A Review of Evidence on Non-invasive Prenatal Diagnosis (NIPD): Tests for Fetal *RHD* Genotype

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CONTENTS

EXECUTIVE SUMMARY

This literature review report is part of the early HTA (Health Technology Assessment) study of NIPD (Non-invasive prenatal diagnostics) RhD technology which, as of 2005, is beginning to diffuse into practice in a number of European countries and the United States. The report is written based on the application of the initial SAFE HTA framework (see www.safenoe.org), developed through a world-wide Delphi process.

The Report

This report concentrates on three main areas.

First and foremost, we set the **background context** for RhD NIPD in prenatal care. While the methodology chapter describes how the literature review was carried out and how additional information was collected, the second chapter provides an overview of the key issues associated with pregnancy of RhD negative women. We present background information based on publications from 1997 to 2006 which describe the genetic condition and its prevalence (RhD negativity) in populations, as well as the frequency of cases of sensitisation and HDN (haemolytic disease of the newborn). We also discuss current service provision for RhD negative women in a number of European countries and look at how the NIPD test might be set within current service contexts.

For general issues relating to the clinical effectiveness and cost-effectiveness of routine antenatal anti-D prophylaxis (RAADP) for pregnant women who are rhesus-negative the reader is referred to an existing review published in 2003 as an HTA report and used to develop NICE guidelines in the UK [1]. This presents a clear description of the use of anti-D prophylaxis (both postnatal and antenatal) in the management of RhD negative pregnancies and prevention of haemolytic disease of the newborn. Figures on prevalence of HDN, incidence of sensitisation etc, are available in this report for the UK. There is no similar published literature for other countries, and this is therefore the reason for an inevitable focus on the UK in some parts of this report, although this data is being sought by the SAFE socio-economic team.

Finally, the background section includes evidence on technologies under development that may be relevant to decisions on the introduction of NIPD RhD testing, either for sensitised women or in the context of RAADP. Recombinant Anti-D prophylaxis, peptide immunotherapy and preimplantation genetic diagnosis are discussed.

Secondly, this report concentrates on the available evidence on the **technical performance and clinical validity of NIPD RhD tests**. The third chapter provides a description of the studies to date which have evaluated the technical performance of RhD NIPD tests, e.g. techniques and material used, testing conditions examined, genotype sensitivity/specificity, reference samples and time of testing. Weaknesses in study design are pointed out which may influence the validity of test performance results. We also highlight various discoveries in the field of non-invasive prenatal diagnostics, mainly in connection with the molecular bases of the RhD gene and its variants, and we report on the first moves

towards test standardisation and external quality control (developed by SAFE partners). In the fourth chapter, we discuss the available information on clinical validity of the NIPD RhD technology and look at evidence from published scientific papers on how the new test might perform once implemented, paying particular attention to the application in multi-ethnic populations.

Finally, the last three chapters look at the **broader picture** and discuss the limited literature available on other issues associated with the implementation of the RhD NIPD technology. Chapter six describes the necessary arrangements for the implementation of the test, assesses evidence on risks and limitations, and reports on possible unintended consequences. Chapter seven briefly describes ongoing (unpublished) research in the field, and in chapter eight we highlight gaps in the published literature and current knowledge. The aim of the last two chapters is to set the direction for future research, as well as focus the attention of policymakers, healthcare providers and the public on the issues that should be considered when make a decision about NIPD RhD introduction.

Main Findings:

Size of the problem

Even though postnatal (postpartum) anti-D prophylaxis has considerably reduced sensitisation, HDN due to RhD incompatibility can still result in prenatal/neonatal death. About 10% of all pregnancies in Europe involve an RhD negative mother and an RhD positive fetus, and at least 30 deaths are associated with HDN every year in England and Wales alone (c.f. 4,482 perinatal deaths in total in 2001). However, this number does not include babies affected by other symptoms of HDN such as anaemia, hydrops fetalis, hyperbilirubinemia and jaundice, seizures, brain damage and deafness. Furthermore, routine prenatal anti-D prophylaxis is not uncontroversial and a reduction in its application could help address issues associated with perceived problems with safety and access to future supplies for this blood product.

Role of NIPD tests in patient management

RhD NIPD can assist in the management of already *sensitised* women. It also has the potential to reduce the application of *unnecessary RAADP* to RhD negative women whose pregnancy is not at risk (i.e. with RhD negative fetuses) and would, therefore, lead to an improvement in the current management of these pregnancies. It might also help to increase the use of antenatal prophylaxis in systems where RAADP is only sporadically administered by health care providers or not practised at all.

Implementation of routine NIPD RhD testing for RAADP

At present, successful universal introduction of this new technology is most likely to be hampered by the following issues:

First, no standardised methodology for NIPD *RHD* genotyping is currently available, although this is being addressed by the SAFE standardisation activity.

Second, current tests may not be sufficiently reliable for individuals with *RHD* gene variants, although, markers are being developed and tested by the SAFE Network of Excellence (NoE) partners.

Third, there is limited evidence to demonstrate cost-effectiveness of pan-European technology introduction. The laboratory cost per test in different European countries is estimated to be between 15 and 27 Euros and the working capital per laboratory is estimated to be ~300,000 Euros for year 1. Also, at present, in terms of organisation there is no indication as to how adapting infrastructure, fully automating procedures, creating universal kits for RhD NIPD, and setting up a centralised laboratory service might influence cost per test in different countries.

Variations in guidelines for prenatal care in member states and alternative health systems might also have an impact on successful implementation. Currently, in European states which have introduced RAADP no policies exist for routine NIPD RhD testing of non-sensitised women. It appears that universal antenatal prophylaxis will not be replaced by NIPD targeted prophylaxis until there is quantifiable evidence demonstrating the costs of introduction and socio-economic and other benefits of this new technology in different country contexts.

RhD NIPD test performance

Through searching the major electronic databases, as well as international literature collections, 21 peer-reviewed papers were identified that in some way evaluate the scientific validity and diagnostic sensitivity and specificity of RhD NIPD tests.

The international STARD checklist (Standards for reporting studies of diagnostic accuracy) was applied to these published articles to assess the quality of studies. It was noted that papers failed to comment on many points of the checklist. It was concluded that the identified studies are largely scientific and because of various shortcomings they should not be used solely to assess the diagnostic accuracy of the new tests.

The list of shortcomings includes: small patient sample numbers in most studies; selective sampling of study populations; exclusion of inconclusive results from the overall calculation of accuracy; lack of consistency in the definitions of sensitivity and specificity; general reporting of 'accuracy' and detection rate; lack of reported measures of statistical certainty/uncertainty; and inconsistent consideration of replicate outcomes in the overall result. This list must raise concerns because the issues mentioned may have a significant effect on the accuracy of tests in the service setting and, as such, the results reported in these studies must be treated with caution.

However, the number of available studies and the countries of origin reflect the global interest in this new technology; and the accuracy results reported in studies illustrate the great potential of NIPD testing. The published studies also demonstrate the rapid developments and technical improvements in the field of NIPD, and more recent reports about clinical experiences indicate that the technology is in the process of being used in an ad hoc manner in several health care systems.

In acknowledgement of the fast development of NIPD *RHD* genotyping, we also included information sources such as grey literature (e.g. unpublished reports) and a number of international interviews conducted with bio-scientists and policymakers. The up-to date evidence contrasts with the conclusions drawn from peer-reviewed published studies and demonstrates that NIPD RhD genotyping is currently advancing in a number of countries in Europe.

Future Outlook

The aim of this report is to provide initial relevant evidence for the initiation of economic modelling of NIPD RhD technology. This will enable us to address questions about the suitability of NIPD tests for implementation i.e. establish what are the optimal conditions within health care systems and clinical practice for the uptake of non-invasive prenatal RhD diagnostics, and evaluate the risks and benefits as well as associated costs, enabling policy makers to forecast the diffusion process into clinical practice across European countries. The ongoing work of the SAFE socio-economic team aims to build on the information in this report in order to produce an early-HTA (e-HTA) report highlighting the economic benefits or disadvantages of this new technology in different settings

Finally, this report is produced to encourage further research, both scientific, in order to perfect the markers for various ethnic groups, and clinical to initiate more population based clinical trials to assess the effectiveness of the technology, as well as socio-economic to address cost-effectiveness in different in health care systems.

LIST OF ABBREVIATIONS

AADP	Prenatal anti-D prophylaxis
ABO	ABO blood group system
BPL	British Products Laboratory
CVS	Chorionic Villus Sampling
DNA	Deoxyribonucleic acid
EQA	External Quality Assurance
HDN	Haemolytic Disease of the Newborn
HTA	Health Technology Assessment
IBGRL	International Blood Group Reference Laboratory
IgG	Immunoglobulin G
IgM	Immunoglobulin M
ISBT	International Society Blood Transfusion
MeSH	Medical Subject Headings
mRNA	Messenger ribonucleic acid
NICE	National Institute of Clinical Excellence
NIPD	Non-invasive Prenatal Diagnostics
NRR	National Research Register
PCR	Polymerase Chain Reaction
QALY	Quality Adjusted Life Year
RAADP	Routine Antenatal anti-D Prophylaxis
rAnti-D	Recombinant Anti-D
RBC	Red Blood Cell
RHAG	Rhesus blood group-associated glycoprotein
RHD/RHCE	Rhesus D/CE gene
ROC	Receiver Operating Characteristic
RQ PCR	Real-time quantitative PCR
SAFE	Special Advances in Fetal and Neonatal Evaluation
SRY	Sex-determining region Y
STARD	Standards for reporting studies of diagnostic accuracy
WP	Work Package

GLOSSARY

Antenatal Anti-D Prophylaxis (AADP)

If an RhD-negative woman has had, or is believed to have had, a potentially sensitising event during pregnancy she will be offered anti-D prophylaxis, an injection of anti-D immunoglobulin, at the time of the event: this is known as antenatal anti-D prophylaxis or AADP.

Health Technology Assessment (HTA)

The systematic evaluation of properties, effects, and/or impacts of health care technology. It may address the indirect, unintended consequences as well as direct, intended consequences of technologies. Its main purpose is to inform technology-related policymaking in health care. HTA is conducted by interdisciplinary groups using explicit analytical frameworks drawing from a variety of methods.

Non-Invasive Prenatal Diagnostics (NIPD)

Prenatal diagnosis is the process of detecting and diagnosing fetal abnormalities before birth. Prenatal diagnosis can be divided into invasive and non-invasive techniques. Non-invasive procedures do not imply any intervention and are harmless to both the fetus and the mother. Non-invasive techniques include maternal serum screening (MSS), ultrasound and analysis of fetal material in the maternal circulation. In this report the term NIPD is used for non-invasive prenatal genetic diagnostic testing using fetal nucleic acids derived from maternal plasma or serum.

Perinatal death

Deaths occurring during late pregnancy (at 22 completed weeks gestation and over), during childbirth and up to seven completed days of life [WHO].

Positive controls

A positive control is a control experiment in which the desired outcome confirms the validity of the experiment. In the case of RhD NIPD, a positive control *confirms the presence of DNA* in a sample that tests RhD negative.

Positive predictive value (PPV) and Negative predictive value (NPV)

PPV is the proportion of those individuals with a positive test result who actually have the condition.
NPV is the proportion of individuals with a negative test result who actually do not have the condition.

Replicates

A replicate is when two or more experimental units carry the same explanatory values.
Technical replicates are repeated measures (statistically). There are four variables that are linked to the number of replicates: variability, effect size, false positive rate, and power, i.e. determining the number of replicates depends on the significance we are looking for, the relative effect size for each gene, and the importance of not missing potential targets.
The number of replicates should be independent of the *gene* because different genes may have different inherent variability [96].

Routine Antenatal Anti-D Prophylaxis (RAADP)

Routine antenatal anti-D prophylaxis (RAADP), an injection of anti-D immunoglobulin, is given by injection to **all** pregnant women who are RhD-negative usually at weeks 28 and 34 (two dose policy UK) or around week 30 (one dose policy other European countries) of their pregnancy.

Sensitivity and Specificity

Sensitivity is defined as the ability of a test to detect a disease or condition when this is present, thus sensitivity is the proportion of diseased patients with a positive test.

Specificity is the ability of a test to correctly exclude the disease or condition in non-diseased populations, thus specificity is the proportion of non-diseased patients with a negative test.

1 METHODOLOGY

1.1 Literature Review

1.1.1 Literature search strategy

Published and grey literature reporting findings on the scientific validity and diagnostic accuracy of RhD NIPD was identified; systematic searches of electronic databases for papers and conference abstracts; browsing international literature collections; and examining bibliographies in key papers. Electronic databases searched included Medline, Embase, the Cochrane Library, Index to Theses, the National Electronic Library for Health (UK), and Conference Papers. Searches were limited to papers published in 1998 and thereafter. This decision is justified by the date of discovery of free fetal DNA in maternal circulation by Lo *et al.* [5] which is the material of choice for non-invasive fetal *RHD* genotyping. Additionally, ongoing studies were sought in the UK National Research Register (NRR). The literature search was updated regularly during the period May 2005 to January 2006. Experts working in the field were also contacted in order to validate the completeness of the evidence sources identified.

1.1.2 Published articles and reports identified

28 published articles were identified. The majority were in English, one was in Polish, one in Dutch and two in French. Papers were identified in the following journals:

- American Journal of Obstetrics and Gynecology
- Annals of the New York Academy of Science
- British Journal of Haematology
- British Journal of Obstetrics and Gynaecology
- European Journal of Obstetrics and Gynecology and Reproductive Biology
- Journal of Histochemistry and Cytochemistry
- Molecular Diagnosis
- New England Journal of Medicine
- Prenatal Diagnosis,
- Transfusion
- Vox Sanguinis

The articles evidence the widespread interest in this new technology with the following 14 countries having published a study in this field: Australia; Belgium; Czech Republic; Denmark; France; Germany; Ireland; Netherlands; Norway; Poland; Spain; Switzerland; UK; USA.

Searching the NRR resulted in the discovery of two additional registered studies. One was carried out at the University of the West of England in Bristol and included 200 multi-ethnic women. The lead researcher was contacted and a report from this study obtained. The second study is still ongoing in Scotland at the time this report was written (100 primigravidae).

Lastly, a study by the Health Care Efficiency Research Programme in the Netherlands was identified [11] and a copy of their final report was provided by the Dutch research group.

