.

***Limitations***

We performed extensive literature searches with an attempt to maximise retrieval of potentially relevant studies. These included electronic searches of a variety of bibliographic databases as well as screening of clinical trial registers and conference proceedings to identify unpublished studies. However, only studies in English were included, therefore some potentially relevant non-English language studies may have been missed. There was some evidence of inconsistency in the meta-analysis of diagnostic accuracy studies. The observed heterogeneity may be due to variations in methods used in the high-throughput NIPT approach (e.g. different diagnostic accuracy thresholds used, and different number and types of exons targeted, gestational age at the time of testing, and different methods of handling inconclusive test results). In addition, there was variation in the reporting of included studies. Particularly, two studies [19] [[7](#_ENREF_5)] did not report the number of inconclusive results of the test and some studies did not report detailed reasons for inconclusive results. The simulation study assumes that the input probabilities are accurate, and does not account for any uncertainty in their estimation. Therefore results of the simulation study should be considered to be illustrative only and not definitive estimates of effect.

***Implications for future research***

Further large prospective cohort studies evaluating diagnostic accuracy of high-throughput NIPT in women of non-white ethnicity are required. This is of particular concern as non-white women are more likely to have less accurate test results. For example, in people with African ethnicity, because of the presence of *RHD*-pseudogene,[25] prenatal detection of fetal RhD type from maternal blood would lead to higher rates of false positives results in this particular population. Further research to improve the NIPT test itself is also warranted, especially for reducing the number of inconclusive test results.

**Conclusions**

The findings from this systematic review have demonstrated high diagnostic performance of high-throughput NIPT testing for the detection of fetal RhD status in RhD negative women, with very low false positive and false negative rates in women tested at or after 11 weeks’ gestation. The use of high-throughput NIPT testing as a routine screening test for fetal RhD status in RhD negative women can largely remove unnecessary exposure to prophylactic anti-D treatment. Due to limited evidence, the accuracy of NIPT in non-white women and multiple pregnancies is unclear.

**Declarations**

***Registration and funding***

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**Research ethics:** No human subjects, human material or human data were involved in this research, which is based on literature review.

**Competing interests:** The authors declare that they have no competing interests.

**Authors’ contribution**

HY, MS and AL developed the protocol with assistance from PS, SG and MH. Study selection, data extraction and quality assessment were conducted by HY, AL and RW. Analysis was conducted by MS. Literature searches were designed and conducted by MH. HY interpreted the evidence and was responsible for writing the manuscript. All authors contributed to the editing of this manuscript. All authors read and approved the final manuscript.

