Prenatal RhD Testing: A Review of Studies Published from 2006 to 2008*

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Key Words  
Fetal DNA · Maternal plasma · SAFE

Summary  
The availability of noninvasive prenatal diagnosis for the fetal RhD status (NIPD RhD) is an obvious benefit for alloimmunized pregnant women. This review gives information about the performance characteristics of current diagnostic technologies and recent promising proof-of-principle studies. Notably, during the past 3 years almost twice as much samples have been investigated with NIPD RhD compared with the studies from 1998 to 2005. Thus we have now a lot more information compared with the knowledge before 2006. There is no doubt that funding of the SAFE Network of Excellence (2004–2009) from the European Commission within the framework 6 program has massively increased the worldwide experience in NIPD RhD. In 2009 European funding has been stopped. Because of this large investment from public funding sources, it is now the duty of policy makers (scientific boards, patient groups, physicians organizations, and health assurances) to discuss if targeted antenatal Rh prophylaxis should be introduced in German-speaking countries or which additional data are required to make a decision and how these additional studies should be funded.

Schlüsselwörter  
Fetale DNA · Mütterliches Plasma · SAFE

Zusammenfassung  

*This work is part of the doctoral thesis of S.P. Müller.
Introduction

Noninvasive prenatal diagnosis for the fetal RhD status (NIPD RhD) by RHD genotyping from maternal plasma is considered a valuable tool in the identification of pregnancies at risk of hemolytic disease of the fetus and newborn (HDFN) [1–6]. Anti-D still remains the most common antibody specificity to cause this severe clinical problem in the Caucasian population [7]. The knowledge of the fetal RhD status aids in the management of alloimmunized pregnancies in RhD-negative women [4]. Advantages are that special management and monitoring, which is required in respective pregnancies, can be initiated in time and concentrated on alloimmunized pregnant women at risk of HDFN due to a fetus positive for the respective antigen.

Pregnancies complicated by RhD alloimmunization have to be monitored for fetal anemia since effective pre- and postnatal transfusion treatment is available to prevent excessive fluid collection in extracellular spaces (hydrops fetalis) and eventually fetal death [4]. Because amniocentesis is associated with a risk of transplacental hemorrhage of up to 17% [8] and the risk of pregnancy loss was found to be 1.9 and 2% after amniocentesis and chorionic villous sampling (CVS) [9], respectively, invasive procedures should be avoided in alloimmunized pregnant women.

Since 2001 NIPD RhD is already offered to immunized women with heterozygous partners as a routine clinical service by the British National Blood Service [2]. In several European countries, including Austria, Germany and Switzerland, NIPD RhD using cell-free fetal DNA (cff-DNA) in maternal peripheral blood is currently available to determine the fetal RhD status. Quality assurance workshops and standardization as well as large-scale validation studies have been coordinated and partially funded from 2004–2009 by the Special Non-Invasive Advances in Fetal and Neonatal Evaluation (SAFE) Network of Excellence.

In parallel with this change towards a noninvasive procedure for molecular fetal blood group determination, the measurement of the change in optical density at a wavelength of 450 nm in amnion fluid (i.e. invasive test for the detection of hemolysis) has been replaced by doppler measurement of the peak velocity of systolic blood flow in the middle cerebral artery (i.e. noninvasive test for the detection of fetal anemia) [10, 11].

NIPD RhD is not only a valuable tool in the management of pregnancies at risk of HDFN, but if implemented in the routine examination of all pregnant women at risk it would also allow to specifically administer Rh immune globulin (RhIg) only in those cases where the fetus is RhD-positive. This would lead to a considerable saving of precious RhIg. In addition, as RhIg is a human blood product and its use is associated with a possible risk of infection, this would lead to reduced exposure to such a risk. We are not aware of a publication which specifically summarizes the risk of RhIg; however, during a SAFE workshop in September 2007 the data from one manufacturer (Bio Products Laboratory, Elstree, UK) were presented. From January 2005 to July 2007, 580,000 vials (250 IU each) were issued. 28 adverse reactions were reported, including 8 rashes, 3 injection site inflammations, 3 patients with vomiting, 1 anaphylactic reaction and 13 isolated reports with no particular pattern. The risk of finding one HBV, HCV or HIV virus particle in one dose was estimated using a probabilistic model covering the whole process from donor to the finished product: This probability was 1 in $2.5 \times 10^3$ for HBV, 1 in $2 \times 10^6$ for HCV, and 1 in $5 \times 10^3$ for HIV.

