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CAT
Critically Appraised Topic

Fetal *RHD* genotyping in maternal blood using cell-free fetal DNA

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CLINICAL BOTTOM LINE

The discovery of cell-free fetal DNA (cffDNA) in maternal plasma has opened up new possibilities for non-invasive prenatal diagnosis (NIPD). Real-time PCR protocols as well as MALDI-TOF mass spectrometry techniques have been developed to determine fetal *RHD* genotype on maternal plasma. In several European centers, NIPD for fetal *RHD* has become the standard of care for evaluation of anti-D allo-immunized pregnant women. Several authors have also suggested the feasibility of fetal *RHD* genotyping in non-immunized women to determine the need for antenatal and perinatal anti-D immunoglobulin. However, up until now there are insufficient data of the cost-effectiveness of large-scale implementation of this test. Most studies were organized in a research/experimental setting in which several replicates were analyzed and inconclusive or unobtainable results have systematically been excluded. In case of large-scale implementation, the number of analyzed replicates should be limited, and inconclusive or unobtainable results need to be taken into consideration. Moreover, up until today, there's still no universal internal control for the presence of cell-free fetal DNA available. This is necessary to avoid false negative results.
International guideline providers such as NICE, ICSI and BCSH await further studies on NIPD using cell-free fetal DNA to issue new guidelines considering the pre- and postnatal management of RhD negative non-immunized pregnant women.

CLINICAL/DIAGNOSTIC SCENARIO

The Rh blood group system is the largest and most complex of the human blood group systems. Of the 49 known antigens, the most important is the Rhesus D (RhD) antigen. In the Caucasic population, about 17% is RhD negative. About 60% of RhD-negative pregnant women carry an RhD positive fetus. These women are at risk of anti-D alloimmunization, which is the most common cause of hemolytic disease of the fetus and newborn (HDFN) (13,30). During pregnancy, small amounts of fetal blood may enter the maternal circulation, which could cause an immune response in the RhD-negative mother. (5). This is most common in the third trimester and during childbirth, however, it can happen at any time during pregnancy, with higher risk in case of medical intervention, abdominal trauma, miscarriage or antepartum hemorrhages (5).

Sensitization has no immediate effects on the mothers health, and doesn't usually affect the pregnancy during which it's caused, but leads to a risk of HDFN in case of a subsequent pregnancy with a RhD positive fetus (5). Circulating maternal IgG anti-D-antibodies can cross the placenta and may bind to the RhD-antigen on the surface of fetal red blood cells (RBC), with subsequent destruction of these antibody-coated RBC (5,15). During pregnancy, this may lead to fetal anemia and, in severe cases, development of fetal heart failure, fluid retention, hydrops and intrauterine death (5). The bilirubin produced as a result of RBC lysis is cleared by the placenta before birth, but after birth the neonatal liver is not capable to clear this excess production of bilirubin, leading to jaundice (5). High bilirubin levels can result in severe brain damage (kernicterus) with a range of neurodevelopmental problems (5). Fetal anemia is treated with intrauterine transfusion; postnatal jaundice can be treated with phototherapy and/or exchange transfusion (5).

National and international guidelines recommend to determine the blood group, the RhD status and the presence or absence of irregular antibodies in the maternal circulation, before pregnancy or at the first prenatal visit (3,5,6-8,9). Repeat D antibody testing is recommended for all unsensitized RhD-negative women at 28 weeks gestation (3,6,8,9). The risk of anti-D alloimmunization between first trimester and week 28 is low (0.18%) but anti-D might not be detected in the first screening (below threshold) but develop during pregnancy and cause HDFN (9).

In non-immunized women, the risk of sensitization can be reduced by administering one dose of 1500 IU anti-D immunoglobulin within 72 hours after a potentially sensitizing event, including child-birth (3,6-8). A similar dose of anti-D immunoglobulin can be administered as a prophylactic treatment in the third trimester of pregnancy (3,5-8,9,30). This last therapy is known as routine antenatal anti-D prophylaxis (RAADP) (3,5,7,8). RAADP neutralizes the fetal RhD antigen in the maternal circulation, which leads to a further reduction of the immunization risk from 1.2% to 0.3% (5,6,24,27). But since only 60% of RhD negative women are pregnant with an RhD-positive fetus, 40% will receive unnecessarily anti-D prophylaxis (30). In Belgium, no national policy about RAADP exists. Due to the limited availability of anti-D immunoglobulin, treatment strategy in the University Hospitals of Leuven is limited to antenatal administration in case of a potentially sensitizing event and postnatal administration to mothers giving birth to an RhD-positive newborn.

When an anti-D-immunized woman is pregnant, intensive follow-up is recommended. Rising anti-D titers or titers over 4 IU/ml are considered to be at risk for HDFN. In that case, Doppler measurement of the middle cerebral artery peak velocity flow is performed to evaluate the degree of fetal anemia (11,26).
Another possible approach is the determination of the fetal *RHD* genotype on amniotic fluid. In case of an *RHD* negative fetus, no further investigations are required. An *RHD* positive fetus requires intensive follow-up. Until recently, the only manner to obtain fetal DNA was through invasive procedures such as amniocentesis or chorionic villus sampling. However, recent developments in noninvasive prenatal diagnosis have changed this.

RHD genotyping using maternal plasma as a source of fetal DNA is introduced in many European laboratories as a noninvasive prenatal test in anti-D allo-immunized women (15). The International Blood Group Reference Laboratory (IBGRL) in Bristol, UK, has been offering this test to immunized women with heterozygous partners since 2001 (13,24,35). In Belgium, non-invasive fetal *RHD* genotyping is proposed to all RhD-negative pregnant women at the University Hospital of Liege, with proposition of RAADP at 28 weeks gestation for all patients bearing RhD-positive fetuses (26).

Starting July first, 2011, the Dutch Sanquin blood supply foundation and the Dutch National Institute for Public Health and Environment (RIVM) have implemented systematic screening of RhD-negative pregnant women in the 27th week of pregnancy with PCR genotyping of the fetal *RHD* on maternal blood (2). Only RhD negative women carrying an *RHD* positive fetus will receive RAADP and postnatal RhIg, providing that the screening for irregular antibodies is negative (2). A pilot study, with parallel testing of RhD serology on umbilical cord blood samples after birth, will run until October 2012. In case of postnatal RhD positivity, anti-D Ig is still offered (2).