1.1.3 Inclusion criteria

Full text articles and reports were retrieved and examined. Papers were examined and excluded if they did not report findings on the scientific validity or diagnostic accuracy of RhD NIPD. Seven papers were excluded at this stage:

- one case reports
- three reviews
- two letters
- one abstract

One grey literature report was included (Netherlands) not only because it reports the largest study undertaken to date (2,543 women), but also because of its useful information on the development of the NIPD test under study.

Papers and reports excluded from the main review were used for additional, supporting information.

1.1.4 Data extraction strategy

A proforma was constructed for data extraction from the 21 included papers. Information was extracted covering the following topics:

- study type
- year and country of publication
- study aim
- number of samples tested
- method and reference test
- testing strategy (exons and introns tested and whether fetal cells or free fetal DNA was used)
- number of **replicates**
- Reported test accuracy/**sensitivity** and **specificity**
- study outcome

1.1.5 Quality assessment strategy

Assessment of the quality of papers reporting studies of diagnostic accuracy was undertaken using the STARD (Standards for reporting studies of diagnostic accuracy) checklist. This checklist (see Annex 1) is the result of an international initiative by scientists and editors to improve the quality of reporting of studies of diagnostic accuracy [73]. The checklist is now required as an annex for all health technology assessment (**HTA**) reports on diagnostic technologies in the UK and increasingly requested by editors of international journals for papers accepted.

Altogether 21 papers were examined and data extracted (see Annex 2). A STARD score out of 25 was also assigned to every paper by a single reviewer.

1.2 Other Information Sources and Delphi Panel

In addition to examination of published articles, stakeholder interviews and surveys were also conducted using different media (online questionnaires, Horizon Wimba, teleconferencing) and in person. These were used to explore specific issues associated with the potential introduction of non-invasive fetal *RHD* genotype testing in different countries.

A Delphi Panel of key experts in 24 countries has also been established. This consists of 90 individuals and includes experts from national HTA agencies/ HTA leads, international HTA networks, clinicians/ laboratory service providers, patient/ user groups, bio-scientists developing these new tests, industrial entrepreneurs/ manufacturers and other individuals with expertise in ethics, law, epidemiology and social sciences.

An initial Delphi exercise has been completed to identify a list of key questions to be addressed in health technology assessments of emerging prenatal genetic tests [12]. This initial framework has been used to help structure the material in this review.

2 BACKGROUND

2.1 Description of the Genetic Character and Sensitisation in Pregnancy

The inheritance of a *genetic character* determined by a single gene, such as Rhesus blood group system, follows a distinctive pattern from generation to generation. In the case of the rhesus system gene, there are two variants (alleles) one associated with rhesus positive *phenotype* (R) and one with rhesus negative phenotype (r). A person with two copies of the r allele (rr) on a pair of chromosomes will be rhesus negative, and those with RR will be rhesus positive. Both are said to be *homozygous* because the two copies of the gene are the same. In cases where they differ (rR, Rr) they are said to be *heterozygous*. Because rhesus negative is recessive it is masked by the dominant rhesus positive, and *heterozygous individuals* are rhesus positive. If the r allele had been dominant, both would be rhesus negative.

In more detail, the *RHD* gene, which encodes for a red blood cell antigen, is one of two highly *homologous* (i.e. 'having a similar structure') genes of the Rh blood group system, the other gene being *RHCE*. A genetic mechanism termed 'crossing over' can result in the *complete deletion* of the *RHD* gene [14]. Individuals, who are homozygous for this deletion, are consequently RhD negative. This is the case for most RhD negative white Caucasians. However, in other population groups, e.g. Africans, the situation is more complex (see [13] for more details). RhD negativity is characterised by the absence of the RhD antigen. Contact of RhD negative blood with RhD antigens, present for example in RhD positive blood, will result in an immunogenic response, in other words the production of antibodies against the antigen. This can occur, for instance, if the RhD positive blood of a fetus comes in contact with the circulation of an RhD negative mother.

The process of antibody formation against the RhD antigen in the RhD negative mother after feto-maternal haemorrhage is termed *sensitisation* and can occur before as well as during the birth of an RhD-positive child [29]. This blood incompatibility can lead to RhD haemolytic disease of the fetus/newborn (HDN) in an RhD positive fetus. This is characterised by the destruction of fetal red blood cells by maternally produced antibodies. Symptoms of HDN range from mild anaemia to the death of the fetus, and can include jaundice, physical disability, and mental retardation. Haemolytic disease does not (normally) occur in *first time pregnancies* because large feto-maternal haemorrhages are most likely to occur in late pregnancy. Furthermore, antibody production takes time and the first antibodies produced - IgM (immunoglobulin of the primary response) - are not able to cross the placenta. However, *if sensitisation does occur during the first pregnancy* all subsequent pregnancies with RhD positive fetuses are at risk of HDN. This is due to IgG, the principal antibody of the secondary immune response being able to cross the placenta.

2.1.1 Evidence on prevalence of RhD negativity across population groups

About 15% of the white Caucasian population is RhD negative. In such populations, approximately 10% of all pregnancies involve a Rhesus negative mother and a Rhesus positive fetus. In a first pregnancy, 60% of Rhesus negative mothers will have a positive child [1, Annex 3]. Due to this occurrence, these pregnancies are potentially at risk of sensitisation.

As pointed out above, RhD negativity occurs in the Caucasian population mainly from the *deletion* of the *RHD* gene. However, it can also be the result of *other rare factors* such as non-functional genes, such as hybrid genes or genes with single nucleotide exchanges, multiple nucleotide exchanges, and deletions or insertions. For example, 0.2-1% of Caucasians have reduced expression of the D-antigen (called weak D). Another group of non-functional genes is the partial D type. D^{VI} is the most common partial D type amongst Caucasians [14]. Non-functional genes may have an impact on the clinical validity of non-invasive fetal *RHD* genotyping due to the resulting disagreement between genotype and phenotype (i.e. rhesus positive or rhesus negative).

Although prevalence of the RhD negative phenotype is less frequent in minority ethnic groups (e.g. 8% in Black Africans and 2% in the South Asian population), the genotype shows greater variability in these groups. Implementation of testing in an ethnically diverse population will therefore require consideration of these variable genotypes. In Asians and Black Africans RhD negativity is often caused by a *silent or inactive RHD* gene rather than a deleted gene. While their phenotype is negative, the presence of parts of the gene sequence can result in a positive result when genotyped. For example, 66% of black Africans have *RHD* genes (*RHDΨ*) that contain a 37-bp insertion which results in a premature stop codon and 15% carry a hybrid *RHD-CE-D* (ce^S), characterised by the expression of weak C and no D antigen which means that carriers of these hybrid genes are phenotypical RhD negative, while genotyping would result in a positive result. Many D-negative Asians, on the other hand, carry the D_{el} gene which shows only a low-level expression of the D antigen while the RHD gene is grossly intact, potentially causing the same kind of mistyping as above [14].

The variety of *RHD* genotypes makes the development of a *universal* non-invasive prenatal diagnostic testing kit, with an application range that includes all ethnic groups, more challenging.

2.1.2 Evidence on incidence of sensitisation in pregnancy

The greatest risks of sensitisation occur at delivery, after abortion and after invasive procedures. Without prophylaxis when these events occur: 5-15% of RhD negative pregnant women will develop anti-D antibodies at delivery; 3-6% after spontaneous abortion; and 2-5% during amniocentesis [15, 16].

In the absence of a high risk event pre-delivery, and if no prenatal prophylaxis is administered, 1-2% of RhD negative pregnant women will become *sensitised* before birth due to feto-maternal haemorrhage [15]. The actual risk attached to sensitisation in an individual pregnancy will depend on various factors. For example, ABO mismatch between mother and fetus has a partial protective effect against RhD sensitisation [14] which explains, at least to some extent, why not all RhD negative women with an RhD positive fetus become sensitised during pregnancy. Also, the gender of the fetus appears to have an influence with male fetuses representing a higher risk of sensitisation [24].

The introduction of postpartum (post-delivery) prophylaxis achieved a reduction of sensitisation events to 1%. Introduction of routine antenatal anti-D prophylaxis (**RAADP**) at weeks 28-30 has further reduced the incidence rate to less than 0.2%, in other words, the number of women sensitised in the UK has reduced from 1,000 per year to less than 140 [17]. In the US and Canada, RAADP at 28 weeks' gestation has similarly reduced the incidence of sensitisation from around 1% to 0.13% of births to RhD negative women [16].

However, even if sensitisation does occur this will not necessarily lead to haemolytic disease of the fetus/newborn (HDN). Therefore, the consideration of a policy of routine antenatal anti-D prophylaxis has prompted several researchers to estimate the measurable benefit of prenatal anti-D and its impact on incidence of HDN as well as sensitisation risk.

2.1.3 Evidence on haemolytic disease of the fetus/newborn (HDN)

Changing incidence of HDN

Before any form of prophylaxis was available, HDN affected 1% of all newborns of second pregnancies to RhD negative women in England and Wales, and even more in subsequent pregnancies. In the mid 1950s, HDN was responsible for one death in 2,180 births in England and Wales. Anti–D prophylaxis (postpartum) was introduced in the late 1960s, and reduced the number of deaths to one tenth of that figure [1]. In the 1970s and 80s, other potentially sensitising events, for instance abortion and ectopic pregnancies, were included in the guidelines ([29] and references therein) with the introduction of antenatal anti-D prophylaxis (**AADP**). Its usage also lowered the incidence of RhD sensitisation to 1% ([17] references therein). Overall, these changes in guidelines have produced a reduction in deaths due to HDN from an initial 310 per year in the mid 1950s to an average of 18 in 1994-1999 (excluding fetal losses which occur before 20 weeks of gestation) [1].

But continuing cases of sensitisation and HDN in the 1990s prompted the introduction of routine antenatal anti-D prophylaxis **(RAADP)** for all RhD negative women prenatally, reducing sensitisations even further to approximately 0.1% [17]. For example, it was reported that about 625 women still continued to become sensitised each year in the UK [1]. This meant that 7 out of 1,000 live born infants were delivered by sensitised mothers [18] mainly due to failure of administration of anti-D either post-partum or prenatally after high risk events such as miscarriage, or due to undetected small prenatal bleeds or due to other red blood cell antigens that can cause sensitisation *for which anti-D immunoglobulin is ineffective*. As a result, it was calculated that 25-30% of fetuses and newborns in second pregnancies of sensitised women would have some degree of haemolytic anaemia and another 20-25% would be hydropic with a significant minority resulting in death. It was estimated that this translated into at least 30 fetal deaths, stillbirths, neonatal and post-neonatal deaths due to HDN in England and Wales [1]. The total number of **perinatal deaths** per annum is approximately 4,482 (2001 figures).

Although the number of deaths due to HDN is relatively small, underreporting of HDN incidences should also be taken into consideration [19]. For example, in Scotland an underreporting of mortality from RhD haemolytic disease has been identified, attributed to the fact that certification data initially excluded stillbirths before 28 weeks. A corrected figure for the number of actual deaths due to HDN was used to support the introduction of RAADP in Scotland.

Morbidity and mortality associated with HDN

HDN is a severe disease which is recognised all over the world. Reports of its epidemiology have been published in a number of different countries. Chavez *et al.* [20] in 1991 commented that Rh HDN continued to contribute significantly to infant morbidity and mortality in the USA. Despite the availability

of preventive measures, the incidence rate in the USA in 2002 was estimated to be about one to six cases in every 1,000 total live births [21].

Likewise, in the 1990s it was reported that HDN was still associated with significant morbidity in Northern Ireland. In a 3-year period (1994-1997), 78 out of 130 babies delivered from 124 sensitised women were affected by rhesus sensitisation. Two stillbirths and one neonatal death were reported among the affected offspring. Another two infants had severe neurodevelopmental delay [22].

A slightly more recent report addresses the epidemiology of HDN in the Netherlands. This presents the results from the screening of 2,392 pregnant women for irregular erythrocyte antibodies over a 3-year period (1995-1998). Antibodies were found in 65 women who were bearing 30 antigen positive children. 12 of these children developed clinical symptoms of HDN, intrauterine death was diagnosed once and one child died immediately after birth. One child had signs of hydrops fetalis and two required exchange transfusion. Phototherapy and blood transfusion were given to 7 babies. Various antibodies can cause HDN (e.g. anti-D, anti-Kell, anti-c, anti-E). Most cases of HDN were caused by anti-D, anti-Kell and anti-c [23].

The survival rate of fetuses with hydrops was investigated by Van Kamp *et al*. [25] who determined the overall survival rate to be 78%. Mild hydrops can be reversed with adequate treatment (intrauterine transfusion) which increases the chance of survival. Early diagnosis of fetal anaemia and referral to a specialised centre are important and enable the start of intrauterine treatment early.

An interesting study reported in 1999 that male fetuses are more severely affected by maternal sensitisation than female fetuses. Perinatal mortality is three times higher in male fetuses, they require more transfusions and have a greater likelihood of developing hydrops fetalis, a major condition of HDN [24].

The papers above show that even though measures to avoid sensitisation are available, HDN has not been eradicated. NIPD *RHD* genotyping as an addition to existing strategies to prevent sensitisation and, therefore, HDN, is a promising development. Many countries have centralised the management of RhD affected pregnancies to guarantee training opportunities and maintenance of expertise [22]. This could also assist in the implementation of RhD NIPD for *sensitised* women, although RAADP would be provided via local maternity services. The findings of the epidemiological research need to be taken into account in any further studies.

2.2 Current Prenatal Testing and Clinical Management

Service provision for prenatal care varies from country to country in Europe. Three recent articles have reported that differences result partly from variations in the structure of antenatal care in different countries, and also the fact that there are different national guidelines, and different ways of providing healthcare. Investigating 25 EU member states Bernloehr *et al*. [89] reported that 20 have national guidelines for antenatal testing. The content of the guidelines, however, varies in respect of the types and number of tests recommended within the antenatal care plan and how often individual tests ought to be carried out. The number of prenatal visits also varies, ranging from a minimum of 4 in Italy to a maximum of 12 in the Netherlands and Germany. The time of the initial visit is also reported to vary

from 6 weeks to 16 weeks of gestational age [90]. A survey of the organisation of antenatal care services also reveals differences in the main care provider, financing, main site of care, integration with other services and uniformity of the organisation of prenatal care [91]. These factors mean that it is impossible to describe a uniform plan of care in uncomplicated pregnancies applicable to all European countries, let alone for pregnancies with complications. Furthermore, differences also exist within individual countries due to regional service provision, unequal access to service by different groups of the population and economic reasons. Thus, the possible integration of NIPD into current EU member states' healthcare systems can only be discussed in general terms, until more detailed national information is available.