Origin of Fetal DNA in Maternal Blood

Most researchers in the field agree that the trophoblast is the source of cff-DNA [12]. This is due to reports indicating that cff-DNA reflects on placental mosaicism [13], and epigenetic similarities to trophoblast tissue [12]. cff-DNA does not appear to be derived form the demission of trafficking fetal cells [14] or the release of cff-DNA in amniotic fluid [15].

The main proportion of cff-DNA molecules seem to have an approximate size smaller than 300 bp and are thus considerably shorter than maternally derived sequences [16, 17]. The oligosomal pattern of cff-DNA suggests that apoptosis may be involved in its process of release. The release of cff-DNA into maternal blood seems to be a constant process and is reflective of material released by normal syncytiotrophoblast turnover where the amount of material released into the maternal circulation has been estimated to be several grams per day at term. The mechanism of release might be apoptosis, aponecrosis, or necrosis [18]. Although, membrane-bound vesicles (apoptotic bodies) which are known to contain nucleosomes have also been proposed as potential vehicle of cff-DNA in maternal plasma [19], it appears that most of the cff-DNA is not associated with such particles [20].

Quantity and Kinetics of Fetal DNA in Maternal Blood

The exact quantity of cff-DNA in maternal plasma is unknown. We anticipate that each DNA extraction method will be less than 100% efficient and downstream amplification/detection methods will also not detect a single copy of DNA with a 100% probability. Most of the studies lack a detailed description of the lower limit of quantification and the precision for samples with low numbers of target sequences. Therefore, all quantitative data have to be interpreted with caution.

The published data illustrate that there is a large interindividual variation. Moreover, in one individual the concentration of cff-DNA varies from 1.4- to 4.5-fold when blood is taken at different time points within a few days [21]. cff-DNA was shown to be detectable from maternal plasma by real-time PCR as early as 5 weeks and 2 days of gestation [22]. Lo et al. [23] initially found 25.4 genome equivalents (geq) per ml
maternal plasma (range 3.3–69.4 geq/ml) in early pregnancy (11th to 17th week of gestation) and 292.2 geq/ml maternal plasma (range, 76.9–769 geq/ml) in late pregnancy (37th to 43rd week of gestation). Rjinders et al. [3] measured on average 89 geq/ml (range 15–703 geq/ml) in 160 plasma samples of RhD-negative women carrying a RhD-positive child at 30th week of gestation. Müller et al. [24] measured on average 15 geq/ml (range 1–1,057 geq/ml) in 574 plasma samples in the second trimester (13th to 28th week of gestation) after cff-DNA extraction with spin columns (QIAamp® DSP Virus Kit; QIAGEN, Hilden, Germany) and on average 101 geq/ml (range 5–5,215 geq/ml) in the same samples when the cff-DNA extraction was performed with magnetic particles and magnetic tips (Chemagic magnetic separation module 1; Chemagen, Baesweiler, Germany).

Kinetic studies have shown that cff-DNA is cleared from maternal plasma after delivery, with a half-life of approximately 15 min [25]. This implies that there is no danger of detecting cff-DNA sequences from previous pregnancies – a small concern when dealing with the analysis of trafficking fetal cells.