In this document, we will review the current knowledge and diagnostic accuracy of fetal *RHD* genotyping on maternal blood. Based on literature data, we will also explore its cost-effectiveness and its potential in clinical practice.

QUESTION(S)

PICO
P: RhD-negative pregnant women
I: *RHD* genotyping on maternal blood using cell-free fetal DNA
C: Serologic RhD phenotyping or RHD genotyping on amniotic fluid or chorionic villus sample
O: Determination of fetal *RHD* genotype in sensitized women / Reducing RAADP in non-sensitized women

Questions

1. What is the current knowledge of fetal *RHD* genotyping on maternal blood, using cell-free fetal DNA?
2. Is it useful to implement fetal *RHD* genotyping on maternal blood in routine clinical practice?

SEARCH TERMS

1) *MeSH Database (PubMed): MeSH term: "(("Rh-Hr Blood-Group System"[Mesh]) AND "Genotype"[Mesh]) AND "Blood"[Mesh]; (("Fetus"[Mesh]) AND "Genotype"[Mesh]) AND "Rh-Hr Blood-Group System"[Mesh] "*
2) *PubMed Clinical Queries (from 1966; http://www.ncbi.nlm.nih.gov/entrez/query.fcgi): Systematic Reviews; Clinical Queries using Research Methodology Filters (diagnosis + specific, diagnosis + sensitive, prognosis + specific)*
3) *Pubmed (Medline; from 1966), SUMSearch (http://sumsearch.uthscsa.edu/), National Guideline Clearinghouse (http://www.ngc.org/), Institute for Clinical Systems Improvement (http://www.icsi.org), The National Institute for Clinical Excellence (http://www.nice.org.uk/), Cochrane (http://www.update-software.com/cochrane, Health Technology Assessment Database (http://www.york.ac.uk/inst/crd/htahp.htm)*
4) *UpToDate Online version 19.3 (2012)*

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APPRAISAL

1) What is the current knowledge on fetal RHD genotyping on maternal blood, using cell-free fetal DNA?

a) Genetic base of Rh phenotypes

Rh phenotypes are controlled by *RHD* and *RHCE*, two homologous genes located on chromosome 1, which have ten exons each and share about 95% identity (13,15,29,30). The RhD-positive phenotype is seen in the majority of the population and is caused by the homozygous or hemizygous presence of the *RHD* gene (13,27,30). The RhD-negative phenotype is quite prevalent in Caucasians (15-17%), moderately prevalent in Africans (3-5%) and rare in Asian populations (<1%) (26,27).

In Caucasians, the RhD-negative phenotype almost always results from homozygosity for a complete deletion of *RHD* (13,15,26,27,30). RhD antigen negative, *RHD* gene positive haplotypes are rare (0.2-1% of all D negative Caucasians) and known to preferentially occur in the Cde and cdE haplotypes. The molecular base is mostly the presence of hybrid *RHD-CE-D* genes with an intact exon 10 (26).

In Africans and Asians, the most common cause of an RhD-negative phenotype is the presence of an inactive *RHD* gene (13,26). 67% of the RhD negative Africans carry the silent *RHD* pseudogene, *RHDψ*, and 15% carry an inactive hybrid *RHD-CE-D*^{S} gene (26,30). Only 18% are homozygous for a *RHD* deletion (30).
RHDψ contains all exons, but is characterized by two inactivating mutations: a 37 bp duplication in exon 4 and a nonsense mutation in exon 6 (13,30). There are also four characteristic single nucleotide changes in exons 4 and 5 (13). Due to these mutations, no RhD antigens are expressed.
The *RHD-CE-D*^{S} hybrid gene contains exons 1, 2, part of 3, 9 and 10 from *RHD* but part of exon 3 and exons 4 to 8 from *RHCE* (30). This hybrid gene doesn't express RhD epitopes either (30).

Next to *RHDψ* and *RHD-CE-D*^{S}, many other rare variant *RHD* haplotypes exist (13,30). These *RHD* variants result either from single nucleotide polymorphisms (SNPs) in *RHD*, encoding amino acid substitutions, or from hybrid RH genes (30). Several of these variants may produce anti-D alloantibodies after immunization by normal RhD-positive cells (26,30).

b) Cell-free fetal DNA (cffDNA)

The past years, molecular biology has proven to be a powerful tool for the prenatal investigation of genetic disorders. However, most methods rely on the use of fetal material which has been obtained through invasive procedures, such as amniocentesis or chorionic villus sampling (19,21,30,34). These procedures are associated with a certain risk (0.5-1%) of fetal loss and a significant risk (17%) of fetomaternal bleeding which could boost the maternal immune response to fetal red blood cell antigens, with possible worsening of HDFN (12,19,21,30,34).

It has been well established that during pregnancy, a small number of fetal nucleated cells passes from the fetus into the maternal circulation (19). This phenomenon makes use of these cells for NIPD possible (37). However, there are two major problems with this strategy, reducing its accuracy. Fetal nucleated cells are only present in maternal blood in extremely low concentrations (19). The average number of fetal cells in maternal blood during the second trimester of a normal pregnancy is 1.2 cells/ml (37). Therefore, this requires very sensitive methods of detection or use of fetal cell isolation procedures (19). Since most techniques are time-consuming and labor-intensive, they are difficult to implement on a large scale (19,37).
The second problem is that fetal cells may persist post partum for many years, sometimes as long as 27 years after delivery (14,17-19,36).

In 1997, Lo et al. started to focus on the acellular fraction of maternal blood, inspired by workers in the field of oncology who discovered the presence of tumor DNA in plasma and

serum of cancer patients (19,38). They demonstrated the presence of cell-free fetal DNA (cffDNA) in plasma and serum of pregnant women by demonstrating the presence of fetus-derived Y sequences (38). Physiological and clinical information suggest that the majority of circulating fetal nucleic acids are derived from the placenta, most probably from apoptotic syncytiotrophoblast (12,17,18).

One year later, the same group demonstrated that the amount of circulating fetal DNA is considerably higher than the amount of fetal nucleated cells in maternal blood. Fetal DNA comprises 3.4% of total plasma DNA in early pregnancy, rising to 6.2% of total plasma DNA in late pregnancy. Fetal cells only comprise 0.0035% in early pregnancy and 0.008% in late pregnancy (37). Cell-free fetal DNA can be detected as early as the 5th week of gestation and in contrast to fetal cells, cffDNA is cleared from the maternal circulation within hours after child-birth (14,17-19,36,37).