2.2.1 Routine antenatal anti-D prophylaxis (RAADP)

It is also evident that variations in services for RhD negative pregnancies currently exist in EU states. In the 1960s, post-partum anti-D prophylaxis was introduced in various developed healthcare systems, administered to RhD negative women after delivery of an RhD positive baby ([17] references therein). Routine antenatal anti-D prophylaxis (RAADP) is usually administered at about 28 weeks of pregnancy. This date is chosen because prophylaxis is effective for up to 12 weeks. Protection is required primarily to cover the third trimester, until the delivery of the baby, because small amounts of fetal blood will usually not stimulate the maternal immune response since fetal cells are usually rapidly cleared by the maternal system. Sensitisation is most likely to occur in the third trimester when the risk of fetomaternal haemorrhages increases to 45% [30].

But there are several disadvantages to a policy of routine antenatal anti-D prophylaxis. Firstly, a policy of RAADP, in addition to AADP and post-partum anti-D, requires an up to fourfold increase in anti-D production [31] which could cause supply issues. This may be the reason why RAADP introduction has been restricted to only a few countries, combined with the Cochrane Database reporting a large 'number needed to treat'. Crowther and Middleton [32] calculated that 213 women need to be treated with prenatal anti-D in order to avoid one case of sensitisation. This calculation is based on all RhD negative women receiving prenatal anti-D because there was no method available to identify the 60% of women with RhD positive babies. Consequently, this number could be reduced to about 128 with the implementation of NIPD *RHD* genotyping to target RAADP.

Secondly, safety issues have been linked with the administration of serum derived anti-D IgG. The possibility remains of infectious agents being present for which there is no test available, or available tests are not sufficiently sensitive e.g. variant Creutzfeldt-Jakob disease. A specific instance of transmission of infection relates to the use of pooled plasma for anti-D production in Ireland where pregnant women were infected with hepatitis C by anti-D IgG between 1991 and 1994 [31].

Thirdly, there may be ethical concerns about boosting donors to maintain high-titre polyclonal anti-D. This deliberate immunisation may result in complications in future therapeutic transfusions due to platelet and leukocyte antibodies or antibodies to non-D erythrocyte antigens [31].

2.2.2 Variation in clinical management of RhD negative pregnant women in different countries

As far as current services for RhD negative pregnant women are concerned, all member states offer post-natal anti-D prophylaxis; this involves prenatal blood typing of pregnant women and a post-natal blood-typing test for the baby if the mother is RhD negative. Similarly, most countries offer AADP (antenatal anti-D prophylaxis) following potentially sensitising events (e.g. amniocentesis, abortion) although guidelines may vary. In contrast, to date not all EU countries have implemented RAADP and even within these countries access to this service may not be universal. Finally, even fewer countries have introduced NIPD RhD tests into routine practice. Where such tests are currently used, they are routinely utilised in the management of *sensitised* women.

Even if a RAADP policy exists in a country, not all women will receive prenatal prophylaxis. One reason may be that the adherence to guidelines is patchy. For example, it has been reported for England and Wales that only 30% of hospitals offer the service of RAADP [7]. A second reason may be that the RAADP policy implemented does not apply to all women. In the Netherlands, for example, only pregnant women with no living child (about 16,000) received prenatal prophylaxis due to the limited supply of home produced anti-D; but 6,400 of these women with an RhD negative fetus receive it unnecessarily [11]. NIPD has the potential to resolve these issues. For example, identification of RhD negative women with an RhD positive fetus during pregnancy by NIPD might increase the number of hospitals providing RAADP to women in England and Wales, making routine prenatal prophylaxis more readily achievable and approaching the theoretical benefit of RAADP. Similarly, in the Netherlands the point is made that 40% of anti-D can be saved by identifying the 40% of women who carry an RhD negative fetus, and this could then be offered to multigravidae, who are currently not included in the RAADP policy. In the Netherlands, prenatal prophylaxis provided to all RhD-negative women might reduce the incidence of new Rh-immunisations in multiparous women from the current 100-125 to 50-70 new cases per year. Because one out of three multiparous women will have another child and 71% of these children will be RhD positive, it has been estimated that around 12 new cases of RhD HDN could be prevented by extending RAADP to these women [11].

2.2.3 Future improvements in management of RhD negative pregnant women

Likely future improvements to the management of RhD negative women are limited. There is currently no therapy available to target the maternal immune response in these women. Once sensitisation has occurred, surveillance and treatment, for instance using intravascular transfusion techniques, is the only option available even though selective administration of maternal intravenous immune globulin and maternal sensitisation to paternal leukocytes have been discussed [21, 26, 27]. The latter is based on the postulation that maternal antibodies to paternal leukocyte antigens that are shared with the fetus cross the placenta and inhibit fetal monocytes, thereby preventing red blood cell destruction [26]. This lack of therapy is the main reason why attention remains directed towards the *prevention of sensitisation.*

NIPD, therefore, has the potential to complement and improve prevention in countries which have introduced RAADP. The alternative to NIPD is to continue with the unchanged, current policy of giving anti-D to all RhD-negative pregnant women during the third trimester.

But in spite of the published evidence that sensitisation is lowered by prenatal anti-D IgG, the actual benefit of prenatal prophylaxis RAADP remains debatable. It has been remarked that the observed decline in incidences is partially attributable to smaller family sizes since HDN increases in severity with increasing birth order. In 1996, the US Preventive Services Task Force argued that the impact of prenatal prophylaxis on the incidences of HDN was relatively small and with that, was 16 times less cost-effective than a postpartum prophylaxis only policy [16].

2.2.4 Current and future RhD NIPD testing

Use of NIPD tests in sensitised RhD-negative pregnancies

RhD NIPD is currently only used routinely in the management of a defined population - *sensitised* women. For example, in the UK the International Blood Group Reference Laboratory (IBGRL) in Bristol offers a fetal *RHD* genotyping service to obstetricians managing the pregnancies of alloimmunised pregnant women via their local blood centre. Blood samples from pregnant women who have tested RhD negative during routine prenatal care and positive for antibodies is genotyped (NIPD) for the fetal *RHD* status in order to decide on further treatment.

NIPD *RHD* genotyping has the potential to replace invasive testing methods like amniocentesis or chorionic villus sampling if these are used for the sole reason of determining the fetal *RHD* genotype. This would appear to be highly desirable because invasive tests are associated with an increased risk of miscarriage as well as an increased incidence of gestational maternal immunisation by inducing fetomaternal haemorrhages in 17% of cases [68, 69]. On the other hand, there is some indication that the perception of end-user might vary slightly. A survey of high-risk pregnant women about to undergo invasive testing revealed that these women see the NIPD test as part of a stepwise testing strategy in which the non-invasive test results will help women to make a decision on whether invasive testing is desirable [38].

When combined with serologic methods, RH molecular testing is already an important tool to resolve discrepancies and ambiguous typing results in transfusion medicine. This should encourage the establishment of RHD markers for RhD NIPD. For fetal RhD testing and prediction of HDN, samples of the mother and father need to be analysed serologically and molecularly [14].

Use of NIPD tests in routine RhD-negative pregnancies

Currently, no country routinely uses NIPD to decide whether non-sensitised women need routine antenatal anti-D prophylaxis or not. At present, this is being considered for introduction in the Netherlands as a universal policy with all RhD negative women tested. Furthermore, there is the possibility that if introduced in Germany women would be given the choice between prophylaxis at about 28 weeks of pregnancy or NIPD *RHD* genotyping [74].

Timing of NIPD tests

If NIPD were to be used to target RAADP, it is sufficient to carry out the test in the third trimester. However, for the management of sensitised women early identification of the fetal genotype would be advantageous. There are, therefore, clinical advantages in being able to perform the test at a woman's booking prenatal visit, usually in the first trimester, rather than in the second half of pregnancy [28]. However, at present testing women in the second trimester increases accuracy rates (see section 4.2).

2.3 Other Technologies under Development

Since any new policy involving NIPD use to target RAADP will be introduced over a number of years (3-6 years), it is also important to consider emerging alternative or competitor technologies and their likely impact on the perceived value for money of RhD NIPD over this period same period.

2.3.1 Recombinant anti-D immunoglobulin

Due to the potential risks associated with the supply and safety issues of existing, serum-derived anti-D products (see section 2.2.1), a potential new product which may be of relevance is recombinant anti-D. This bio-engineered product may resolve supply and safety issues of human anti-D used for prevention of sensitisation and HDN. However, it is unclear when the product is likely to reach the market.

In order to gather information on this, European and Australian companies involved in the development of recombinant anti-D were identified and approached for information [33, 34, 35, 36]. The information provided indicates that dosage and treatment regimens are expected to be similar to existing anti-D products, potentially providing an alternative safe and steady supply of anti-D. The companies themselves report excellent early clinical data and a more cost-effective product although no peer-reviewed publications are available. They also appear optimistic that their product will make it to the market in the near future (3 years).

Scientific experts we surveyed do not share the optimistic views of the biotechnology companies. Experts commented that it is particularly difficult to produce a recombinant anti-D since the RhD antigen is known to express several different antigenic epitopes. For a complete pattern of reactivity, and in order to be successful in prophylaxis, all epitopes need to be included. In addition to antigen specificity, antibodies also need to be able to interact with effector cells in order for IgG coated RBCs to be cleared from the maternal circulation [31].

Dosage and treatment regimens may also need to be adapted from the regimens currently used for existing anti-D products. For example, in 2000 Miescher *et al.* [37] published the result of a recombinant monoclonal anti-RhD antibody with a novel reactivity pattern which was expected to meet the stringent regulatory and safety requirements demanded for the use of recombinant products in humans. Testing the new product, it was discovered that one testing strategy required more recombinant anti-D than with chromatographically purified rhesus (D) immunoglobulin - Rhophylac [17] to attain the same activity. The relevance of these quantitative in-vitro differences for clinical efficacy needs to be addressed and might be contradictory to the claim that dosage and treatment regimens are expected to be the same with both types of anti-D antibodies.

It appears that, at this stage, the performance of monoclonal anti-D antibodies in various *in vitro* assays cannot be readily transferred to predict *in vivo* efficacy [31]. Furthermore, there are obvious issues with the public perception associated with recombinant anti-D IgG. Administering a biotechnology product to young healthy pregnant women has been seen as a high commercial risk and it has been suggested that people's judgement of relative risk needs to change for patients to make a reasonable decision on the potential risks from biotechnology products as opposed to blood products [31]. These views contrast with the over optimistic statements and timeframes of the biotechnology companies. Further information and evidence on this issue are currently being collected.

2.3.2 Peptide immunotherapy

Another promising alternative technology which would avoid the risks associated with blood products entirely, is tolerance induction to the RhD antigen by mucosal delivery of synthetic RhD peptides [82]. This would make women "immune" to sensitisation. Mucosal administration of peptides has been previously used to induce systemic tolerance to antigens in different models [82] and acts by active immune regulation. Hall *et al*. [82] demonstrated that the activation of a specific immune response can be prevented by the manipulation of T-helper (Th) cell recognition of the RhD protein in a transgenic mouse model.

Four dominant RhD peptides, which stimulate Th cells in alloimmunised RhD-negative donors, have been tested for their tolerogenic potential in a humanised mouse model, transgenic for the molecule conferring RhD specificity of Th cells. Hall *et al*. [82] report that these 4 peptides induce tolerance when the RhD peptide was administered 2 weeks prior to RhD immunoglobulin challenge. This study shows that humanised mice can make IgG antibodies specific for the RhD protein and that this can be prevented by nasal administration of synthetic peptides containing dominant Th epitopes. This research represents an important first step in the development of immunotherapy for HDN and would provide several advantages in relation to passive RhD immune globulin, which is transient and needs to be administered after each exposure to the antigen. Furthermore, in the latter case the antibody dose needs to be optimised to match the size of the feto-maternal haemorrhage for maximum efficacy.

The study of Hall *et al*. [82] provides the basis for further research into the mechanism of tolerance induction, and phase-1-trials of peptide immunotherapy to prevent anti-RhD responses in humans are currently being planned. This product is unlikely to reach the market in the medium-term future (3-6 years).

2.3.3 Preimplantation genetic diagnosis

For the small number of severely sensitised women, preimplant genetic diagnosis (PGD) may be considered rather than NIPD in a natural pregnancy. In 1995 single cell analysis of the RhD status was carried out on cultured lymphoblasts. It was suggested that this technique can be used in PGD in sensitised women for the prevention of HDN [83]. Avner *et al*. [84] determined the RhD status of two embryos for the selective transfer of RhD negative embryos in a family of a sensitised woman and a heterozygote partner. Typing resulted in the conclusion that both embryos were RhD negative. However, the result could not be confirmed since pregnancy was not achieved.

In 2005 Seeho *et al*. [85] reported the first case of an unaffected RhD negative baby being born to an RhD-alloimmunised mother using preimplantation diagnosis. A couple with two children, the second child affected by HDN, was counselled that RhD screening prior to implantation using PGD could allow selective transfer of only RhD negative embryos. Two out of twelve embryos were typed RhD negative. Transfer resulted into a clinical pregnancy and RhD negativity could be confirmed postnatally [85].

3 NIPD TEST DEVELOPMENT AND PERFORMANCE

3.1 Emergence of Non-invasive prenatal diagnosis based on free fetal DNA

In the middle of the 1990s, fetal cells [4] and free fetal DNA in maternal circulation [5] were recognised as a potential route for non-invasive prenatal diagnosis. Scientists believed that it should be possible to detect fetal cells in maternal blood as early as four weeks of gestation when the fetal heart commences beating and the villi are vascularised. The earliest detection so far is around week 4 and 5 using PCR based amplification of Y-specific DNA sequences [6]. The number of fetal cells in maternal circulation increases with gestation age, but they are still very rare even in late pregnancy [6]. There are also potential problems associated with the reported persistence of fetal cells from previous pregnancies which can lead to false positive results [8]. While the work on the detection of fetal cells in maternal circulation continues, several laboratories are now working on the evaluation of fetal DNA extracted from maternal plasma first discovered in 1997 [5]. Fetal DNA may arise directly from various sources (the syncytiotrophoblast, from shed apoptotic cells or result from lysis of fetal cells transferred across the placenta [9]) and can be detected as early as 7 weeks [10]. Rapid removal of fetal DNA from the maternal circulation suggests that it might be less susceptible to false positive results than using fetal cells [9].