### Preanalytics

Although concentrations of cff-DNA in maternal plasma have shown to be considerably stable over a course of 24 h, amounts of total DNA have been proven to increase with time before processing [26]. This finding was examined in more detail in the study of Müller et al. [24]: in one of 662 (0.15%) pregnancies with a RhD-positive fetus, NIPD RhD failed in the first test and only was positive in a repeat test (approximately 14 pg/ml). Transport at room temperature in that case lasted 6 days, resulting in a maternal DNA concentration high above average. Similarly Finning et al. [27] observed in samples older than 14 days an increase of false-negative and inconclusive results. Minon et al. [28] and Rouillac-Le Sciel-lour et al. [29] worked with samples not older than 48 h only. In order to guarantee a reliable test result, the usage of samples where the transport of whole blood at room temperature lasted more than 3 days is therefore not recommended. In our experience plasma separated from cells within 3 days can be stored for several weeks below –70 °C and tested after storage for the presence of cff-DNA.

In three cases (0.83% of pregnancies with RhD-negative fetus) in the study of Müller et al. [24], false-positive determination of the fetal RhD status occurred, regardless of which DNA extraction method was applied. Here, not the DNA extraction procedure but most likely the centrifugation and manual handling of the sample material before storage was the critical factor. To avoid sample contamination and to reduce sample handling steps, DNA could be extracted directly from the primary tube as recently demonstrated by Finning et al. [27].

### Table 1. NIPD RhD studies published from 2006–2008

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Sensitivity, %</th>
<th>Specificity, %</th>
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<th>Number of samples tested</th>
<th>Extraction</th>
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*Only controls specific for cff-DNA are mentioned here.
Sensitivity, Specificity and Accuracy of Prenatal RhD Tests

Before NIPD RhD can change the current practice of anti-D administration to all at-risk pregnant women, the accuracy of thecff-DNA assay needs to be rigorously assessed.

A comprehensive meta-analysis of Geifman-Holtzman et al. [30] comprises 37 English-written publications from 1993 to 2005 describing 44 different protocols for the conduction of NIPD RhD from maternal blood. There were a total of 3,261 samples that were tested for the fetal RhD status in peripheral maternal blood. This meta-analysis determined an overall diagnostic accuracy of 94.8%. However, individual accuracies varied substantially from 31.8 to 100%, depending on which protocol and study design was applied. For the present review PubMed (US National Library of Medicine) was searched from 2005 to December 9, 2008 using the words ‘fetal’, ‘RhD’, ‘maternal’, and ‘DNA’. Articles published in 2005 and listed in the meta-analysis of Geifman-Holtzman were excluded in order to avoid an overlap of these two reviews.

We identified 11 studies published from 2006 to 2008 describing 6,548 tests for the prenatal fetal RhD genotype using 14 different protocols (table 1). Reporting 100% sensitivity or specificity does not take the sample size into account. We therefore report findings with no false-negative or no false-positive observations differently so that the reader can estimate the statistical power of each study. Because false-negative results will cause the most concern (due to new cases of HDFN), we sorted the studies according to the sensitivity. Two kinds of studies could be identified: Protocols 1–6 were evaluated in a relatively large patient cohort comprising 300–1,869 specimens in order to determine the clinical utility of NIPD RhD. Protocols 7–14 were evaluated in relatively small samples and can thus only be regarded as proof-of-principle studies.

The sensitivity in the large-scale studies ranged from 99.5 to 99.8%, and specificities reported ranged from 94.0 to 99.5% (table 1). Compared with other protocols, Finning et al. [27] reported the lowest specificity (94.0%) and accuracy (95.7%). The Finning study was designed to include a relatively high percentage of individuals from various ethnic origins. Therefore, the difference in specificity might be explained by different ethnicity of patient groups.