The same year, Lo et al. also developed a real-time Taqman PCR assay for determination of fetal *RHD* genotype on maternal plasma (36).

c) Fetal *RHD* genotyping on maternal blood using cffDNA

1. Analytical performance

1.1. Pre-analytical considerations

1.1.1 Patient variables

There is no consensus on the optimal gestational age for blood sampling. There is also a difference in approach of sensitized and non-sensitized pregnancies. As stated above, fetal DNA can already be detected at the 5th week of gestation and increases with gestational age (attachment 1). In sensitized women, early diagnosis is desirable in order to plan further diagnostic procedures and therapy. However, if sampling takes place too early in pregnancy, a false negative or inconclusive result is possible due to low levels of cffDNA. The leaflets of the commercially available kits advise a minimal gestational age of 12 weeks with, in case of a negative result, confirmation with a second sample collected at least two weeks later (42,43).

In non-sensitized women, the result of the fetal *RHD* genotyping test will be decisive on whether or not administering RAADP. Therefore, sampling can be delayed until later in pregnancy. Since RAADP is administered at week 28, the sample may be taken one week earlier, as it's done in the Netherlands (2)

1.1.2 Sample requirements

When comparing plasma and serum samples, Lo et al. discovered that the absolute concentration of fetal DNA in maternal plasma is similar to that in maternal serum, but the main difference is the presence of a larger quantity of background maternal DNA in serum as compared to plasma, possibly caused by liberation of DNA during the clotting process (attachment 2) (14,37). Most studies are performed with EDTA plasma but citrate plasma may also be used (13,20,21,23,25,26,29,34,35,38,42). As it is generally known, heparin samples are not useful given the inhibitory effect on the PCR assay.

Some centers require a paternal sample as well to determine specific markers which can be used as internal controls (21).

1.1.3 Sample stability and processing

Samples should be processed within 48 hours of venipuncture to reduce the amount of maternal DNA in the plasma resulting from breakdown of maternal white cells (13,21,26,29,34,35). Samples must be centrifuged, followed by careful removal of the plasma. The plasma may be stored at -20°C or lower pending further processing (20,21,26,29,34,35). The buffy coat can also be removed and frozen for determination of silent maternal *RHD* variants (26,29,34).

1.2. Analytical considerations

1.2.1 (Lack of) standardization

As stated above, cffDNA extracted from maternal plasma is now recognized as a potential source for prenatal diagnosis, but the methodology is currently not well standardized (29,31). There are considerable differences in the used techniques for DNA extraction, primer choice, PCR conditions and detection of amplified products, resulting in considerable variations in diagnostic performance between different laboratories (29).

The SAFE network (Special Advances in Fetal and Neonatal Evaluation, funded by the European Community network of excellence), proposes the QIAamp DSP Virus Kit (Qiagen, Hilden, Germany) for cffDNA extraction, since this kit has proven to have the highest yield of cffDNA (14,17,31).

There is a commercial kit available, offered by L'Institut de Biotechnologies Jacques Boy and BioRad/DiaMed, namely the Free DNA Fetal Kit® RhD (42,43). Rouillac-Le Sciellour et al. compared the commercial kit with their in-house developed PCR on 300 RhD negative women and found 100% concordance (29).

1.2.2 Size fractionation

Fetal DNA fragments are smaller than maternal DNA, so size-fractionation is a possible approach to reduce background maternal DNA (14). Most of circulating fetal DNA molecules are 145-201 bp long whereas most maternal DNA is over 300 bp long (17). This might be used to enrich fetal DNA (17,25).

1.2.3 Primer choice

Primers should be chosen so that only *RHD* and not *RHCE* is amplified (30). Based on the knowledge that the majority of RhD negative phenotypes is caused by a deletion of the *RHD* gene, first generation genotyping tests were based on a single *RHD* gene region amplification, most often exon 10 (13,17,26,34,36). The discovery of variant *RHD* genes has hampered the test interpretation. Nonfunctional *RHD* alleles such as *RHDψ* and *RHD-CE-D*^{S} are serologically typed as RhD negative. However, since they contain an intact *RHD* exon 10, this inactive maternal RHD gene will be amplified, leading to false positive results (13,26). Two or more 'diagnostic sites', with at least inclusion of an *RHD*-specific PCR that is negative on the *RHD* pseudogene, should be tested to limit the rate of false positives (17,26,30). This often requires multiplex PCR assays in which several regions of the *RHD* gene are amplified (17).

Several combinations have been proposed by the different groups. Details can be found in attachment 3.

1.2.4 Test principle

Real-Time quantitative PCR using Taqman chemistry is a possible diagnostic approach because of its ease of use and ability to automate and thus avoid contamination (15,34). Taqman RT PCR relies upon PCR primers, to define the specificity of the reaction, and a probe with reporter and quencher dyes attached (13). If the target DNA sequence is present, the increase in PCR product formation is monitored by the increase in probe reporter dye fluorescence throughout each cycle and converted by the software into an amplification plot (increase in reporter dye fluorescence vs. PCR cycle) (13,20). The cycle at which the reporter dye reaches a threshold level of fluorescence (Ct) is dependent on the starting amount of target DNA present (13). The more target DNA is present in the sample at the start of the PCR, the lower the Ct value (13,26).

MALDI-TOF MS has recently emerged as a new platform for highly sensitive and accurate analysis of DNA, especially cell-free DNA (cf-DNA) (33). This

technique combines flexibility, accuracy, automated analysis and high-throughput data generation (15,22,25,28). Sequenom Inc. (San Diego, CA) developed a MassARRAY genotyping platform combining PCR, base extension reaction (SABER) and MALDI-TOF MS and offering all necessary software for efficient and accurate analysis (25,28,40). After initial DNA amplification by PCR, an additional linear amplification step called single allele base extension reaction is performed with an extension primer designed to anneal to the region immediately upstream of the mutation site (25). SABER has been shown to improve the detection of fetal-derived subtle mutations (25).
The MassARRAY platform can be used for SNP genotyping, methylation detection and quantitative gene expression analysis. Sequenom also manufactures clinical tests, such as the SensiGene™ Fetal RHD genotyping test. They are currently conducting a clinical trial for the evaluation of the performance of a noninvasive first trimester fetal *RHD* genotyping test, using MALDI-TOF mass spectrometry (40). Estimated study completion date is March 2012 (40).