The amount of free fetal DNA in maternal blood is a limiting factor for most forms of fetal genotyping (including fetal RhD). While fetal DNA amounts increase during pregnancy [10], there are notable variations from pregnancy to pregnancy and also there are day-to-day variations which would have to be taken into consideration since certain non-invasive tests need to be performed at specific gestation age to bring useful information early enough in pregnancy. To date, fetal DNA typing has been limited to the diagnosis of mutations of paternal origin, i.e. cases in which a specific and unique gene from the father is sought (autosomal dominant disorders and X-linked disorders, in which the father and mother carry different identifiable mutations). Fetal cells lack nuclei and therefore do not allow standard prenatal karyotyping which would be required to detect all fetal chromosome disorders [9]. However, fetal DNA typing is useful for detecting some genetic characteristics and the D gene specifically.

The development of assays for non-invasive *RHD* genotyping first became feasible once it was possible to detect non-invasively the SRY gene (sex-determining region of the Y chromosome) which is found on the Y chromosome. With the growing understanding of the molecular basis of the Rh blood group system and advances in determining fetal RhD blood group status using PCR on amniotic or chorionic villus cells, the development of assays for non-invasive *RHD* genotyping became possible.

3.2 Emergence of RhD NIPD technology

In 1994, Lo *et al.* [39] reported the investigation of fetal RhD status in three sensitised RhD negative women by non-invasive means demonstrating the feasibility of fetal RhD sequence detection from circulating RhD-positive fetal cells. This was the beginning of NIPD *RHD* genotyping.

Following the discovery of *free fetal DNA* in the maternal circulation [5], this (rather than fetal cells) became the material of choice for non-invasive fetal *RHD* genotyping because RhD positivity or negativity depends on the presence or absence of one single gene. Only one of the studies we identified did not exploit the presence of free fetal DNA but used mRNA from erythroblasts instead.

Cunningham *et al*. [6] postulate three advantages in using mRNA. Firstly, using mRNA reduces the likelihood of contamination because it is more sensitive to degradation. Secondly, presence of a certain mRNA is limited to the cells that produce it which are, in this case, fetal erythroblasts?what does this mean?. Finally, mRNA is present in multiple copies. It was also suggested that reverse transcription PCR might be a more sensitive alternative to standard PCR.

Extracting DNA from either serum or plasma does not seem to have a significant influence on diagnostic accuracy in fetal sexing, the early application of NIPD [40]. Results of earlier studies, however, suggest a *preference for plasma* [41, 42]. Tufan *et al*. [40] reported slightly higher accuracy when using plasma for sex genotyping rather than serum. This might be explained by a concentration difference of cell free fetal DNA in maternal plasma versus serum [10]. This tendency to prefer plasma was carried through to RhD NIPD. In general plasma is the favoured material for DNA extraction even though some research groups have used serum and one group whole blood. Turner *et al*. [8] have argued that when using whole blood, the sensitivity of the assay is increased because it represents a larger volume of blood for DNA extraction. But Finning *et al* [43] replied to this statement by pointing out that plasma contains a higher proportion of fetal DNA than the cellular fraction of maternal blood. Furthermore, the background of maternal DNA is reduced when using plasma, which increases sensitivity of the test.

Randen *et al*. [46] have examined the impact of different temperatures and duplexing (i.e. simultaneous amplification of markers of the *RHD* and *SRY* genes in one test tube) on the test result and reported that temperature had an impact on the performance of DNA extraction. There was a statistically significant reduction of fetal DNA in samples stored at room temperature. Duplex PCR of *RHD* and *SRY* reduced the sensitivity for *SRY* gene amplification and it had to be concluded that this duplex PCR was not suitable for routine analysis. Zhong *et al*. [47], on the other hand, have reported the same efficiency when determining multiple fetal loci from free fetal DNA. A further factor influencing test performance appears to be the time that samples are stored. It has been advised recently that samples need to be processed as quickly as possible [48].

3.3 Papers Reporting Studies of RhD NIPD Diagnostic Accuracy

Altogether 21 papers were examined and data extracted (see Annex 2). A STARD score was assigned to every paper in the following areas.

3.3.1 Mesh headings

Articles were generally poorly indexed. Only 3 early articles identified the paper as a study on diagnostic accuracy using the recommended MeSH heading Sensitivity and Specificity.

3.3.2 Research questions or study aims

When stating the research questions or study aims, papers also did not refer to sensitivity and specificity. Furthermore, when reporting study findings (see Annex 2), ‘accuracy’ and ‘detection rate’ were more frequently used expressions. Genotypic and phenotypic sensitivity/specificity could not be distinguished in most papers under these two specified headings. The majority of studies that aimed to

report on test diagnostic accuracy were actually scientific studies exploring the new test and testing its limitations.

Finally, occasional use of terms such as sensitivity and specificity did not equate well with the accepted definitions of test sensitivity and specificity. Authors appeared to use different definitions which meant that where such results were presented they were not directly comparable. This fact complicated the assessment of these papers against the checklist. According to the STARD checklist, the identified studies were for that reason generally either poorly designed or poorly reported.

3.3.3 Study populations

When presenting the study methods and reporting results, papers were generally found not to describe the study population in depth, including whether a consecutive series of participants was used, the beginning and end dates of recruitment, or the recruitment centres used. Reporting of the number of participants satisfying the inclusion criteria who did or did not undergo the index tests and/or the reference standard; and an explanation of why participants failed to receive either test were rarely covered.

The number of samples tested in the studies reflects the movement in the progress of a new technology. Early studies used a smaller number of samples, rarely exceeding 100 pregnant RhD negative women. Promising results from these studies prompted researchers to be more positive about the potential transition into clinical practice of this non-invasive technology, consequently establishing larger trials. In 2004, a French study investigated the feasibility and accuracy of large scale RhD fetal diagnosis, including 893 RhD-negative women [44]. In 2005, Minon *et al.* [87] reported the results of 223 samples evaluating two years of routine practice of RhD NIPD in Belgium. The largest trial to date has been conducted in the Netherlands. An abstract published in 2004 describes a Dutch study which recruited 2,543 D-negative pregnant women [49]. The final report is currently being finalised [11]. This was the first large scale trial to provide evidence for clinicians and, very importantly, health care funders on the real potential of the new test in use.

3.3.4 Test method

Reference standard

The reference standard used in studies varies. In cases where invasive procedures were carried out, fluid or cells from amniocentesis or chorionic villus sampling were used for PCR and the results compared with those of the index test. In all other cases, postpartum serological blood typing was used as the reference standard for the validation of the results. Data collection was generally planned before the index test and reference standard were performed (prospective studies).

Technical specification of material and methods

Description of the technical materials and methods was generally well covered in the studies throughout. Quantitative real-time fluorescent PCR was the method of choice. Although the expense of the equipment may limit use in a routine setting [40]. Real-time PCR, in contrast to conventional PCR, collects data in the exponential growth phase of the reaction which is the most specific and precise phase of the reaction. Traditional PCR, however, is measured at the end-point requiring post PCR

processing. This allows real-time PCR to be more precise and more sensitive and it can be automated. Fluorescence dyes attached to a probe are added to the reaction mix which change their fluorescence either in intensity or colour as the PCR reported in 2004 product is synthesised. The increase in fluorescence is directly proportional to the number of product copies generated and can be measured while amplification is occurring.

An attempt to compare conventional with real time PCR discovered no greater sensitivity when using real time PCR but a greater specificity [44]. Furthermore, real time PCR is less time consuming and possesses an extra level of protection against contamination. At the same time, conventional PCR was thought to be unsuitable for diagnostic use [45]. But Tufan et al reported in 2005 that optimisation of a multiplex PCR could disprove this attitude towards conventional PCR, and this would be a manageable task for any clinical laboratory where conventional PCR is available [40]. However, conventional PCR has not prevailed and quantitative real-time PCR is the method of choice in the majority of published studies. The first report of its automation was published in 2004 by Van der Schoot *et al*. [49].

Besides the choice of material (see 3.2 above), other factors have been shown to influence the success of PCR-based non-invasive prenatal genetic diagnosis. DNA extraction methods and PCR cycle numbers, as well as the risk of contamination, are major success-limiting factors and therefore need to be optimised. It has been suggested that the QIAamp DNA Blood Mini extraction kit, in combination with a 40-cycle PCR, gives the optimum results. It has also been suggested that the risk of contamination can be reduced by using ready-to-use PCR mixtures which contain all components therefore reducing necessary pipetting steps to a minimum [40].

When measurements were taken

The time in pregnancy at which samples were taken varied between studies. The Czech group Hromadnikova *et al.* [65] tested mainly women in the second and third trimester, while populations of other research groups covered all trimesters [60, 44, 46, 67, 10]. Targeting a specific category of pregnant women, i.e. women about to undergo invasive procedures or blood samples taken for maternal triple serum screening, predetermined the time of testing to the second trimester in two studies [47, 9]. Women undergoing genetic counselling were restricted to first trimester pregnancies in one study [63]. Turner *et al.* recruited women before 20 weeks gestation, bearing in mind the likely point of time of the first prenatal visit. Similarly, Gonzalez-Gonzalez *et al.* [48] tested 11 to 16-week pregnant women.

In the Netherlands, where the reduction of anti-D application in RAADP seems to be the driving force for adoption into clinical practice, early genotyping was not an issue. Here, women that were 28-30 weeks pregnant were tested.

Other descriptors

The number, training and expertise of the people carrying out and reading the tests was poorly reported. The 'masking' of test readers was specified in only one paper i.e. readers of the index test and reference standard were masked to the result of the other test. Definitions and rationale for the units, cut-offs and/or categories of the results of the index test(s) and the reference standard were not applicable in *RHD* genotyping since this is a qualitative test. Test reproducibility was not reported as such, but the number of replicates required per sample for an accurate prediction of fetal RHD status was discussed (see section 3.3.7).

3.3.5 Test results

The variations in sample characteristics and test parameters reported in papers complicated the evaluation and comparison of study quality and the generalising of results. Reported test 'accuracy' ranged from 75% to 100%.

Time interval from index test & distribution of severity of disease

The time interval from index test to reference standard was only reported indirectly when stated that the reference standard was the postpartum serological blood test. This STARD item, and details of any treatments administered between, are not relevant to RhD status, which cannot change. Similarly the distribution of severity of disease in those with the target condition; and other diagnoses in participants without the target condition is not relevant in RhD NIPD because presence and absence of a gene is tested for.

Cross tabulation of results

Most studies reported a cross tabulation of study outcomes of the index test and the reference standard, and discrepancies between the two. However, some studies did not include indeterminate results in the calculation of accuracy, i.e. cases in which a woman is suspected to have a non-functional gene [60, 44]. This will influence the reliability of the study results. Studies also did not generally report how missing responses and outliers of index tests were handled.

Measures of statistical certainty

Methods for calculating or comparing measures of diagnostic accuracy, and the statistical methods used to quantify uncertainty, were not described in any study. Measures of statistical certainty/uncertainty e.g. 95% confidence intervals, were not reported by any study. In fact, as well as no attempts to minimise bias by masking of test readers, the absence of any statistical methods to estimate uncertainty were two consistently weak points of these studies. Estimations of variability of diagnostic accuracy between subgroups of participants, readers or centres were also not reported in any study.

Other

Finally, the STARD item relating to reporting of adverse events of index tests and reference standard was not relevant in the context of RhD NIPD.

3.3.6 Markers for RhD NIPD

For *RHD* genotyping, regions of highest divergence between the *RHD* and *RHCE* genes are required for NIPD. These were reported in 1993 to be located in exons 4, 5, 7, and 10 as well as intron 4 which has a 600bp deletion in the *RHD* gene in contrast to the *RHCE* gene [57]. But gene variants in the *RHD* gene place some doubt on the accuracy of a PCR reaction using only one primer pair. This led Aubin *et al*. [50] in 1997to evaluate and compare 4 different PCR methods of *RHD* gene detection using different primer sets. Blood samples of blood donors, including a number of different gene variants and amniotic fluid from 92 fetuses, were collected for the study. The authors reported that the method using exon 7 was the most sensitive out of the four (exon 10, exon 7, exon 4, intron 4).

Using blood samples from RhD-negative pregnant women, only one method (exon 10) gave 6 false positive results in Caucasians. A much higher false positive rate was obtained if the blood samples were of African or Asian origin. Three of the four methods were reported to have the potential to give false negative results when some rare variants were examined. These are clinically more relevant than false positives. The methods were those using exon 7, 4, and intron 4. From these results, Aubin *et al.* [50] recommended as a minimum that an assay for routine use be based on two methods which should include use of exon 7 because this is the most sensitive. This combination would be able to detect all D variants investigated. However, sensitivity of exon 10 could be improved by changing one *RHD* sequence specific oligonucleotide. In 2004, this new exon 10 PCR was reported to reach the same sensitivity as exon 7 PCR [44].

Applying this approach for non-invasive *RHD* genotyping, Johnson *et al.* [45] in 2003 and Hromadnikova *et al.* [58] in 2005 reached the same conclusion about the usefulness of using at least two RhD specific products for amplification in order to increase accuracy but the markers were not consistent. Exons 7 and/or 10 were the most commonly used markers for *RHD* genotyping in the studies under investigation. Exons 6 and/or 4 and 5 have been included to prevent positive results if a non-functional gene (*RHD*Ψ)was present [28, 87]. The Czech group included two different markers, namely intron 2 and exon 5, in order to additionally genotype for the closely related *RHCE* gene [59].

3.3.7 Number of repeated measurements required per specimen

In addition to **positive controls**, replicates (i.e. repeated measures) are needed for an accurate prediction of fetal RHD status [67] and to ensure the reliability of test results. Between two and seven replicates were reported in the studies reviewed. However, some studies either did not include replicates or did not report them. Conversion of replicate outcomes into an overall test result was handled differently across the studies. Hromadnikova *et al.* [65] considered a sample to be positive if at least 1 out of 5 replicates was positive. In contrast, Legler *et al.* [64] demanded at least 2 positive results out of 4 replicates for an overall positive test result. Similarly, Clausen *et al*. [88] only considered a sample as positive when at least two replicates out of 4 were positive for two different exons. A Norwegian study has reported the discrepancy between index test and reference standard in two different ways; considering all replicates in one analysis and demonstrating a reduction in false result when only requiring one positive replicate for an overall positive result [46].