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**Tables and Figures**

**Table 1 Characteristics of the diagnostic accuracy studies**

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Study**  **(First author / year)** | **Location** | **DNA extraction tool** | **Exons targeted** | **Controls** | **Reference standard** | **Gestational age at time of NIPT (median/range)** | **Sample sizeα** | **Confirmed RhD positive** | **Confirmed RhD negative** | **Inconclusive test results (%)** | **Reasons for inconclusive results (n of cases)** |
| Akolekar 2011 [18] | UK (London) | MDx BioRobot (Qiagen) | 5 & 7 | RhD+, RhD–,  RHDΨ +, No DNA | “Serologically at delivery” | 12.4 (11 – 14) | 586 | 410 | 176 | 84 (14.3) | Maternal or fetal RHD variant and insufficient maternal plasma for further analysis (NR)! |
| Banch Clausen 2014 [6] | Denmark | QIAsymphony SP; MagNA Pure LC; MagNA Pure Compact Instrument (Roche) | 5 & 10, or 5 & 7, or 7 & 10 | RhD+, RhD–£ | Cord blood serology.~ | 25 (23 – 28) | 12668 | 7830 | 4838 | 274 (2.2) | Maternal weak D (93); maternal silent RHD variant (38); high level of maternal background DNA (29); technical problems (19); maternal DVI (14); weak PCR signal (13); suspected maternal RHD positive (3); no reported cause (65) |
| Chitty 2014 [15] | UK (Bristol) | MDx BioRobot (Qiagen) | 5 & 7 | RhD+, RhD–,  RHDΨ+, No DNA | Cord blood serology | 19 (5 – 35) | 4913 | 2890 | 2023 | 393 (8.0) | NR |
| Finning 2008 [16] | UK (Bristol) | MDx BioRobot (Qiagen) | 5 & 7 | RhD+, RhD–,  RHDΨ+, No DNA | Cord blood serology> | 28 (8 – 38) | 1869 | 1156 | 713 | 56 (3.0) | Insufficient DNA (30); suspected maternal RHD gene (25); failure to extract DNA from plasma (1) |
| Grande 2013 [19] | Spain | COBAS AmpliPrep (Roche) | 5 & 7, 10$ | RhD+, RhD– | Cord blood serology | 24 - 26 | 282 | 186 | 96 | NR | NR |
| Soothill 2015[17] | UK (Bristol) | MDx BioRobot (Qiagen) | 5 & 7 | RhD+, RhD–,  RHDΨ+, No DNA | Cord blood serology | 15 – 17  (mostly) | 499\* | 315 | 184 | 61 (12.2) | NR |
| Thurik 2015 [7] | Netherlands | MagNa Pure 96 (Roche) | 5 & 7 | RhD+, RhD– | Cord blood serology^ | 26 | 18383\* | 11283 | 7100 | NR | NR |
| Wikman 2012 [20] | Sweden | MagNA Pure LC (Roche) | 4 | GAPDH | Serology from cord blood or citrate samples from newborns+ | 8 - 40 | 3291# | 2073 | 1218 | 32 (1.0) | RHD variant (14); no second sample (18, of which 13 were spontaneous abortions & miscarriages) |

***α*** *Number of blood samples unless otherwise specified; \* number of participants; # excludes pre-8 weeks gestation pregnancies; $ on 2nd DNA extraction, only to confirm RHD negative results; £ Multiple controls without template were also included using sterile H2O; ! 5 mothers with insufficient DNA were excluded from the analyses and not classed as inconclusive*

*NR: not reported; RhD+: RhD positive; RhD-: RhD negative; PCR: Polymerase chain reaction; GAPDH: glyceraldehyde-3-phosphate dehydrogena; ~ Region 1: ID-Card DiaClon ABD-Confirmation for donors, (DiaMed GmbH 1785 Cressier FR Switzerland) with monoclonal anti-D (cell lines ESD-1M, 175-2) that detects most weak RhD types and partial DVI phenotype; Region 2: direct agglutination in a gel matrix test with IgM monoclonal anti-D clone 175-2 (DiaMed); further tests in gel matrix test with in-house Dw1 anti-D for initial negative tests. For discrepancies, DNA extracted from the cord blood and tested for RHD exon 10 and further analyzed by PCR-SSP using the RH-type kit (Biologische Analysensystem GmbH, Lich, Germany); Region 3: Direct agglutination with monoclonal antibody Diagast anti-D IgM (ref. no. 71000) for RHD positive. For unexpectedly negative reactions, an additional IAT with anti-D IgG LOR17 was performed. IAT with anti-D IgG LOR17 was used for RHD negative; Region 4: Serological testing of cord blood RBC was done by using Seraclone Anti-D (RH1) Blend; Ref 802032 (Biotest, Germany); Region 5: Serological testing of cord blood RBC with 2 complete anti-Ds (Medion Diagnostics, IgM anti-D[MS201] and Seraclone, [Rh1] 226) in saline. For reactions of less than 3+ for both reagents, further investigation for D expression by IAT with two IgG anti-Ds. > No DNA extraction of cord blood samples. ^ Cord blood serology with WA-Diana system (DiaMed GmbH) using two monoclonal anti-D reagents, LHM 59/20 (LDM3) + 175–2 and ESD- 1M+ 175–2. Maternal and fetal RHD variant genes were analyzed with an RHD-multiplex ligation-dependent probe amplification (MLPA) assay on genomic DNA. Mutation analysis and copy number variation investigated via RHD MLPA. Discordant positive results due to maternal or fetal RHD variants were identified and excluded from the study. Samples with weak PCR signals were excluded. The Kleihauer–Betke test and multiplex short tandem repeat (STR)-PCRs on 15 systems on leukocyte-derived DNA were used exclude errors around cord blood collection. + Blood typing using DiaClon ABO/Rh for Newborns DVI+ gelcards.*