1.2.5 Internal controls

One issue that has been repeatedly discussed is the need for an internal control to demonstrate the presence of circulating cell-free fetal DNA in the maternal plasma sample when PCR results are negative (10,16,34). Up until now, there is no such universal control available (10,29). False-negative PCR results caused by a low level of fetal DNA at time of blood sampling is especially a problem in early pregnancy (< 13 weeks) (29).
An overview of possible internal controls for the presence of cffDNA is given in attachment 4, with the SRY gene, epigenetic markers such as RASSF1A methylation and SNP detection by Maldi-TOF MS being the most promising.

1.2.6 Number of replicates and test interpretation

A recurring item in all studies is the poor repeatability of this test. Minon et al. found that, of 360 women with an RhD-positive fetus, 77 (21.4%) had at least one negative replicate (26). This emphasizes the importance of performing several replicates from each maternal sample and of testing several *RHD*-specific sequences to increase the probability of fetal DNA detection (26).
The number of replicates, analyzed by the different study groups, varied from 1 to 4 replicates per exon and 2 to 12 replicates per sample (13,17,21,23,26,34,35). Each group developed different interpretation schemes based on the type and number of positive replicates (13,23,26,29,34,35). However, this approach is labor intensive and expensive and thus not suitable for mass screening (23,27).
Considering the low total amount of fetal DNA present in the maternal plasma, Ct values for positive fetal *RHD* are high (range of 32-40) (29). Low Ct values, < 30 cycles, indicate a very high amount of DNA and thus suggest the presence of a silent variant *RHD* gene in the maternal genome (29). This can be confirmed by *RHD* genotyping of genomic DNA extracted from maternal leukocytes, isolated from the buffy coat (22,29).

2. Diagnostic performance

2.1. False positive results

False-positive results have been attributed to the presence of afunctional/dysfunctional maternal or fetal genes, resulting in a serologically RhD-negative status or, in one case, to a mother who previously received an organ transplantation from an RhD-positive donor (11,15,17,26,34).
In case of false-positive results, the mother will receive unnecessary anti-D immunoglobulin which is the 'preferred' mistake since now, 40% of RhD-negative mothers receive unnecessary prophylaxis (11).

2.2. False negative results

False-negative results were mainly caused by an insufficient amount of fetal DNA in the maternal sample, often due to early gestation (<13W) (10,11,15,17,34). This emphasizes the need for a universal internal control for the presence of fetal DNA. Other potential causes of false-negative results are suboptimal test sensitivity or genetic mutations in the *RHD* gene, localized within the PCR primer or probe binding sites (10,11,15,17).

2.3. Sensitivity, Specificity, PPV, NPV, accuracy

Attachment 5 shows a detailed overview of diagnostic performance characteristics found by 24 different study groups. Data were partially adopted from the table published in the meta-analysis by Geifman-Holtzman (11). The mean reported diagnostic accuracy was 97.9% (range 80.8%-100%). Lowest diagnostic accuracy was reported in two small studies from 2003, performed on 28 and 31 patients respectively. Sensitivity and specificity values ranged from 82.4%-100% and 66.7%-100% respectively, with positive and negative predictive values ranging from 89.5%-100% and 57.1%-100% respectively.

Several of these studies were carried out on a small patient population. The total number of included patients ranged from 20 to 1869.

Geifman-Holtzman et al. concluded from their analysis that best diagnostic accuracy was reached when obtaining the sample during the first trimester (11). However, only 1 out of 3 published studies reported gestational age (11). Opposite results were reported by Akolekar et al., who evaluated 591 samples from first trimester pregnancies (23). They noticed a significantly higher false negative rate at 11-13 weeks (3.5%) than the rate of < 0.2% at 26-32 weeks, reported by Finning et al. (23,27). This is most likely due to the failure to detect cffDNA in early pregnancy (23). A potential solution is a repeat test at 26 weeks to reassess the need for prophylactic anti-D (23). This implies the additional cost of an extra test, but women with a false negative result will not be deprived of receiving prophylactic anti-D (23).

In 2009, Freeman et al. performed a systematic review of the quality of reporting of diagnostic accuracy in published studies, an item which was not considered by Geifman-Holtzman et al. (10). 27 studies were assessed using the STARD (standards for reporting studies of diagnostic accuracy) checklist (attachment 6) (10). Scores ranged from 5/25 to 13/25 points, illustrating a generally poor quality of reporting (attachment 7) (10). There is a widespread exclusion of inconclusive and unobtainable results due to insufficient fetal material or maternal variant genes. Both will have implications for population wide implementation (10). In clinical practice, all such samples excluded from analysis would represent individuals for whom the test cannot produce a result (10). Inconclusive/unobtainable results should be managed as *RHD* positive since statistically, the majority will indeed be *RHD* positive (23).

The systematic exclusion of inconclusive or unobtainable results continues to exist in recent publications. In 2011, Bombard et al. reported a diagnostic accuracy of 97.1%, decreasing to 85.2% if the 29 excluded samples (12% of all samples) were taken into account (22). Akolekar et al. found a diagnostic accuracy of 98.8% when performing the PCR on 591 samples obtained during the first trimester of pregnancy (11-13 weeks gestational age) (23). However, when inconclusive or unobtainable results were included, accuracy decreased to a meager 84.6%. As many as 15% of all performed PCRs produced inconclusive results, which the authors attributed to the relatively high proportion of African women (19.3%) (23).

Another issue is the lack of large-scale peer-reviewed studies reporting on high-throughput testing of non-sensitized women (10). The proposal for the large-scale implementation of this test is based on results from small-scale studies (10,21). Most data from studies have been obtained in a research setting rather than a clinical setting and lacked a control for the presence of fetal DNA in case of negative results. However, the cost of the test, as well as its reliability, will be determined by the number of retests (10). Finning et al. were the only group that published a report on the effect of high

throughput *RHD* typing (27). A total of 1869 patients was included, with the majority (92%) having a gestational age of 26-30 weeks (range 8-38W) (27). Accuracy was determined at 95.7% with a false negative rate of 0.16% (27). The authors concluded that conclusive negative results were obtained in 36% of the women tested, which, in England and Wales, would represent 35.000 to 40.000 mothers per year (attachment 8) (27,30).

Sanquin Diagnostic Services offers noninvasive fetal blood group genotyping for rhesus D in maternal plasma for alloimmunized pregnant women since the beginning of 2003, using a stringent diagnostic algorithm with the inclusion of fetal DNA identifiers to exclude false-negative results (21). Through the analysis of both exon 5 and 7, false positive results due to variant genes could be avoided.