Comparing conventional with real time PCR, Rouillac *et al*. [44] only classed a sample positive if both assays reported a positive result of a least one of the two markers, i.e. exon 7, while Johnson *et al.* [45] used three markers and reported their specificity and sensitivity in case of concordance of at least two exons.

3.3.8 Performance in twin pregnancies

Multiple pregnancies have been investigated in 5 reports [28, 44, 59, 86, 87]. Pregnancies with at least one RhD positive fetus could be successfully identified as RhD positive in 3 studies [59, 86, 87]. The correct identification of the other fetus(es) is of less importance since prophylaxis needs to be administered in the case of one positive fetus. However, two studies reported discrepancies between genotyping and amniocentesis. In one case RhD positive DNA was amplified at 13 weeks gestation,

while genotyping of amniotic cells resulted in a negative result for both fetuses [86]. RhD negativity was confirmed by postnatal serology. Since this pregnancy was the result of IVF, it was suspected that a third non-evolutive RhD-positive embryo could have caused the result. In contrast, Finning *et al*. [28] correctly identified at least one RhD positive fetus by genotyping, while amniocentesis identified two RhD negative fetuses. At birth, serologic tests confirmed that one boy was indeed RhD positive and it was concluded that amniotic fluid for amniocentesis must have been drawn from only one amniotic sac.

3.4 Results Obtained in Multiple Laboratories and Quality Assurance

To date, only a limited number of inter-laboratory studies have been carried out in order to assess the feasibility of NIPD delivery in the service setting. In 2004, five participating centres in a National Institute of Child Health and Human Development Consortium developed and validated a standard protocol for DNA extraction for fetal DNA amplification [71]. Using the SRY gene, variations in sensitivity measures were greater among the laboratories than measures of specificity. Freezing and shipping did not affect the results. It was concluded that a robust and carefully optimised protocol is needed for use of fetal DNA testing in routine clinical practice.

More recently, the SAFE NoE organised two workshops in order to assess the consistency of performance of NIPD *RHD* genotyping across laboratories. The first workshop demonstrated 'excellent reproducibility' with consistency across 12 laboratories in Europe [72], while the results of the second workshop are currently awaited. Protocols for DNA extraction, as well as sequences for primers and probes, were standardised in these workshops. More work is still needed in this area to establish internal laboratory quality control programmes and external quality assurance schemes for NIPD.

A need for external quality assurance (EQA) has also been recognised by the International Society of Blood Transfusion (ISBT) and International Council for Standardization in Haematology (ICSH) in the general area of molecular blood group genotyping. Even though this has become routine practice in many laboratories around the world, there is little regulation in the form of external quality assurance [70]. In order to establish an EQA scheme, an international workshop was organised to identify methods used in different laboratories and ascertain accuracy and reliability of these methods. The 2005 report records that two out of six samples, which were distributed among 30 participating laboratories, were prepared for *RHD* genotyping. A variety of different methods were recorded for RhD testing in these laboratories; RHDΨ was a major cause of error for these samples, as was simple misreporting. Another two samples consisted of plasma from RhD negative pregnant women with plasma from a heterozygous RhD positive male added. Only 15 out of 24 laboratories reported correct results for RHD in these samples. Four laboratories did not obtain conclusive results, while five did not test the samples. Of the 15 laboratories that reported correct results, real-time quantitative PCR was used in 11 laboratories; 4 participants employed conventional PCR. Different amounts of plasma for DNA extraction and different centrifugation steps of the plasma were used. This international workshop has produced a list of recommendations on how to perform molecular blood group genotyping tests and how to present the results. Further workshops will be held every two years.

4 CLINICAL VALIDITY

In the papers reviewed, discussion of the future clinical application of NIPD was one of the areas generally well covered. However, papers were less clear when considering the relationship between genotype and RhD phenotype, phenotype sensitivity/specificity, and population sampling procedures.

4.1. RhD genotype/Phenotype Relationship

The general background literature indicates that the relationship between the genotype (the genetic makeup) and the phenotype (the RhD status) of individuals depends on the ethnic population to which the individual belongs. Almost all white Caucasian RhD negative individuals lack the *RHD* gene completely, presumably due to 'crossing over' which results in the *complete deletion* of the *RHD* gene [14]. However, other RhD negative phenotypes can result from a *defective*, rather than a deleted, *RHD* gene. Within 8,000 RhD negative blood samples from Europeans, 14 different *RHD* positive antigen D negative and 3 different D_{el} alleles were found. The cumulative frequency of the first was calculated to be 1:1,500 and of the second to be 1:3,000 [2]. Such non-functional genes are more likely to occur in certain other population groups e.g. Africans [13]. Several variants have been identified by complex serological observations and confirmed and characterised by molecular typing. Quantitative, as well as qualitative, variants have been discovered (Annex 4).

These variants mean that genotyping may not reliably predict the RhD phenotype. For example, RhD variants can result in *false RhD positives* if the mother carries a non-functional gene which is detected by the real-time PCR assay. In a 2004 feasibility study, fetal *RHD* genotyping results on maternal plasma had to be invalidated in 34 cases out of 893 for this reason [44]. Additional PCRs in exon 4, intron 4 and exon 6 were able to identify only 26/34 of the non-functional maternal genes as *RHD*Ψ pseudogenes. Another study did not carry out follow ups of inconclusive results but cited RhD polymorphism in general for discrepancies in study results [9].

The major differences between the *RHD* and *RHCE* genes suggest that exon 10 and intron 4 tests should provide a suitable testing strategy for the majority of white Caucasians. A multiplex PCR of these two regions would allow a distinction to be made between normal RHD and the clinically most relevant partial D category D^{VI}. This cannot be identified when a duplex exon 7 and exon 10 PCR is carried out and, therefore, the result would be noted as *inconclusive* in these cases [44]. Furthermore, amplification of exon 7 will type *RHD*Ψ pseudogenes *falsely as RhD positive*. Therefore, testing diverse race populations, as opposed to white caucasion populations, requires further adjustments to be made. It has been reported that sequence specific primer pairs for *RHD* exon 4, *RHD* exon 5 and *RHD* exon 6 could successfully distinguish a *RHD*Ψ from *RHD* and would correctly type the individual as RhD negative [28]. However, information about the ethnic origin of the parents might be helpful since *RHD*Ψ or *RHD-CE-Ds* would give the same result as D^{VI} variants with exons 4, 5 and 10. This may prove difficult in practice. For example, use of a standard questionnaire to identify ethnic origin proved to have low sensitivity/ specificity in a UK trial undertaken to inform policy on the introduction of a national haemoglobinopathy screening programme [97].

A small number of later studies with modifications to allow for D-variants in fetal genotyping in a testing strategy were included. However, it has been suggested that for mass-scale genotyping to be effective and for genotyping to replace conventional serotyping of blood, all *RHD* alleles would need to be fully

characterised and, thus, detected [56]. This would be particularly important if NIPD tests were to eventually replace postpartum serological blood typing.

4.2. NIPD Test Phenotype Sensitivity/ Specificity

The papers reviewed did not generally distinguish clearly between phenotypic and genotypic sensitivity/specificity when reporting results. Clinical validity could, therefore, only be addressed by looking at the overall test 'accuracy' reported in these studies. Also, as explained above (section 3.3.2) the lack of reports of sensitivity and specificity rendered many papers of poor quality according to the STARD checklist. Furthermore, alternative reported measures of test diagnostic performance such as 'detection rate' and 'accuracy' have to be treated with some caution in the papers reviewed, even more so since some studies did not include indeterminate results in the calculation of 'accuracy'. Where sensitivity and/or specificity were reported these were sometimes calculated based on a low number of samples, for instance 56 RhD-negative pregnant women [88]. Positive and negative predicted values were not reported in any of the studies reviewed.

The first clinical report was published by Lo *et al.* in 1998 [10]. The authors tested 57 pregnant women in the UK and reported a 'detection rate' of 100% in the second and third trimester but encountered two *false negatives* in the first trimester due to lack of DNA in the sample. This was the first report to identify this new technology as promising for fetal *RHD* genotyping. In the following years, several groups investigated the performance of this new test. In 1999, Bischoff *et al* in the USA detected 70% of 20 RhD positive fetuses correctly using frozen serum without any false positives [61]. In the same year a UK study using reverse transcription of mRNA achieved 75% accuracy [6]. The authors encountered 18 false positives among 96 RhD negative pregnant women at various stages of gestation and discovered a clear trend of increased accuracy with rising gestational age. Two years later, in 2001, Nelson *et al.* in Australia reported achieving 100% accuracy prospectively in 26 RhD negative women 9-34 weeks pregnant with RhD positive fetuses [62].

Determination of multiple fetal loci from cell free DNA was first described in 2001 by Zhong *et al*. [47]. 34 second trimester maternal plasma samples were investigated for the *RHD* and SRY gene. The authors failed to detect the *RHD* gene in one male fetus. The following year, Finning *et al*. [28] reported on a study aimed at developing a clinically useful fetal *RHD* genotyping assay that does not give positive results in the case of a *RHD* gene variant. The authors combined the sensitivity of real-time PCR with an improved RhD typing assay to distinguish *RHD* from *RHDΨ*. This strategy enabled them to correctly predict the fetal *RHD* genotype in 100% of 137 women, 8-42 weeks pregnant. They utilised exons 4, 5 and 6 as opposed to exon 7 and 10 which were generally used by other studies. In the same year (2002), the development of a new quantitative real-time PCR enabled Costa *et al.* [63] to achieve a 100% detection rate in 102 RhD negative women in the first trimester. Several further studies with small numbers and similar success rates were published in the following years [8, 9, 45, 46, 64, 75, 88] in countries like Germany, Norway, Ireland, Denmark, Poland and Switzerland. These early studies reported the promising features of the NIPD test but a number of barriers to uptake into clinical practice were also pointed out. These included: the lack of positive controls for RhD negative female fetuses [46]; the considerable number of serological RhD negative individuals with non-functional or rearranged *RHD* genes (4% of RhD negative women) [44]; and the need for larger trials [8].

The first large-scale study was published in 2004 and comprised 893 RhD negative pregnant women [44]. This was also the first paper to distinctly mention the inclusion of 'Asian' as well as Caucasian women in the study. Using markers for exons 7 and 10 the researchers announced an accuracy rate of 99.5% with only 5 false positives and 4 false negatives. However, they also reported that 42 samples (5%) had to be excluded due to a non-functional or rearranged *RHD* gene. Due to the presence of this *RHD* gene, even though it is not functional, fetuses of women with a variant *RHD* gene could not be *RHD* genotyped non-invasively.

In 2005, a 2-year-experience study of sensitised women was reported from Belgium. A total of 223 fetuses of 218 pregnant women, including 13 Africans and 1 Asian woman, were accurately genotyped [87]. A combination of exons 4 and 5 with exon 10 in the testing strategy enabled the group to successfully identify fetuses with non-functional *RHD* genes, of which they encountered four. As a result, non-invasive prenatal determination of fetal RhD has already been introduced into clinical practice for *sensitised* women and has prompted modification of the management of these pregnancies.

Similarly, the provision of a clinical *RHD* genotyping service for *sensitised* women in the UK (see section 2.2.3) was announced in 2004 as a result of a 2002 evaluation study [28]. The objective was to effectively reduce invasive procedures by using non-invasive RhD-genotyping. Up to that point, 283 sensitised women had been tested and it was reported that the number of invasive procedures could be significantly reduced [67]. The test diagnosed the genotype of 223 out of 233 fetuses correctly. This study of sensitised women also identified a further reason for discrepancies between PCR and serology results. In 3 cases, serology showed an RhD-negative genotype while PCR indicated an RhD-positive fetus. It was concluded that serial intrauterine transfusion had possibly led to this discrepancy.

In France in 2005 the use of NIPD tests to more effectively utilise prenatal RhD prophylaxis was reported; only patients with an RhD positive fetus were provided with anti-D prophylaxis. Gautier *et al*. [60] reported that 285 RhD negative, non-sensitised pregnant women of various gestational ages had been tested and a 99.3% 'success rate' achieved. Only two samples could not be tested because these were cases of incomplete gene deletions in the mother. A sensitivity and specificity of 100% was reported, based on the 272 patients (96%) who were available for follow up.

In the Czech republic, Hromadnikova *et al*. [59, 65] have pursued a different aspect of *RHD* genotyping, assessing the feasibility of fetal *RHD* and *RHCE* genotyping. Their 2005 paper reported 100% accuracy, but they considered a sample positive if only one of 5 or 7 replicates was positive. The study was restricted to Caucasian pregnant women.

Even though the overall test results look promising and an accuracy rate of 100% has been reported in several studies, statements on reported accuracy need to be treated with some caution. Selective participant sampling, variations in the number of replicates taken into consideration for overall test results, as well as the exclusion of samples with non-functional or rearranged *RHD* genes, may have led to an overestimation of the reported accuracy rate in several studies [59, 44, 60, 63, 47, 61, 92]. In a recent article on design-related bias in studies of diagnostic tests, Lijmer *et al*. [66] emphasise that omitting cases that are difficult to diagnose causes an overestimation of sensitivity and specificity in many published papers.

4.3 Population Sampling Procedures

Most papers described their population sampling as a consecutive, prospective patient series, although a small number of studies did not clearly state how they recruited participants. Two studies recruited their study population retrospectively. The characteristics of the populations recruited varied quite considerably between studies and sampling did not always guard against bias. Some smaller studies even carried out selective participant sampling by choosing their participants against pre-specified criteria. One study selected 24 negative pregnant women and 4 patients with weak D phenotypes as the study population [58]. This implies an anticipation of identifying the four D variants. Other studies recruited women who were about to undergo invasive procedures [6, 47] or were about to receive genetic counselling [63]. This indicates that these studies concentrated on at risk pregnancies, which may not resemble the general pregnant population. Some studies concentrated on sensitised women [67], while others tested only non-alloimmunised pregnant women [60]. Selective participant sampling was taken even further by Nelson *et al*. [62], who recruited only RhD-negative pregnant women with RhD-positive fetuses and Bischoff *et al.* [61], who used retrospectively frozen serum of sensitised RhD negative women with RhD-positive fetuses. The ability of these studies to address clinical validity and test accuracy in the general population has to be questioned.