**Table 2** **Risk of Bias of included studies**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  | **Risk of bias** | | | | **Applicability concerns** | | |
| **Study** | **Patient selection** | **Index test** | **Reference standard** | **Flow and timing** | **Patient selection** | **Index test** | **Reference standard** |
| Akolekar (2011) [18] | High | High | Unclear | Unclear | High | Low | Unclear |
| Banch- Clausen (2014) [6] | Low | Low | Low | Low | Unclear | Low | Low |
| Chitty (2014) [15] | Low | Low | Low | Low | Low | Low | Low |
| Finning (2008) [16] | Low | Low | Low | Low | Low | Low | Low |
| Grande (2013) [19] | Low | Low | Low | Low | Low | Low | Low |
| Soothill (2015) [17] | Low | Unclear | Low | Low | Low | Low | Low |
| Thurik (2015) [7] | Low | High | Low | High | Low | Low | Low |
| Wikman (2012) [20] | Low | Low | Low | Low | Unclear | High | Low |

High: High risk of bias; Low: low risk of bias

Table 3: Bivariate meta-analyses of false positive and negative rates

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Analysis case | Number of studies | False negative rate  (at risk of sensitisation) | | False positive rate  (unnecessary anti-D) | |
|  |  | Estimate (%) | 95% CI | Estimate (%) | 95% CI |
| Inconclusive tests treated as test positive | 8 | 0.34 | 0.15– 0.76 | 3.86 | 2.54 – 5.82 |
| Excluding all women with inconclusive test results | 8 | 0.35 | 0.15 – 0.82 | 1.26 | 0.87 – 1.83 |
| Studies conducted in Bristol only | 3 | 0.21 | 0.09 – 0.48 | 5.73 | 4.58 – 7.16 |

**Figure legends**

**Figure 1: Flow diagram: Study selection process**

**Figure 2: HSROC and bivariate meta-analysis**

**Figure 3: False negative rate by gestational age at time of NIPT**

**Appendix figure 1: False positive rate by gestational age at time of NIPT**

**Appendix figure 2: Inconclusive results by test timing**

**Appendix 1:** **Search strategy**

**MEDLINE**

1 Rh-Hr Blood-Group System/ (10006)

2 (RhD or "rhesus D" or "Rh(D)" or "Rh-(D)" or Rh D).ti,ab. (3323)

3 (Rh-negative or Rh-positive).ti,ab. (898)

4 (Rhesus negative or Rhesus positive).ti,ab. (228)

5 ((rh or rhesus) adj2 (factor or factors or antigen$ or system or group)).ti,ab. (3438)

6 or/1-5 (13812)

7 Rh Isoimmunization/ (1505)

8 ((isoimmuni$ or iso-immuni$ or isoimmune or iso-immune) adj6 (rh or rhesus or maternal or pregnan$)).ti,ab. (1164)

9 ((alloimmuni$ or allo-immuni$ or alloimmune or allo-immune) adj6 (rh or rhesus or maternal or pregnan$)).ti,ab. (870)

10 ((unsensiti#ed or un-sensiti#ed or non-sensiti#ed) adj6 (rh or rhesus or maternal or pregnan$)).ti,ab. (25)

11 ((sensiti#ation$ or sensiti#ed) adj6 (rh or rhesus or maternal or pregnan$)).ti,ab. (1074)

12 ((fetomaternal or feto-maternal or foetomaternal or foeto-maternal) adj2 immuni#ation).ti,ab. (80)

13 ((rh or rhesus) adj2 (immuni#ation or autoimmuni#ation)).ti,ab. (695)

14 or/7-13 (4428)

15 exp Erythroblastosis, Fetal/ (11006)