Bombard et al. evaluated analysis of cffDNA using MALDI-TOF MS (22). They used two cohorts of patients: in the first cohort of 236 patients, they compared the result with serological RhD determination on cord blood; in the second cohort of 205 patients, they compared the result with PCR determined *RHD* genotype (22).
The reported diagnostic accuracy was 97.1% in cohort 1 and 99.5% in cohort 2 (22). But again, in this study, accuracy was calculated after exclusion of inconclusive or unobtainable results. After correction for this, diagnostic accuracy decreased to 85.2% in cohort 1 and 96.6% in cohort 2. Positive and negative predictive values were 98.6% and 94% respectively in cohort 1 and 99.3% and 100% respectively in cohort 2, demonstrating an excellent concordance between the two methods (22)

2) Is it useful to implement fetal RHD genotyping in routine clinical practice?

1. Clinical impact
   A. Diagnostic aspect

   The use of cffDNA avoids invasive procedures such as amniocentesis and chorionic villus sampling to determine fetal *RHD* status in anti-D sensitized women (13,24,36). If the fetus is *RHD* negative, it is not at risk for HDFN and no further antenatal monitoring or invasive procedure is required (12,23). In cases where the fetus is *RHD* positive, the woman should be referred to a tertiary fetal medicine unit for appropriate management. Serial assessment of maternal antibody titers and monitoring for fetal anemia are advisable (12,15,21,23).

   The lack of a universal internal control to demonstrate the presence of cffDNA in the maternal plasma is the main cause for concern. Negative results should be interpreted with caution since a small proportion will be labeled false negative. Therefore, it is suggested to analyze a repeat sample 2-4 weeks later in case of a negative result, especially when the sample was obtained at less than 12 weeks of gestation.

   Next to fetal *RHD* typing, the use of cffDNA has also opened new possibilities for testing other blood group antigens, such as Kell, RhC/c and RhE/e (12,21).

   B. Treatment

   NIPD for *RHD* has no real therapeutic impact, since the decision to treat for HDFN is based on clinical or echographic signs of fetal anemia or HDN. If significant anemia is detected, intra-uterine transfusion by cordocentesis will be performed using antigen negative blood (1,4,15). After birth, phototherapy or exchange transfusion may be necessary (1,4).

   C. Prevention

   Introduction of postnatal anti-D prophylaxis within 72h of delivery in the late 1960s reduced sensitization and HDFN rates considerably internationally (10,24,29). A further reduction was obtained by the introduction of RAADP in non-sensitized RhD negative women (10,24,27). However, 40% of RhD-negative women carry a RhD negative fetus, leading to unnecessary administration of anti-D prophylaxis (10,23,26,27,29). Fetal *RHD* genotyping on maternal plasma can reduce the number of unnecessary anti-D injections.

   Advantages of avoidance of unnecessary RAADP are listed below (8,11,24,26,27,30,34).

   - No unnecessary injections
   - Avoidance of exposure to blood products / ethical considerations:
     Anti-D Ig is a blood-derived product, obtained by immunization of RhD negative male volunteers. Although considered safe for routine antenatal use, administration of this biological substance is associated with a theoretical risk of transferring (unrecognized) viruses or prions. Scientists are currently working on recombinant anti-D Ig.
   - Allergic reactions to RAADP administration are described
   - Low supplies of anti-D immunoglobulin: Promotes efficient use of the limited pool of anti-D immunoglobulin.
   - Reduced costs

2. Organizational impact

   Mass testing will require an accurate high-throughput automated laboratory procedure with a very high test sensitivity (24). Up to date, there is still no universal control to confirm the presence of fetal DNA available. The current available methods have a low throughput and are labor intensive, so mass screening is not feasible unless development of highly automated procedures which require as little replicates as possible (24).

3. Cost impact
    A. Intellectual property rights

    The use of cell-free fetal nucleic acids from maternal plasma has been patent-protected by European patent EP 0994963B1 and the international US patent 6,258,540. The patents were granted in 2001 (US) and 2003 (UK) and are owned by the University of Oxford's ISIS Innovations Ltd, with Dennis Lo as one of the inventors (39). The patents include all detection methods in prenatal diagnosis, performed on maternal serum or plasma samples from a pregnant female, which comprise detection of the presence of a nucleic acid of fetal origin (39). In 2005, ISIS granted an exclusive license to the US-based company Sequenom Inc., giving it rights to control the technology claimed in both patents, with exception of the use of QF-PCR for RhD genotyping in Europe, which had previously been exclusively licensed to L'Institut de Biotechnologies Jacques Boy in France (39).

    In most European countries, there are rules that no liability arises if the patent infringement is done privately for purposes which are not commercial, or for experimental purposes (39). If the technology would be offered as a service, however, this exception would no longer apply and, in the absence of a permission or license, the service provider would be infringing the patent (39).

    B. Commercial situation

    The largest and most dominant company in this area is Sequenom Inc, based in San Diego. Studies with their Mass-ARRAY platform are ongoing.

    Other companies offering commercial tests for fetal RHD genotyping are L'Institut de Biotechnologies Jacques Boy (Free DNA Fetal Kit® RhD) and BioRad/DiaMed (Free DNA Fetal Kit® RhD, €5040 for 87 reactions) (39).

    One dose of Rhogam costs 42.16 euro. Scientists are currently working on the development of recombinant anti-D IgG as a replacement for human plasma products (24,27). A phase 2 clinical trial is in progress (24,27).

    C. Cost-effectiveness

    In most countries, *RHD* genotyping on maternal plasma is only offered in case of anti-D alloimmunization. Szczepura et al. performed an economic evaluation of mass testing for fetal *RHD* genotype on maternal blood in England and Wales (24). They evaluated the costs of an in-house test as well as a commercially available test (Jacques Boy) and evaluated the impact on national savings in case of introduction of large-scale testing (24). Use of the commercial kit would make NIPD more expensive as compared to RAADP (24). Use of the in-house developed kit would lead to a national saving from 0.46-1.6% as long as zero royalty fee applies (24). If a royalty fee is obliged, NIPD targeted antenatal prophylaxis would no longer be cost-effective (24). They conclude that their findings do not support large-scale introduction of *RHD* NIPD testing for non-sensitized pregnancies (24).