Restriction of patient sampling to Caucasians also reduces the clinical validity of findings, as does failure to report the make-up of the study population. Both were observed in the papers reviewed. In fact, most studies included in this report did not state the ethnic background of the sample population. Three studies specified their study population as Caucasian only [59, 60, 65], while only two studies described their study population in more detail. One study population was simply defined as a mixed population of Caucasians and Asians [44]. In this study, both sensitised and non-sensitised RhD negative pregnant women were tested. The other reported the inclusion of 13 Africans in the study population, of which 3 were of North African origin, and one Asian woman [87]. Although the study populations in this study probably reflects the likely testing clientele in Europe, numbers are too small to allow generalisation.

Three studies [11, 28, 67] may have included mixed populations because they reported random participant sampling. Finning *et al.* [28, 67] specifically adapted primer combinations in order to allow differentiation between various D variants including those prominent in people of African origin (see Annex 4). The negative phenotype in Blacks is more commonly caused by an RHD pseudogene (*RHD*Ψ) or a hybrid gene (*RHD-CE-D*^{S}). Overall, 67% of RhD negative black individuals have the pseudogene and 15% the hybrid gene. In African-Americans, the corresponding figures are 24% and 22% [54]. NIPD test diagnostic accuracy in US ethnic minorities (e.g. African Americans) has not been reported in any published studies.

5 SOCIO-ECONOMIC IMPLICATIONS

5.1 RhD NIPD Economic Evaluations

Economic evaluation of NIPD tests used in the context of RAADP for RhD negative pregnancies has been initiated in the Netherlands [11]. In their 2005 (unpublished) report, three scenarios are considered: 1) Current RAADP policy (i.e. only pregnant women with no living child, about 16,000, receive prenatal prophylaxis); 2) NIPD (PCR-test) employed to target RAADP in nulliparous women only; 3) NIPD PCR-test employed in all nulliparous and multiparous women. In a cost analysis, it was concluded, based on the Dutch model: that scenario 2) is 4.5% less expensive than current RAADP practice in the Netherlands; and that scenario 3) is 11.5% more expensive than current practice. There are no full economic evaluations e.g. including cost-utility measures. Further evidence on cost-effectiveness is required if wider implementation of RAADP plus RhD NIPD is to be supported by policy makers and health insurers. There are similarly no published economic evaluations of NIPD use in sensitised pregnancies.

In contrast, a cost-effectiveness analysis of the introduction of RAADP for pregnant women who are rhesus-negative is presented by Chilcott *et al.* in a UK report published in 2003 [1]. The authors concluded that RAADP could provide a cost-effective intervention when prevention of haemolytic disease of the newborn is taken into account. The report estimated that RAADP (2 doses) given to all pregnant women who are RhD-negative would be economically attractive, using a maximum acceptable cost-utility ratio of £30,000 per quality adjusted life year (QALY) as indicated by NICE [1]. Furthermore, because NHS list price (2002) for anti-D was used in these calculations, the authors suggested that in clinical practice, due to price variability, cost-effectiveness might be even better.

The ongoing work of the SAFE Socio-economic group includes co-ordinated economic modelling of RhD NIPD introduction into five selected countries (UK, France, Germany, the Netherlands and India).

5.2 RhD NIPD Technology Implementation

The first large scale clinical trial with automated high throughput screening of RhD negative women, to prove the suitability of the technology for mass screening, was also carried out in the Netherlands [11]. Using only the exon 7 PCR assay, a diagnostic 'accuracy' of 99.4% was reported with ‘equivalent’ test performance in all ethnic groups. Test accuracy in the specialist centre was reported to be higher than the serological test in a multi-centre routine setting.

This study is important since it shows development and progress from diagnostic evaluation studies to implementation in a clinical context. The report also provides a picture of how test delivery might best fit into current prenatal care in order for it to be cost-effective. The Dutch study reveals that only centralised implementation (i.e. 1-2 labs nationally) would be economically justified and the authors also suggest combining the time of testing with the 30th week antibody screening to reduce costs.

Further improvements in test performance for diverse populations can be expected after a PCR assay multiplexing exon 7 PCR with exon 5 has been validated by the same group. This assay will enable the

provision of results for women with an *RHD* pseudogene and the *RHD* variant type VI in the testing population. These are the most common variant genes in Africans and Caucasians, respectively. The improved test will result in a negative PCR in both cases and will not produce false positives.

Centres in Bristol, UK, Göttingen, Germany and Paris and Marseilles, France have started to offer RhD NIPD tests in clinical practice (by request) for non-sensitised as well as sensitised women. However, no publications on the former have been produced to date.

5.3 Risks and Limitations of Current NIPD Technology

The major risk associated with the introduction of RhD NIPD technology relates to the accuracy of the test in a mixed population. While a number of 2004 and 2005 publications indicate 99-100% accuracy rates [44, 58, 60], the true accuracy rate at the population level might be lower. The large number of variations in the *RHD* gene makes it difficult for primers to detect 100% of RhD variants using currently available technologies. As of now, a small percentage of misdiagnoses are unavoidable. The main disadvantage is linked to *false negatives*, with the associated risk that a small number of women may not receive prenatal anti-D, even though the fetal-maternal gene set-up would require it. Thus, unless 100% sensitivity (ability of test to *detect* RhD positive fetus when present) can be achieved these women might become sensitised with a likelihood of <2% [16], thereby increasing the total number of sensitised cases in a country. Furthermore, until 100% sensitivity *and* specificity (ability of test to exclude RhD positive fetus) rates are achieved, the post-birth blood cord sampling procedure is unlikely to be avoided even if the woman's fetus has been tested prenatally. The ability to abandon blood cord sampling would be an important factor influencing cost-effectiveness in favour of RhD NIPD implementation.

In terms of limitations, studies identify that at present testing women in the second trimester increases accuracy rates. Thus, the optimum time (as of now) for RhD NIPD testing is around week 26. This may require an additional appointment for a woman in different healthcare systems. However, it is anticipated that when further markers are developed, testing could be moved to earlier in the pregnancy and possibly be combined with other tests during the first prenatal appointment.

A further limitation of the RhD NIPD technology is that it requires specialist skills and laboratory infrastructure in order to conduct population level testing. While the necessary technology exists, the organisation of laboratory services, provision of high-throughput equipment, development of internal laboratory QA measures and an external quality assurance (EQA) scheme, and the training of laboratory personnel, physicians, midwives and nurses will require significant financial investment.

Finally, a potential unintended consequence of NIPD test development generally was highlighted in 2005 with articles reporting a home-based prenatal fetal gender test being offered by an online pregnancy store [77, 78]. The Baby Gender Mentor test involves self-collection of blood spot samples that are shipped directly to the manufacturer's laboratory for rapid turn-around. The manufacturers claimed the test could be used as early as 5 weeks into pregnancy and reported >99.9% accuracy [79]. The caution expressed by a number of scientists about the quoted test performance appears to be justified as wrong diagnoses have been revealed [80]. These have led to a class-action lawsuit being filed against the manufacturer (further information see www.in-gender.com). Non-invasive prenatal tests are also being

developed for the establishment of paternity during the term of pregnancy, with non-invasive paternity testing being offered throughout the world [81].

6 ONGOING RESEARCH

Further research is currently ongoing in all areas concerned with RhD NIPD test accuracy and reliability, as well as the development of large scale clinical trials.

A Scottish project was launched in 2002 to contribute to the general investigation of the feasibility of *RHD* fetal genotyping [98]. The aim was to test 100 primigravidae women and record reductions in diagnostic amniocentesis. This study is still ongoing. In 2004, the same group reported results of their ongoing development of the real-time PCR assay, in which they target exons 4, 5 and 7 of the *RHD* gene [92]. They have also expanded the field of application to the KELL blood group [93].

In terms of test development, standardisation of real-time PCR, the development of suitable positive controls for RhD negative female DNA samples, and alternative DNA sampling methods (i.e. transcervical canal aspirates) addressed in England [94]. There are also reports to indicate that MLPA (multiplex ligation-dependent probe amplification) may become a cost-effective alternative to real-time PCR. Although this technique lacks the sensitivity of real-time PCR, it possesses greater specificity due to the ability to test for a great number of markers simultaneously. A comprehensive *RHD* diagnostic MLPA test has been reported that includes probes for 7 out of the 10 *RHD* exons. Markers were selected to enable the test to identify several RhD polymorphisms. This new approach appears very promising since the combination of MLPA with real-time PCR (in the form of the novel LigAmp technique) would combine the sensitivity of one technology with the specificity of the other [94].

Confirmation of the technical performance of the RhD NIPD test method in prospective trials in mixed populations is still outstanding. In England, the IBGRL has set out to address this gap in the evidence. A trial will be mounted, adhering to the STARD checklist, to include 5,000 samples starting in 2006 [67]. Spare blood samples from women at about 28 weeks gestation will be made available from 5 antenatal laboratories in the UK. The samples will be tested for exon 5 and 7 in order to include *RHDΨ* detection.

Finally, a recent (unpublished) poster has presented the first meta-analysis of studies reporting the accuracy of non-invasive fetal *RHD* genotyping from maternal blood [95]. In their analysis the authors used a random effect model to compare results obtained from a number of studies with a total of 3,078 samples. A composite sensitivity of 0.954, specificity of 0.986, positive predictive value (**PPV**) of 0.990 and negative predictive value (**NPV**) of 0.921 were reported. Studies appear not to have been assessed for quality against the STARD or other checklist.

7 GAPS IN THE LITERATURE

At present a number of gaps exist in the literature on the given topic since the technology is new. As far as the fundamental science is concerned, it may take a few more years before the answers to some of these questions are available. On the other hand, the RhD NIPD technology is moving quickly towards the phase of clinical trials and we expect to see reports on test performance in large populations and different ethnic groups in the near future.

Evidence on test accuracy

Currently, the majority of reports on RhD NIPD performance are based on a small number of samples (N<100), with only the Dutch study based on a very large sample population of 2,543 women [11]. There is also a need to assess the performance of the technology in a diverse population in order to be able to evaluate the likely sensitivity and specificity of the test at a population level. Studies to date have included relatively small numbers of ethnic minority participants.

As can be seen in Appendix 2, application of the STARD checklist to currently published scientific papers leads to the conclusion that most studies of diagnostic accuracy are of poor design and quality. For more thorough evaluation of NIPD tests, we need studies which not only investigate a greater number of women but also report on clinical genotypic and phenotypic sensitivity/specificity, as well as specifying the statistical certainty and uncertainty of any results obtained. Furthermore, elimination of any potential bias due to sampling of participants and a generally more rigorous approach towards the study design is desirable, including blinding of test readers, recording of time and place of recruitment, and inclusion of 'booster' samples for black and ethnic minority groups if necessary.

Evidence on quality assurance

An optimum testing protocol (test standardisation) in conjunction with internal laboratory QA measures needs to be finalised. External quality assurance (EQA) schemes also need to be developed for RhD NIPD. As part of this process, further comparisons of results in multiple laboratories would be valuable, as well as reports of the skill levels required for implementation of the new test.

Guidelines on the management of RhD negative pregnancies differ from country to country and, for the most part, these are not published in international literature. Information is needed on current management of these pregnancies; incidence of sensitisation and HDN, and laboratory expertise and organisation.

Broader socio-economic implications

As Section 5 makes clear, there are very few studies covering the broader socio-economic aspects of the new technology and the medical condition concerned. Further studies of cost-effectiveness and economic modelling in different countries are essential. An international overview of practices in prenatal care, costs, types of healthcare system and usage of non-invasive technologies would be an invaluable document in this respect. Also, even though the risks and ethical issues associated with the introduction of NIPD RhD tests into clinical practice may be viewed as of lower priority than for other genetic tests, they cannot be ignored. From the risk perspective, issues associated with the sensitivity and specificity of the test need to be communicated to women, as do possible (even though unlikely) side effects from the use of anti-D products (serum or recombinant). From the ethical perspective, issues of population diversity and women's choice will become relevant as the test enters the population

level of adoption. Furthermore, the potential use of the new technology for fetal gender identification and, therefore, possible sex selection, should not be ignored.

The available evidence on risk communication to pregnant women and informed choice are being examined by two other SAFE workpackages (WP5 on Psychosocial aspects of risk communication and WP7 on Ethical aspects). Their activity will hopefully provide some information concerning risks and ethical aspects relevant to NIPD for *RHD* genotyping. At present, this technology has not been evaluated from either the risk or ethics perspective in the literature.

Finally, in terms of organisational implications, no publications exist which describe the necessary arrangements, i.e. infrastructure and finance in order to fully automate procedures, create universal kits for RhD NIPD and set up central laboratories. The factors which will influence market entry need to be researched.

8 CONCLUSIONS

It is evident from this literature review that NIPD RhD tests have entered, or are about to enter, clinical practice in a number of countries. The evidence base remains, at present, incomplete in many respects.

For *sensitised* women, it is recognised that the current use of prenatal invasive technologies is associated with an increased risk of miscarriage. Prenatal diagnostics for these women would therefore benefit from a move away from the use of invasive tests towards the use of non-invasive techniques. Non-invasive fetal *RHD* genotype testing is already being used in combination with other tests for the care of sensitised women in the UK, Czech Republic, the Netherlands, and some other countries. This can be interpreted as a successful introduction of NIPD tests into prenatal care. However, NIPD tests are currently more consistently accurate in the 2nd trimester (around week 26). Non-invasive testing during the first trimester would alleviate worry and remove procedures in pregnancies where the fetus is found to be RhD negative. Studies to further develop the test have been undertaken in laboratories in the UK, the Netherlands, Belgium, France, Italy, Hong Kong and the USA.
Since 2005, trials have also been underway of non-invasive diagnosis of fetal *RHD* genotype in routine pregnancies of RhD negative women - a much larger group of women. For *non-sensitised* women, the use of NIPD to target routine antenatal anti-D prophylaxis is more complex. At present, conclusions cannot be drawn purely from the peer reviewed literature covering non-invasive prenatal *RHD* genotyping. Additional information is needed to reveal the present state of development of the NIPD technology. Overall, the technology continues to be in the experimental stage rather than clinical practice. Efforts to collect more data from large scale population studies continue and the number of tests performed is increasing rapidly. It is clear that introduction of NIPD testing at a national level will require the establishment of centralised, automated laboratories, additional training for physicians, midwives and laboratory technicians, as well as information dissemination to patients in each country. To what extent and at what cost has not been quantified at the present date.