16 ((hemolytic or haemolytic) adj2 (disease$ or disorder$)).ti,ab. (4465)

17 HDFN.ti,ab. (95)

18 ((rhesus or rh) adj2 (disease$ or disorder$)).ti,ab. (742)

19 ((rhesus or rh or RhD) adj2 (incompatib$ or antagonism)).ti,ab. (750)

20 ((erythroblastoses or erythroblastosis) adj2 f?etal$).ti,ab. (760)

21 or/15-20 (13551)

22 6 or 14 or 21 (25723)

23 Prenatal Diagnosis/ (33273)

24 Maternal Serum Screening Tests/ (153)

25 Hematologic Tests/ (5564)

26 ((prenatal or pre-natal or antenatal or ante-natal) adj3 (test$ or screen$ or diagnos$ or determin$ or detect$)).ti,ab. (32925)

27 ((fetal or foetal or fetus$ or foetus$) adj3 (test$ or screen$ or diagnos$ or determin$ or detect$)).ti,ab. (20036)

28 (NIPD or NIPT).ti,ab. (328)

29 or/23-28 (69981)

30 Genotyping Techniques/ (2761)

31 ((genotype$ or genotyping) adj2 (fetal or foetal or fetus$ or foetus$ or prenatal or pre-natal or antenatal or ante-natal)).ti,ab. (606)

32 ((genotype$ or genotyping) adj2 (maternal or pregnan$)).ti,ab. (789)

33 ((genotype$ or genotyping) adj2 (noninvasive or non-invasive)).ti,ab. (71)

34 cell-free f?etal DNA.ti,ab. (489)

35 cffDNA.ti,ab. (87)

36 or/30-35 (4483)

37 22 and 29 (1795)

38 22 and 36 (276)

39 37 or 38 (1869)

40 (editorial or comment).pt. (946538)

41 39 not 40 (1824)

42 exp animals/ not humans/ (4137930)

43 41 not 42 (1815)

Appendix 2: **Probability estimates derived from published data, used in the simulation study**

|  |  |  |
| --- | --- | --- |
| **Probability** | **Estimate** | **Source** |
|  |  |  |
| Rhesus positive fetus | 60.7% | Bristol-based diagnostic studies |
| Rhesus positive fetus (with inconclusive NIPT) | 70.7% | Bristol-based diagnostic studies |
| False negative NIPT test | 0.21% | Diagnostic meta-analysis (Bristol studies) |
| Inconclusive NIPT test | 6.7% | Bristol-based diagnostic studies |
| False-positive test (if conclusive) | 1.5% | Diagnostic meta-analysis (Bristol studies) \* |
|  |  |  |
| Compliance with antenatal anti-D (without NIPT)  (received at least one dose of anti-D) | 99% | UK 2013 audit |
| Uptake of NIPT | 96% | De Haas 2012 (clinical effectiveness review) |
| Compliance with postpartum anti-D | 99% | UK 2013 audit |
| Compliance with antenatal anti-D  (if NIPT test refused or missed) | 80% | Soothill (2015) (clinical effectiveness review) |
| Compliance with antenatal anti-D  (if NIPT inconclusive) | 99% | Soothill (2015) |
| Uptake of antenatal anti-D in women with negative NIPT | 6% | Soothill (2015) |
| Compliance with postpartum anti-D after NIPT process | 99% | No data. Assumed same as without NIPT |
|  |  |  |
| Sensitisation with antenatal anti-D and post-partum anti-D | 0.35% | Pilgrim et al 2009 HTA report |
| Sensitisation with only postpartum anti-D | 0.95% | Pilgrim et al 2009 HTA report |
| Sensitisation with no anti-D | 10.7% | From Pilgrim, and Crowther et al. 1997 |
|  |  |  |
| Subsequent pregnancy in sensitised women | 62% | Used by Chitty (2014), no source given |
| Death of RhD negative fetus in sensitised women | 5% | Used by Chitty (2014), no source given |
|  |  |  |

*\* This result is based on a diagnostic meta-analysis of the Bristol-based studies, excluding women with inconclusive test results.*