    However, in the Netherlands, an economic evaluation apparently showed cost-effectiveness of large-scale *RHD* genotyping on maternal plasma in non-immunized women, leading to the aforementioned national implementation (2,17).

    If a reliable recombinant alternative for human anti-D immunoglobulin is produced, NIPD may become financially more attractive since this recombinant product will probably be more expensive (24,27).

4. Decision making

    There is currently a refund provided by the Belgian health care system for fetal *RHD* genotyping in a pregnant woman, in case of anti-D alloimmunization or prior to an invasive procedure (B5000) (44). The test is offered by the Centre for Human Genetics (CME) of the University Hospitals of Leuven, who perform an in-house developed real-time PCR using exon 10.

    With regard to large-scale implementation in Belgium for non-sensitized women, there are several remarks to be made. Most studies were set in a research setting rather than a clinical-diagnostic setting, with exclusion of inconclusive or unobtainable results. However, these results should be considered as *RHD* positive, thus anti-D prophylaxis should be administered. This should be taken into account in cost-effectiveness studies.

    Furthermore, due to the lack of an internal control for fetal DNA, analysis of several replicates is necessary to improve the accuracy and sensitivity of the test. Current diagnostic

strategies are expensive, time-consuming and labor-intensive and do not permit high-throughput testing. As long as the presence of fetal DNA cannot be proved, negative results should be interpreted with caution since they may represent false negative results due to insufficient fetal DNA. Finally, to this date, there are insufficient data of the cost-effectiveness of large-scale implementation of this test. Therefore, at this time, there will be no implementation of large-scale testing in the laboratories of the Belgian Red Cross. However, new developments, more specifically the clinical trial with MALDI-TOF MS, as well as the results of the pilot study in the Netherlands, should be followed.

COMMENTS

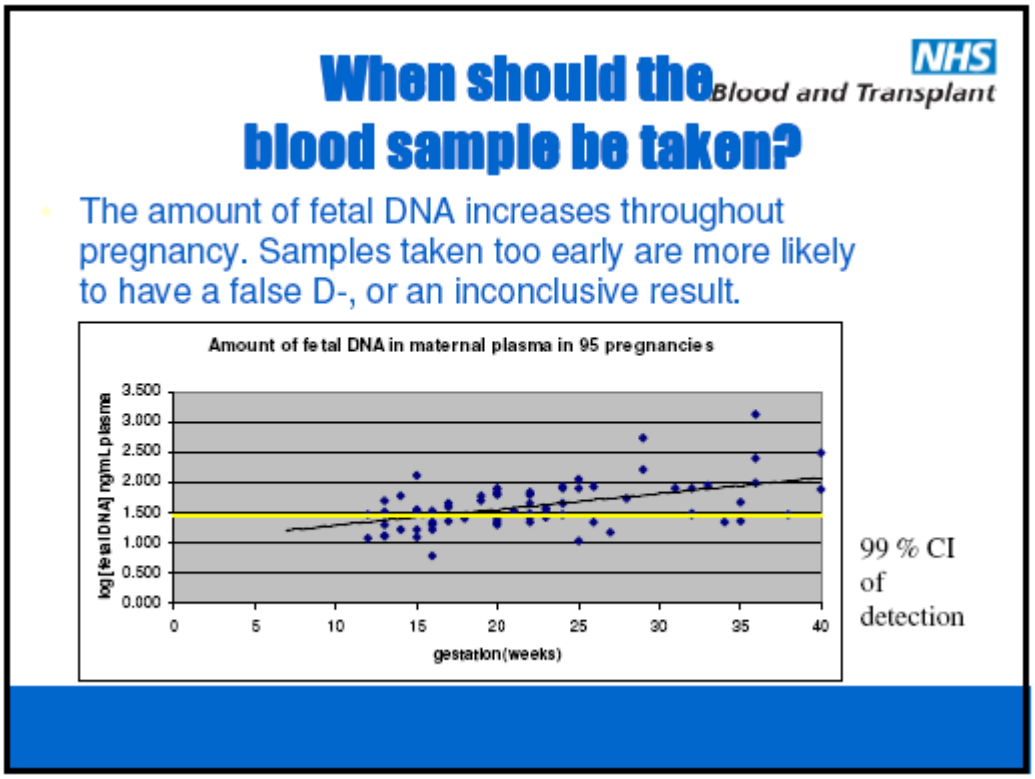
Fetal *RHD* genotyping on maternal plasma using cffDNA is currently offered by the Centre for Human Genetics (CME) of the University Hospitals of Leuven. In Leuven, only about 10-15 analyses/year are requested.

TO DO/ACTIONS

1) Follow-up of results of pilot study in the Netherlands
2) Follow-up of results of clinical trials with MALDI-TOF MS

ATTACHMENTS

Attachment 1 (41)



Attachment 2: Evaluation of the total amount (maternal + fetal) of extracted DNA from plasma en serum samples from 50 pregnant women, using the β-globin TaqMan Assay (37).

Lo et al.: Quantitative Analysis of Fetal DNA

Table 1

Quantitative Analysis of Maternal Plasma and Serum by the β-globin TaqMan Assay

	Mean (copies/ml)	Median (copies/ml)	Range (copies/ml)
Plasma (early + late pregnancy)	3,466	1,594	356–31,875
Serum (early + late pregnancy)	50,651	34,688	5,813–243,750
Plasma (early pregnancy)	986	975	356–1,856
Plasma (late pregnancy)	5,945	4,313	1,125–31,875

Attachment 3: Choice of primers by different groups

Group	Targeted exons	Detailed information	References
International Blood Group Reference Laboratory (IBGRL), Bristol, UK	4, 5, 10	Primers of exons 4 and 5 are designed to amplify only the *RHD* gene, not *RHDψ* and *RHD-CE-D^s*	13,30,35
	5, 7	Evaluated for large-scale testing purposes. Exon 5 amplifies *RHD* only, exon 7 amplifies *RHD* and the inactive *RHDψ* gene	23,27
Minon et al., Liege, Belgium	4, 5, 10	Primers of exons 4 and 5 are designed to amplify only the *RHD* gene, not *RHDψ* and *RHD-CE-D^s*	26
Rouillac-Le Sciellour et al., France	5, 7, 10	Initially only two exons (7 and 10) but false positive results due to *RHDψ*; Added an additional *RHD* exon 5 PCR which amplifies a 82 bp fragment of *RHD* but not *RHDψ*	29,34
SAFE network (The Special Non-Invasive Advances in Fetal and Neonatal Evaluation Network of Excellence)	5, 7	No product of *RHD* exon 5 is generated when a nonfunctional *RHD* pseudogene or a *RHD-CE-D^s* gene is present	15
Sanquin Diagnostic Services, The Netherlands	5, 7	No product of *RHD* exon 5 is generated when a nonfunctional *RHD* pseudogene or a *RHD-CE-D^s* gene is present	21
Bombard et al., USA	4, 5, 7	Added an additional PCR for a 37-base pair insertion found in exon 4 which is indicative of the presence of *RHDψ*	22