Taking this into consideration, there is no clear evidence as yet to demonstrate whether this new technology will be significantly more cost-effective than current practice for non-sensitised pregnancies. Further cost-effectiveness studies, building on the Netherlands study, which include all known cost factors, are urgently required and should include an attempt to identify currently unknown costs. This is the aim of the SAFE NoE (WP6 on Socio-economics) and work on economic modelling has already commenced. To date, the introduction of RhD NIPD has not occurred on grounds of cost-effectiveness and the future development will be followed with great interest.

Aside from the issue of direct costs, there may be other opportunity costs associated with different approaches. The use of donor blood for the production of anti-D immunoglobulin, for example, could be reduced by approximately 40% or less and used for other products or purposes, if RhD NIPD tests were introduced.

However, while the introduction of RhD NIPD tests will help to identify the need for routine antenatal anti-D prophylaxis (RAADP), overall this test is unlikely to significantly decrease the total number of sensitisations and cases of HDN compared to the impact achieved by a universal RAADP.

Furthermore, despite the reported benefits of RAADP in decreasing the number of sensitisations and cases of HDN further than postnatal prophylaxis alone, only postnatal administration of anti-D is

practised in many countries. The development of RhD NIPD tests may have the potential to influence the implementation of RAADP in these countries, if cost-effectiveness can be demonstrated. In other countries like the UK where policy guidelines exist for RAADP, but are not uniformly followed, NIPD may improve uptake.

Finally, it might be argued that the clinical benefits are too insignificant for RhD NIPD to be introduced into the routine prenatal care of non-sensitised women. However, successful introduction of these tests may be advantageous in allowing other NIPD tests, based on similar techniques, to be incorporated into the system of prenatal diagnostics in countries. Thus the RhD test may have a role as a pioneering technology in the area of NIPD testing and could help to establish techniques and quality assurance mechanisms, and set up laboratories and required infrastructure, thus generating a platform from which implementation of other NIPD tests can easily take off. A goal for the future might be one sample for a series of NIPD analyses carried out in the first trimester of pregancy. RhD NIPD would then be just one component in an overall test package that could help to determine pregnancy status, future fetal development and likely pregnancy outcome. In this context, RhD NIPD implementation can be viewed as essential to kick-start the introduction of NIPD technology more broadly.

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Annex 1: STARD checklist for reporting NIPD study diagnostic accuracy

Section and topic	No	Checklist items	Yes/ No
TITLE/ABSTRACT/ KEYWORDS	1	Identify the article as a study on diagnostic accuracy (recommend MeSH heading 'sensitivity and specificity').	
INTRODUCTION	2	State the research questions or study aims, such as estimating diagnostic accuracy or comparing accuracy between tests or across participant groups.	
METHODS			
Participants	3	Describe the study population: The inclusion and exclusion criteria, setting and locations where the data were collected.	
	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?	
	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.	
	6	Describe data collection: Was data collection planned before the index test and reference standard were performed (prospective study) or after (retrospective study)?	
Test method	7	Describe the reference standard and its rationale	
	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.	
	9	Describe definition and rationale for the units, cutoffs and/or categories of the results of the index test(s) and the reference standard.	
	10	Describe the number, training and expertise of the persons executing and reading the index tests and the reference standard.	
	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.	
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).	
	13	Describe methods for calculating test reproducibility, if done.	
RESULTS			
Participants	14	Report when study was done, including beginning and ending dates of recruitment.	
	15	Report clinical and demographic characteristics of the study population (e.g. age, sex, spectrum of presenting symptoms, comorbidity, current treatments, recruitment centers).	
	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).	
Test results	17	Report time interval from the index tests to the reference standard, and any treatment administered between.	
	18	Report distribution of severity of disease (define criteria) in those with the target condition; other diagnoses in participants without the target condition.	
	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.	
	20	Report any adverse events of index tests and reference standard	
	21	Report estimates of diagnostic accuracy and measures of statistical certainty (e.g. 95% confidence intervals).	
	22	Report how indeterminate results, missing responses and outliers of index tests were handled.	
	23	Report estimates of variability of diagnostic accuracy between subgroups of participants, readers or centers, if done.	
	24	Report estimates of test reproducibility, if done	
DISCUSSION			
Discussion	25	Discuss the clinical applicability of the study findings	

Annex 2: Overview of scientific papers reviewed

Literature Summary of scientific papers: technical evaluation of NIPD RhD

Year and country	Study Type	Study aim	Title	Method / Ref test	Foetal cells (FC) / free foetal DNA (ffDNA)	Exons / Introns tested	Replicates	Sample no /gestation	Sensitivity/Specificity	Outcome	Author and Publication	Score *
Netherlands 2005 (ZonMw)	Prospective	Enhance the efficiency of immunoprophylaxis in RhD- pregnancies by selective, PCR-guided, prenatal administration rather than universal administration of anti D-IgG	Prenatal foetal RhD blood group typing to enhance efficiency of prenatal anti-D immunoprophylaxis in RHD negative pregnant women	Real time PCR / serology		Exon 7, additionally exon 5 if RhD variant is suspected	3 replicates, only samples with 2 or 3 positive wells were scored as a positive test	Random cohort of 2500 (2380) blood samples of RhD-negative 30th week pregnant women sent in for allo-antibody screening (Sept - Dec 2003)	5 false positives, 3 false negatives, 99.4% accuracy	Diagnostic accuracy was 99.4%, test performed well in all ethnic groups. A small economic benefit is achieved. No disadvantages were observed; only centralised implementation (including registration, surveillance, and PCR adaptation) is economically efficient.	Bonsel, H. *et al.* ZonMw	**No peer-reviewed report**
Denmark 2005	Prospective	To establish a reliable test for prenatal prediction of foetal RhD type	Reliable test for prenatal prediction of foetal RhD type using maternal plasma from RhD negative women	Novel real time PCR-based exon 7 assay combined with exon 10 assay / serology	ffDNA from plasma	Exons 7 and 10	4 replicates	56 RhD negative pregnant women 15-36 weeks pregnant including 18 stored samples from UK for final validation of assay	Specificity: 94.7% Sensitivity: 100% 100% concordance from 16th week, one sample from 15th week was inconclusive, sample was considered positive when at least two replicates were positive for both exons	Set up was very reliable	Clausen, F.B. *et al.* Prenatal Diagn (in press)	**9.5/25**
Belgium 2005	in French	To evaluate the predictive value of RhD foetal genotyping	Prenatal determination of foetal RHD in maternal plasma: two-years experience of routine clinical use	Real-time multiplex PCR / serology	ffDNA from plasma	Exons 4, 5, and 10		218 pregnant women of various gestational ages including 5 twin pregnancies	100% concordance, 3 positive cases for exon 10 due to RhD psi in mother	Using this method for two years in routine practice has led us to modify out management scheme for sensitised RhD negative pregnant women	Minon, J.-M. *et al.* J Gynecol Obstet Biol Reprod **34**:448-453	**11/25**
Czech Republic 2005	Prospective		Non-invasive foetal RHD and RHCE genotyping from maternal plasma in alloimmunised pregnancies	Real time PCR / serology	ffDNA from plasma	RHD: exons 7 and 10 RHCE: intron 2 and exon 5	7 replicates	23 alloimmunised Caucasian pregnant women (16 anti-D; 5 anti-D+C; 2 anti E) 11 to 37 weeks pregnant	100% (in three samples foetal DNA very low. 2 cases developed severe HDN)	Present data present compelling argument that this type of testing may be incorporated into our clinical diagnostic algorithm for following pregnancies at risk for HDN.	Hromadnikova, I. *et al.* Prenatal Diagnosis **25(12)**: 1079-1083	**10.5/25**
Czech Republic 2005	Prospective	Assess feasibility of foetal RHD and RHCE genotyping	Non-invasive Foetal RhD and RhCE Genotyping using real-time PCR testing of maternal plasma in RhD negative pregnancies	Real time PCR / serology	ffDNA from plasma	RHD: exons 7 and 10 RHCE: exon 2 and 5	5 replicates	45 RhD negative Caucasian pregnant women 11to 40 weeks pregnant	100% accuracy rate sample was considered positive if at least one replicate was positive	Non-invasive RhD is recommended together with foetal sex determination in alloimmunised D-negative pregnancies	Hromadnikova, I. *et al*. Journal of Histochemistry and Cytochemistry **53**(3): 301-5	**11/25**
Czech Republic 2005	Prospective		Non-invasive foetal RhD exon 7 and exon 10 genotyping using real-time PCR testing of foetal DNA in maternal plasma	Real time PCR / serology	ffDNA from plasma	Exons 7 and 10	5 replicates	24 RhD-negative pregnant women and 4 patients with weak D phenotypes, 11 to 38 weeks pregnant (mainly II and III trimester)	Specificity approaching 100%; sensitivity 100% for exon 7; 2 exon 10 not detected despite positive exon 7 results (1 could be shown to be absent)	Non-invasive prenatal RhD genotyping should involve the amplification of at least two RhD-specific products.	Hromadnikova, I. *et al*. Foetal Diagn Ther **20**(4): 275-280	**9.5/25**
Spain 2005	Prospective	Evaluate the use of foetal DNA in maternal plasma for clinical application	Application of foetal DNA detection in maternal plasma: a prenatal diagnosis unit experience	Real-time multiplex PCR / PCR from CVS or amniotic fluid	ffDNA from plasma	Exon 7		20 RhD negative pregnant women 11 to 16 weeks pregnant	90% sensitivity	Successful application of QF-PCR to diagnose Huntington disease and cystic fibrosis, 90% for foetal RhD were correctly diagnosed. Foetal RhD status detection has been considered a useful tool in cases of sensitised women with heterozygous partners. An assay with a larger number of patients is being performed to determine sensitivity and usefulness. DNA samples should be processed as quickly as possible.	Gonzalez-Gonzalez,C. *et al.* J. Histochem Cytomchem **53** (3): 307-14	**4.5/25**

* Assessment against STARD checklist by a second independent reviewer is still required

France 2005	Prospective		Foetal RhD genotyping by maternal serum analysis: a two year experience	Real-time PCR / PCR from amniotic cells or serology	ffDNA from serum	exon 10	4 replicates	285 RhD negative pregnant women (Caucasians, non sensitised), 8-35 weeks pregnant	In 2 cases RhD status could not be determined due to uncomplete RHD gene deletion in mother (gene variant) 100% accuracy in 272 patients that were available for follow up specificity and sensitivity of the assay were 100% overall success rate 99.3%	Prophylaxis was not provided to patients with RhD negative fetus. RhD genotyping assay can be systematically proposed to all RhD-negative women in order to more effectively utilize RhD prophylaxis.	Gautier, E. and Costa, J-M. *et al.* American Journal of Obstetrics and Gynaecology 192: 666-9	**15/25**
Netherlands 2004	Abstract		Screening for foetal RHD-genotype by plasma PCR in all D-negative pregnant women is feasible	Real-time exon 7 PCR / serology	ffDNA from plasma			2543 D-negative pregnant women, 28-30 weeks pregnant	Specificity: 98.2% sensitivity: 98.2% in 7 cases genotype suggests D-positivity maybe due to weak-D or null alleles. In 7 cases no RHD-sequence were detected but cord blood was typed positive.	First large scale study demonstrating the feasibility of screening D-negative women to restrict prenatal anti-D to women carrying D-positive fetuses. Using an automated assay.	Van der Schoot, C.E. *et al.* Vox Sang 87 (Suppl3): Abstract (Tu07.04)	**Short abstract**
UK 2004	Prospective		A clinical service in the UK to predict foetal Rh (Rhesus) D blood group using free foetal DNA in maternal plasma	Real time multiplex PCR / serology	ffDNA from plasma	Exons 4,5 (excluding RhD (psi)) and 10	4 replicates	283 immunised pregnant women with heterozygous partner	50 confirmatory results awaited, 223 correctly predicted, no result in 7 cases, 3 cases with discrepant serology (possibly due to intrauterine transfusions)	Providing the non-invasive test for foetal genotyping has significantly reduced the number of invasive procedures carried out in the UK. Future application: to reduce anti-D application (currently 10500 RhD negative pregnant women per year in England and Wales)	Finning, K. *et al.* Ann N Y Acad Sci 1022: 119-123	**5.5/25**
UK 2004	2 case studies		Use of maternal plasma for non-invasive determination of foetal RhD status	PCR / amniocentesis / serology in case two	ffDNA of plasma			2 samples from RhD Sensitized patients 18 weeks pregnant	Both cases RhD status accurately determined	Maternal plasma testing for foetal RhD status represents a new tool in the management of cases of RhD sensitisation in pregnancy. RhD negative girl in first case prompted amniocentesis in week 20.	Harper, T.C. American J. of Obstet & Gyn **191:** 1730-2	**Case study**
France 2004	Prospective	Feasibility and accuracy of large scale RhD foetal diagnosis in RhD negative women and to validate the diagnostic use of foetal RHD genotyping	Large-scale pre-diagnosis study of foetal RHD genotyping by PCR on plasma DNA from RhD-negative pregnant women	Conventional and real time PCR / PCR from amniotic cells or serology	ffDNA from plasma	Exons 7 and 10	2 replicates (one conventional PCR one real time PCR), positive result if at least exon 7 was positive in both PCRs	893 RhD-negative women (Caucasians and Asians) immunised and non-immunised 7 to 40 weeks pregnant	42 samples were excluded due to non-functional or rearranged RHD gene (4% of RhD negative women); accuracy 99.5%, 5 false positives, 4 false negatives	Exons 7 and 10 of RhD gene were amplified. A strategy is proposed to avoid small number of false positives and negatives. Real time PCR is not more sensitive than conventional PCR but has an extra level of protection against contamination, less time consuming, best specificity	Rouillac-Le Sciellour, C. *et al.* Mol Diagn. **8**(1): 23-31	**11/25**
Poland 2004	prospective	Detection of fetal *RHD* gene in the plasma of RhD negative mother and comparison with RhD of the newborn	Non-invasive foetal RhD typing and RhD negative pregnant women - prelim. Observations	Real-time PCR	ff DNA from plasma	Exons 7 and 10		45 plasma samples of 28 RhD negative women, various gestation age	All 23 RhD positive fetuses were detected, in 5 RhD negative samples other fetal genes were detected	Real-time PCR is an appropriate non-invasive method for foetal RhD examination, 2 RhD exons should be examined	Guz, K. *et al.* Ginekol Pol **75**: 21-5	**Polish**
Ireland 2003	Prospective		Detection of foetal Rhesus D gene in a whole blood of women booking for routine prenatal care	Real time quantitative PCR / serology	ffDNA from whole blood	Exon 10		31RhD negative women before 20 weeks gestation	82% accuracy rate no false positives	Whole blood used, larger volume of blood for DNA extraction increased sensitivity of RhD assay, non-invasive procedure is feasible, more studies needed. Real time quantitative PCR can be used for RhD genotyping of fetus if false negative results can be eliminated. 18% of RhD+ babies would not be detected at the first prenatal visit. Foetal DNA analysis was not revealed until after delivery.	Turner, M.J. *et al.* European J. of Obstetrics and Gynaecology and Reproductive Biology **108**: 29-32	**13/25**

Australia 2003	Prospective / retrospective		Evaluation of the clinical usefulness of isolation of foetal DNA from the maternal circulation	PCR / serology	ffDNA from plasma or serum	Exons 7 and 10		28 blood samples of RHD-negative women 15-17 weeks pregnant collected for maternal triple serum screening	3 false negative and 2 false positive results	Both amplifications of the Rh genes were performed in the same tube. It is necessary to safeguard against contamination and cover the polymorphisms present within the RhD gene. False positives may result from gene rearrangements and false negatives due to non-functioning genes. Out of 24 samples 21 were correctly sexed.	Siva, S.C. *et al.* Aust N Z J Obstet Gynaecol **43**(1): 10-15	**12.5/25**
Norway 2003	Prospective	Performing foetal RhD genotyping investigating the effect of storage temp on DNA concentration	Prenatal genotyping of RHD and SRY using maternal blood	Monoplex and duplex fluorescent PCR / serology	ffDNA from blood	Exon 7	2 – 5 replicates	114 RhD negative women 6-38 weeks pregnant 22 samples in duplex with SRY gene	290 samples: 32 false negatives, 4 false positives discrepancy: 12%, 8% if one positive replicate counts as positive result	There was a statistically significant reduction of foetal DNA in samples stored at room temperature. Duplex PCR showed reduced sensitivity for SRY gene amplification. Lack of positive control for RHD negative female fetuses. Therefore this duplex PCR is not suitable for routine analysis.	Randen, I. *Et al.* Vox Sanguinis **85**: 300-306	**9.5/25**
Australia 2003	Prospective letter		Variation in the reliability of RHD prenatal genotyping using the polymerase chain reaction and targeting multiple exons of the RHD gene	Conventional PCR / serology	ffDNA from plasma	Exons 7 and 10		47 RhD negative pregnant women (II and III)	Exon 4 5 10 Sensitivity 62 50 94 Specificity 91 100 36 for multiple exons (at least two exons concordant : sensitivity:100 specificity: 91	More than one exon of the RhD gene must be examined. Conventional PCR might be unsuitable for diagnostic use.	Johnson, L. *et al.* Vox Sanguinis **85**: 222-223	**Case study**
Germany 2002	Retrospective	Evaluation of real-time PCR method for the detection of RHD, C, c, E and e	Prediction of foetal RhD and Rh CcEe phenotype from maternal plasma with real-time PCR	Real-time PCR / serology	ff DNA from thawed excess plasma	Exon 7, RHD psi - and RHD psi	4 replicates	16 mostly alloimmunised pregnant women 11 - 38 weeks pregnant	1 false negative, at least two positive results needed for overall positive tests result	D status determined in 26 out of 27 cases	Legler, T. *et al.* Transfus Apheresis Sci **27**(3): 217-23	**10/25**
France 2002	Prospective		Foetal RhD genotyping in maternal serum during the first trimester of pregnancy	New quantitative real-time PCR/ PCR of DNA from invasive procedure or serology	ffDNA from serum		2 replicates	102 RhD- women in first trimester undergoing genetic counselling	100% detection rate / 62 positive and 40 negative fetuses identified	Sera used: Recommend technique for routine application	Costa, J.-M. *et al.* Brit J. of Haem **119:** 255-260	**11/25**
UK 2002	Prospective	To develop a clinically useful foetal RHD genotyping assay that detects only RHD and not RHD psi or RHD-CE-Ds	Prediction of foetal D status from maternal plasma: introduction of a new non-invasive foetal RhD genotyping service	Real-time PCR/ PCR of DNA from invasive procedure or serology	ffDNA from plasma	Exons 4, 5 and 6		137 RhD- women 8-42 weeks pregnant	100% detection	Plasma used: non-invasive combination of the sensitivity of real-time PCR with an improved RhD typing assay to distinguish RhD from RhDψ enables highly accurate prediction of foetal D status. More DNA found when fetus was female than in male pregnancies. Non-invasive technique more accurate in twin pregnancy than invasive.	Finning, K.M. *et al.* Transfusion, **42**:1079-1085	**14/25**
Switzerland 2001	Prospective		Risk free simultaneous prenatal identification of foetal Rhesus D status and sex by multiplex real-time PCR using cell free foetal DNA in maternal plasma	Novel multiplex real-time PCR / PCR of DNA from invasive procedure	ffDNA from plasma			34 maternal plasma samples from rhesus D negative pregnant women, II trimester about to undergo invasive procedure	Correctly: 12/13 Rhesus D positive males, 5/5 Rhesus negative males, 100% female RhD positives or negatives	First report describing the determination of multiple foetal loci from cell free foetal DNA. Both loci are amplified with the same efficacy; Rhesus D could be detected in highly diluted samples. Suitable for automation.	Zhong, X.Y. *et al.* Swiss Med Wkly **131** (5-6): 70-74	**10.5/25**

Australia 2001	Prospective		Genotyping foetal DNA by non-invasive means: extraction from maternal plasma	Real-time PCR / serology	ffDNA from plasma			26 RhD negative women pregnant with RhD positive fetuses 9-34 weeks pregnant	100% accuracy, all RhD genes could be amplified	Experiment might not have been carried out blind. Different times of clearance of foetal DNA postnataly have been discovered.	Nelson, M. *et al.* Vox Sang **80**: 112-116	**4/25**
UK 1999	Prospective	To develop a non-invasive method for determining foetal RhD status in order to provide improved care for women most at risk	Non-invasive RNA-based determination of foetal Rhesus D type: a prospective study based on 96 pregnancies	Reverse transcription PCR / serology	mRNA from erythroblasts	Intron 4 of RhD and CE (control), exon 10		96 RhD negative women: various stages of gestation about to undergo invasive procedures	Overall 75% accuracy rates III: 85%; II: 82%; I: 48% 18 false negatives	Suggest that reverse transcription may be a useful and perhaps more sensitive alternative to standard genomic PCR	Cunningham, J. e*t al.* Brit. J. of Obstet Gynaecol **106**:1023-8	**10.5/25**
USA 1999	retrospective	To investigate whether foetal RhD status can be determined in sensitised RhD negative pregnant women with RhD heterozygous partners.	Non-invasive determination of foetal RhD status using foetal DNA in maternal serum and PCR	PCR and nested PCR / serology	ffDNA from serum	Exon 7		Frozen serum of 20 sensitised RhD-women 15-36 weeks pregnant with RhD+ fetus	in 70% (14) RhD+ fetuses were detected, no false positives PCR: 10/20 nested PCR: 10/16	Detection of foetal RhD sequence in maternal serum of sensitised women	Bischoff, F.Z. *et al.* J of Social Gynecol. Investig **6**: 64-9.	**9.5/25**
UK 1998	Prospective	Assess the feasibility of fetal *RHD* genotyping using fetal DNA extracted from plasma samples	Prenatal diagnosis of foetal RhD status by molecular analysis of maternal plasma	Fluorescence based PCR / serology or PCR of amniotic fluid	ffDNA from plasma	Exon 10		57 RhD- (12-I), (30-II), (15-III)	II and III: 100% detection rate I: 2 RhD+ fetuses undetected (no RhD DNA), 10 samples correctly genotyped	Plasma used: genotyping can be performed rapidly and reliably beginning with the 2nd trimester	Lo, D.Y.M. *et al.* NEJM, **339**: 1734-8	**11/25**
Netherlands 1998	Prospective letter		Detection of foetal RhD-specific sequences in maternal plasma	PCR of serum / PCR of amniotic cell DNA	ffDNA from plasma	Exon 7		31 RhD- 16-17 week and 12 RhD-not pregnant (control)	All negatives were correctly identified / 18 (=all) RhD+ fetuses correctly identified	Plasma PCR is promising in prenatal tests	Faas, B.H.W. *et al.* The Lancet **352**:1196	**11/25**
Ongoing research/unpublished												
UK			Further developments of non-invasive prenatal diagnosis using maternal plasma? TCC and a source of foetal DNA					200+ multi-ethnic women,			Avent, N. *et al*.	
UK			Identification of foetal genotype in uteri using DNA extracted from maternal plasma.					100 primigravidae with unknown Rh status			Urbaniak, S. *et al*.	
Paternal RhD genotyping												
Switzerland 2003			Determination of RhD zygosity using real-time quantitative PCR							Real-time PCR developed for the determination of RhD gene and the fetal Y chromosome from maternal plasma can be used to determine the paternal RhD genotype	Li, Y.et al Swiss Medical Weekly, 133(31-32): 442-5	

Annex 3: Numbers and ratio of RhD-positive and RhD-negative babies reported in 18 studies

	Sample size	RhD positive		RhD negative		Reference List Number
		Number	%	Number	%	
	213	137	64.32	76	35.68	87
	31	17	54.84	14	45.16	2
	24	12	50.00	12	50.00	58
	17	12	70.59	5	29.41	59
	283	179	63.25	104	36.75	60
	114	82	71.93	32	28.07	46
	102	62	60.78	40	39.22	63
	20	14	70.00	6	30.00	48
	57	39	68.42	18	31.58	10
	2543	1549	60.91	994	39.09	49
	31	18	58.06	13	41.94	86
	47	36	76.60	11	23.40	45
	45	24	53.33	21	46.67	65
	96	56	58.33	40	41.67	6
	842	649	77.08	193	22.92	44
	58	34	58.62	24	41.38	88
	28	23	82.14	5	17.86	75
	2380	1465	61.5	915	38.5	11
Total	**6931**	**4408**	**63.60**	**2523**	**36.40**	

Annex 4: Molecular bases of the RHD gene

The *RHD* and the *RHCE* gene were cloned in 1990, which allowed thorough investigation and characterisation of the molecular bases of these two highly homologous (93.8%) genes. *RHD* and *RHCE* are situated in tail-to-tail configuration on chromosome one. They are arranged in 10 exons each and the *RHD* gene is flanked by two 9-kb regions of 98.6% identity, the *Rh boxes.* The gene products, the Rh antigens, are 416 amino acid transmembrane proteins presumably involved in ammonia and ammonium transport. The Rh proteins have 12 hydrophobic transmembrane domains and 6 extracellular loops [13, 14].

The homology between the two genes challenges RhD genotyping because the RHCE gene is nearly universally present and sequences have to be carefully selected for RHD genotyping to be specific. Two major differences between RHD and RHCE have been discovered and subsequently exploited for the development of PCR reactions. These are a 3' untranslated region of exon 10 of RHD, which is not present in RHCE, and a 600-bp difference in intron 4 between RHD and RHCE [50].

The two proteins differ by 32 to 35 amino acids. Nine of these RhD specific amino acids are situated at the extracellular surface of the red blood cells, defining the numerous epitopes on the six extracellular loops of the RhD protein explaining the high serological complexity of the RhD antigen [51].

The absence of one or more D epitopes in *RhD positive individuals* can lead to qualitative variants, which are able to produce antibodies to the missing parts of the antigen when exposed to the whole D antigen. These D variants are called partial D. Seven different categories of partial D have been established. D^{IV} lacks the most epitopes and is the most frequent type, with gene frequencies of 0.02-0.05% ([52] and references therein). The D^{IV} phenotype is the *only known D variant phenotype* that can make anti-D antibodies that are clinically significant in that they are capable of causing severe HDN and neonatal death. Serology and genotyping are required to type partial D women D-negative since a RhD-positive fetus will be problematic in these pregnancies. But when polyclonal antibodies are used for RhD typing these women will type RHD positive [52].

There are three genetic mechanisms that result in partial-D and D-variants.

- Substitution of part of the RHD gene with RHCE
- Deletion of part of the RHD gene
- Mutations resulting in single amino-acid substitutions in the extracellular parts of the polypeptide

A further group of D variants is the weak D type. This is characterised by a qualitatively normal D antigen, which is expressed at a low level. These variants are caused by nucleotide changes resulting mostly in point mutations in the transmembrane or cytoplasmic region of the polypeptide. This presumably affects efficient expression or correct insertion into the membrane resulting in a reduced number of copies on the red cell surfaces.

Weak D individuals are required to be typed as D-positive as some of them are able to cause alloanti-D immunisation [53]. This is important for fetal genotyping as weak D fetuses might cause sensitisation of the maternal circulation.

Very weak forms of D are D_{el}, which can be found in individuals in the Far East, and Rh_{null} as well as Rh_{mod}. The latter two are caused by mutations in the RHAG gene, a homologous gene involved in guiding the RhD and RhCE proteins in the red cell membrane.

The RhD negative phenotype occurs in Caucasians with a frequency of ~15% and is most commonly characterised by homozygosity for a deletion of RHD. RhD negativity is less frequent in Africans or Asian. But the negative phenotype in Blacks is more commonly caused by two specific variant genes:

- *RHD*Ψ an RHD pseudogene which contains a 37bp insertion in exon 4 and a nonsense mutation in exon 6 and
- *RHD-CE-D*^{S}, a hybrid gene comprising exons 1, 2, and the 3' end of exon 3 of *RHD*, the 5' end of exon 3 and exons 4–7 of *RHCE*, exons 9 and 10 of *RHD,* and exon 8 is undetermined

Individuals with these gene variants possess at least part of the RHD gene but lack the antigen on their erythrocytes. Testing for these genes in a mixed population requires a test that either positively distinguishes RHDΨ from RHD, or gives a D– result when RHDΨ is present. Overall, 67% of RhD negative black individuals have *RHD*Ψ and 15% *RHD-CE-D*^{s}. In African-Americans, the corresponding figures are 24% and 22% ([54] and references within).

See [14, 50, 54, 55] for more detailed reviews on RHD variant genes.