Attachment 4: Overview of internal controls for the presence of cffDNA

Control	Detailed information	Function/technique	Remarks	Reference
CCR5	Ubiquitous gene, which is amplified from maternal and fetal DNA.	- Confirmation of successful DNA extraction - Quantification of total plasma DNA - Control for the quantity of maternal DNA		11,13,30,35
SRY	Y chromosome sequences		Only applicable in case of a male fetus	13,16,19,23, 26,29,30,34, 35
biallelic insertion/deletion polymorphisms	Allow the detection of fetal-derived, paternally-inherited polymorphisms.	1. Testing of maternal buffy coat for polymorphism 2. Maternal plasma is then tested for those polymorphisms that were absent from the maternal genome	- Increased complexity of the diagnostic assay - Time-consuming - Labor-intensive - Not informative in 4% of cases	11,16,21,23, 35
RASSF1A hypermethylation	RASSF1A is a promoter region of the tumor suppressor gene Ras association domain family 1A gene. RASSF1A gene is hypermethylated in placental cells and hypomethylated in maternal blood cells.	Methylation analysis is performed using methylation-sensitive endonucleases, thus cutting hypomethylated RASSF1A background sequences from maternal origin, while leaving hypermethylated placental RASSF1A sequences intact for amplification.	- Aberrant RASSF1A methylation in malignancies → not useful if history of cancer - Methylation-specific PCR assays are difficult to design and time-consuming to optimize - Mass spectrometry-based methods for methylation analysis have been described.	16,21,26,29, 30,32
Other blood group antigen	RT PCR for other paternally derived blood group antigens (C/c; E/e, K)	Implies serological testing of both parents for D, C/c, E/e and K/k to identify paternal blood group antigens that could serve as a genetic control marker to confirm the presence of fetal DNA		21
Single Nucleotide Polymorphism (SNP)	Detection of fetal alleles of SNPs in maternal plasma to detect difference between the minority fetal and the majority maternal DNA molecules	Uses special primer extension method (SABER, single allele base extension reaction) followed by analysis by Maldi-TOF MS.	- Amenable for multiplexing - Expensive mass spectrometry equipment	16

Attachment 5: diagnostic performance reported by several published studies on RhD genotyping from maternal plasma (partially adopted from (20))

Table: published studies on RhD genotyping from fetal plasma in maternal plasma (partially adapted from (20))

References	Gestation (weeks)	Methods	RhD	No. Tested	No. Included	# correct	Accuracy	Total accuracy	Sensitivity	Specificity	PPV	NPV
Lo et al, 1998	7-41	Real-time PCR	exon 10	57	57	55	96,5%	96,5%	94,9%	100,0%	100,0%	90,0%
Faas et al, 1998	16-17	PCR	exon 7	31	31	31	100,0%	100,0%	100,0%	100,0%	100,0%	100,0%
Zhang et al, 2000	All trimesters	Real-time PCR	exon 7	58	58	57	98,3%	98,3%	96,9%	100,0%	100,0%	96,3%
Nelson et al, 2001	9-34	PCR	exon 10	26	26	26	100,0%	100,0%	100,0%	100,0%	100,0%	100,0%
Finning et al, 2002	8-42	Real-time PCR	exons 4,5,6,10	158	137	137	100,0%	86,7%	100,0%	100,0%	100,0%	100,0%
Costa et al, 2002	8-14	Real-time PCR	exon 10	102	102	102	100,0%	100,0%	100,0%	100,0%	100,0%	100,0%
Legler et al, 2002	11-38	Real-time PCR	exons 4,7	28	27	26	96,3%	92,9%	93,0%	100,0%	100,0%	92,3%
Turner et al, 2003	6-20	Real-time PCR	exon 10	31	31	28	90,3%	90,3%	82,4%	100,0%	100,0%	82,4%
Siva et al, 2003	15-17	PCR	exons 7,10	28	26	21	80,8%	75,0%	85,0%	66,7%	89,5%	57,1%
Rijnders et al, 2004	11-19	Real-time PCR	exon 7	72	71	71	98,6%	98,6%	100,0%	96,6%		
Rouillac et al, 2004	7-36	Real-time PCR	exons 7,10	893	851	842	98,9%	94,3%	99,4%	97,5%	99,2%	98,0%
Finning et al, 2004	All trimesters	Real-time PCR	exons 4,5,10	233	226	223	98,7%	95,7%	100,0%	96,2%	98,0%	100,0%
Clausen et al, 2005	15-16	Real-time PCR	exons 7,10	59	59	58	98,3%	98,3%	100,0%	100,0%	100,0%	100,0%
Zhou et al, 2005	14-42	Real-time PCR	exons 4,5,10	98	96	92	95,8%	93,9%	94,4%	100,0%	100,0%	85,7%
Gautier et al, 2005	8-35	Real-time PCR	exon 10	274	272	272	100,0%	99,3%	100,0%	100,0%	100,0%	100,0%
Gonzalez et al, 2005	11-16	Real-time PCR	exon 7	20	20	20	100,0%	100,0%	100,0%	100,0%	100,0%	100,0%
Brojer et al, 2005	All trimesters	Real-time PCR	exons 7,10; intron 4	255	230	229	99,6%	89,8%	100,0%	100,0%	100,0%	100,0%
Rouillac et al, 2007	10-34	Real-time PCR		300	300	298	99,3%	99,3%	100,0%	97,5%	99,1%	100,0%
Minon et al, 2008	10-38	Real-time PCR	exons 4,5,10	563	545	544	99,8%	96,6%	100,0%	99,5%	99,7%	100,0%
Finning et al, 2008	8-38 (92% between 26-30 W)	Real-time PCR	exons 5,7	1869	1869	1788	95,7%	95,7%	96,7%	94,0%	98,8%	99,5%
Bombard et al, 2011	11-13	Maldi-TOF MS	exons 4,5,7	236	207	201	97,1%	85,2%	97,2%	96,9%	98,6%	94,0%
Bombard et al, 2011	6-30	Maldi-TOF MS	exons 4,5,7	205	199	198	99,5%	96,6%	100,0%	98,3%	99,3%	100,0%
Akolekar et al, 2011	11-14	Real-time PCR	exons 5,7	591	502	496	98,8%	83,9%	98,2%	100,0%	100,0%	96,5%
Scheffer et al, 2011	7-38	Real-time PCR	exons 5,7	140*	133	133	100,0%	95,0%	100,0%	100,0%	100,0%	100,0%
OVERALL				6349	6075	5948	97,9%	93,7%				

*: total number in which serology was available + results inconclusive

Discordance reported accuracy - total accuracy

Attachment 6: STARD checklist (10)

Table 1
Coverage of the STARD checklist items by 27 papers reporting the diagnostic accuracy of RhD NIPD.

Section and topic	No.	Checklist items	Coverage[a] (%)
Title/abstract/ keywords	1	Identify the article as a study on diagnostic accuracy (recommend MeSH heading 'sensitivity and specificity').	61[b]
Introduction	2	State the research questions or study aims, such as estimating diagnostic accuracy or comparing accuracy between tests or across participant groups.	92[c]
Methods			
Participants	3	Describe the study population: the inclusion and exclusion criteria, setting and locations where the data were collected.	32
	4	Describe participant recruitment: was recruitment based on presenting symptoms, results from previous tests, or the fact that the participants had received the index tests or the reference standard?	78
	5	Describe participant sampling: was the study population a consecutive series of participants defined by selection criteria in items 3 and 4? If not, specify how participants were further selected.	12
	6	Describe data collection: was data collection planned before the index test and reference standard were performed (prospective study) or after (retrospective study)?	34
Test method	7	Describe the reference standard and its rationale.	54
	8	Describe technical specification of material and methods involved including how and when measurements were taken, and/or cite references for index tests and reference standard.	98
	9	Describe definition and rationale for the units, cutoffs and/or categories of the results of the index test(s) and the reference standard.	0
	10	Describe the number, training and expertise of the persons executing and reading the index tests and the reference standard.	4
	11	Describe whether or not the readers of the index tests and reference standard were blind (masked) to the results of the other test and describe any other clinical information available to the readers.	28
Statistical methods	12	Describe methods for calculating or comparing measures of diagnostic accuracy, and the statistical methods used to quantify uncertainty (e.g. 95% confidence intervals).	8
	13	Describe methods for calculating test reproducibility, if done.	0
Results			
Participants	14	Report when study was done, including beginning and ending dates of recruitment.	8
	15	Report clinical and demographic characteristics of the study population (e.g. age, sex, spectrum of presenting symptoms, comorbidity, current treatments and recruitment centers).	68
	16	Report the number of participants satisfying the criteria for inclusion that did or did not undergo the index tests and/or the reference standard; describe why participants failed to receive either test (a flow diagram is strongly recommended).	16
Test results	17	Report time interval from the index tests to the reference standard, and any treatment administered between.	48
	18	Report distribution of severity of disease (define criteria) in those with the target condition; other diagnoses in participants without the target condition.	0
	19	Report a cross tabulation of the results of the index tests (including indeterminate and missing results) by the results of the reference standard; for continuous results, the distribution of the test results by the results of the reference standard.	88
	20	Report any adverse events of index tests and reference standard.	4
	21	Report estimates of diagnostic accuracy and measures of statistical certainty (e.g. 95% confidence intervals).	12
	22	Report how indeterminate results, missing responses and outliers of index tests were handled.	62
	23	Report estimates of variability of diagnostic accuracy between subgroups of participants, readers or centers, if done.	0
	24	Report estimates of test reproducibility, if done.	0
Discussion			
Discussion	25	Discuss the clinical applicability of the study findings.	98

[a] Coverage is a measure of how well the studies have adhered to items of STARD checklist (coverage = ∑(scores)/total papers), where studies covering checklist item well score 1 point, partially 1/2 point and no coverage 0 points).
[b] Phrases used to identify studies as diagnostic papers included: accuracy/detection rate, sensitivity and specificity, evaluation of test reliability, efficiency and feasibility.
[c] Studies scored as long as they mentioned: evaluation or determination of test accuracy, evaluation of test reliability, efficiency and feasibility of test.

Attachment 7: weaknesses affecting the generalisability of reported accuracy rates of RhD NIPD in 27 studies (10).

Table 2
Weaknesses affecting the generalisability of reported accuracy rates of RhD NIPD in 27 studies.

Study	Study population		Study conduct		Data analysis		Study design		Refs.
	Selective participant sampling	Lack of reporting ethnicity and/or sensitisation status of participants	Lack of reporting no. of replicates (if done) used for overall study outcome	Lack of reporting failure rate	Lack of inclusion of reported failure rate into analysis	Difference in reported and adjusted accuracy	Lack of control for presence of fetal DNA	Lack of known genotypes in study as control	
1					✓	✓	✓		[13]
2		✓			✓	✓		✓	[14]
3		✓			✓	✓	✓	✓	[35]
4	✓				✓	✓	✓		[15]
5	✓	✓		✓	✓		✓	✓	[26]
6					✓	✓	✓	✓	[30]
7		✓		✓	✓		✓	✓	[33]
8				✓	✓	✓	✓	✓	[28]
9							SRY only		[32]
10		✓						✓	[31]
11		✓			✓	✓		✓	[16]
12	✓	✓			✓	✓	✓		[36]
13		✓						✓	[25]
14		✓		✓	✓		✓	✓	[34]
15		✓	✓				✓		[4]
16		✓					✓		[39]
17	✓	✓	✓		✓	✓	✓		[5]
18	✓	✓	✓	✓	✓		SRY only	✓	[9]
19		✓					✓	✓	[6]
20		✓					✓		[37]
21	✓	✓					✓	✓	[23]
22		✓		✓	✓	✓	✓	✓	[7]
23		✓			✓	✓	✓		[29]
24	✓	✓		✓	✓		✓	✓	[8]
25		✓	✓	✓	✓		✓		[38]
26	✓	✓		✓	✓		✓	✓	[24]
27		✓		✓	✓	✓	✓	✓	[2]

Attachment 8: estimation of impact of large-scale screening in England and Wales (41)