Minon et al. [28] reported a very high specificity (99.5%) compared with other large-scale studies. The authors tested alloimmunized women, a clinical indication for fetal RhD genotyping which requires accuracy, whereas Müller et al. [24], van der Schoot et al. [31], and Finning et al. [27] looked at cost-effective strategies to screen all RhD-negative women for the fetal RhD status in order to change the current antenatal prophylaxis strategy towards a targeted antenatal prophylaxis. The high specificity (and sensitivity) in the Minon study was obtained by performing the DNA extraction twice on each sample and testing 3 different exons in duplicate [28]. If there were discrepancies between the three RhD exons, real-time PCR assays targeting the exons with negative results were repeated on two additional replicates prepared from a new extract of the last preserved maternal plasma. The maternal (and paternal)uffy coats were tested with the same assay. If the discrepancy persisted without any amplification of maternal genomic DNA, the result was considered inconclusive and a new blood sample was obtained for re-analysis. Such an approach seems to be effective in alloimmunized pregnancies, but probably will not be applicable for large-scale screening programs to target antenatal Rh prophylaxis. Costs for repeat tests have to be balanced with costs for RhIg which could be recommended in all inconclusive cases.

The robustness of the assays can be determined from those large-scale studies which report inconclusive results or percentage of additional tests performed on the initial specimens or on subsequent specimens from mother and/or father. Very few repeat tests (1.4%) were required when the Chemagen extraction method was used [24]. 3.0% inconclusive results were reported by Finning et al. [27] using the Bi-Robot MDx (QIAGEN). Amplification failures for exons 4, 5 and 10 were 13.2, 2.9 and 3.1%, respectively, in the report of Minon et al. [28] using the Cobas Ampliprep (Roche Diagnostics, Vilvoorde, Belgium) for DNA extraction, which is within the range of the other reports for exons 5 and 10, but demonstrates a low efficiency of the exon 4 PCR. Compared with the automated extraction methods (1.4% repeat tests), rather more (5.4%) repeat tests were required when the manual QIAamp DSP Virus Kit was used [24]. This observation is in line with the finding that a higher yield of cff-DNA was observed when the Chemagen extraction method was applied [24]. In summary robustness, sensitivity, and specificity are influenced by the cff-DNA extraction method as well as by the amplification/detection method as it is discussed below.

cff-DNA Extraction Methods

Interestingly each group developed their own protocol(s) before performing the validation studies. However, a few similarities can be identified: Clausen et al. [32], Rouilhac-Le Scielour et al. [33], and Müller et al. [24] independently identified the two almost identical test kits, QIAamp DSP Virus Kit and MinElute Virus Kit (QIAGEN), as good candidates for the manual extraction of cff-DNA from maternal plasma. One difference was that Müller et al. [24] performed DNA extraction from 1 ml plasma, whereas the other two groups used 500 µl. The utility of this DNA extraction method was further demonstrated in an international workshop [34].

The direct comparison of one manual and one automated method in the study of Müller et al. [24] did not reveal any striking difference for sensitivity and specificity. Therefore each laboratory has to decide on the basis of sample number processed in one run, if it is worth to spend a large amount of investment in high-throughput instruments. Currently the
industry is offering new instruments, but before they can replace any established existing nucleic acid extraction procedure, these instruments need careful validation. For such a validation the development of sensitivity standard material is one important tool. SAFE was part of the 6th EC framework program and was funded for the key mission to implement routine NIPD RhD and cost-effective neonatal screening throughout the EU and beyond [35]. SAFE partners have identified the necessity for sensitivity standard material in past workshops and are supporting the development of such a material (S. Urbaniak, personal communication).

Another important validation tool is the comparison of diagnostic sensitivity and specificity by sharing samples and testing with different protocols. We performed such a technical platform assessment as a two-center study (Basel and Göttingen) which is reported here for the first time:

In Basel, DNA was extracted from 1 ml plasma using the MagNA Pure® LC DNA Isolation System (Roche Applied Science, Mannheim, Germany), and a duplicate RHD exon 7 real-time PCR (RHDex7 real-time PCR) was performed (table 2). A repeat PCR was run if the results of the first one differed from each other, if both showed a negative result or if cycle threshold (Ct) values were > 38. Altogether a maximum number of 4 PCR runs was carried out, and a Ct value > 40 was determined as cut-off. In Göttingen, DNA was extracted from 500 µl plasma using the QIAamp DSP Virus Kit and from 1 ml plasma when applying the Chemagen Magnetic Separation Module 1. A multiplex RHDex5 and RHDex7 real-time PCR was performed in duplicate, and the PCR run was repeated if results differed from each other, whereupon a maximum number of 4 runs per sample were carried out. 279 samples were tested, gestational weeks ranged between 6 and 39 (median week 25). The results were compared with the data obtained from cord blood using serology. Postnatal cord blood serology specified 190 (68.1%) newborns to be RhD-positive and 89 (31.9%) to be RhD-negative. The spin column method as reference technology was the only one to be in complete concordance with serology (table 3). The Chemagen method gave one false-positive result. The MagNA Pure LC method led to one false-positive and four false-negative results. From these experiments comparing two different automated extraction technologies for the first time in a relatively large cohort we conclude that the Chemagen extraction method using 150 µl plasma-equivalent/PCR seems to be more robust than the MagNA Pure LC instrument where 40 µl plasma-equivalent are tested only (table 2). Furthermore, nucleic acid extraction with the QIAamp DSP Virus Kit seems to be a good reference extraction method.

**RHD Exons Selected for Prenatal RHD Tests**

Because of the high frequency of the non-coding *RHDΨ* allele and the presence of exon 10 in many other non-coding *RHD* alleles (e.g. *RHD*-CE(4–7)-D in Blacks or *RHD*-CE(3–9)-D in Asians), SAFE partners tested with primers which do not detect *RHDΨ* and did not test with primers specific for *RHD* exon 10 [24, 27, 36]. The groups of Rouillac-Le Scellour et al. [33] and Minon et al. [28] who used primers for exon 10 developed flow charts how to resolve discrepancies when the exon 10 real-time PCR gives a positive result and the other exons (4, 5 or 7) show a negative result. But this will cause a delay of diagnosis and frequently requires testing of the paternal DNA, maternal DNA, and a repeat plasma sample. Thus avoiding exon 10 real-time PCR will also avoid unnecessary follow-up investigations and inconclusive results.

When e.g. exons 5 and 7 are used for detection only, DBT might be one of the rare *RHD* alleles which will be missed. DBT is a rare partial D phenotype in Germany, and only a few families have been reported in the international literature [7].

### Table 2. Comparison of three different methods for cff-DNA extraction

<table>
<thead>
<tr>
<th>Method</th>
<th>Plasma input, µl</th>
<th>Elution volume, µl</th>
<th>DNA eluate per total reaction volume, µl</th>
<th>Plasma-equivalent/PCR, µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>QIAamp DSP Virus Kit</td>
<td>500</td>
<td>40</td>
<td>15/50</td>
<td>187.5</td>
</tr>
<tr>
<td>Chemagen</td>
<td>1,000</td>
<td>100</td>
<td>15/50</td>
<td>150</td>
</tr>
<tr>
<td>MagNA Pure LC</td>
<td>1,000</td>
<td>100</td>
<td>8/25</td>
<td>40</td>
</tr>
</tbody>
</table>

### Table 3. Characteristics of three fetal RHD genotyping methods evaluated in 279 cases

<table>
<thead>
<tr>
<th>Method</th>
<th>Sensitivity, % (n = 190)</th>
<th>Specificity, % (n = 89)</th>
<th>Accuracy, %</th>
<th>False-positives, n</th>
<th>False-negatives, n</th>
</tr>
</thead>
<tbody>
<tr>
<td>QIAamp DSP Virus Kit</td>
<td>&gt;99.5</td>
<td>&gt;99.5</td>
<td>&gt;99.6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Chemagen</td>
<td>&gt;98.9</td>
<td>98.9</td>
<td>99.6</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>MagNA Pure LC</td>
<td>97.9</td>
<td>98.9</td>
<td>98.2</td>
<td>1</td>
<td>4</td>
</tr>
</tbody>
</table>

**Sequence Detection Instruments**

All large-scale studies evaluated real-time PCR for the detection of fetal DNA. In the proof-of-principle studies mass
spectrometry, capillary electrophoresis, and gel electrophoresis were also used. Whereas mass spectrometry and capillary electrophoresis have advantages compared with real-time PCR when multiple PCR fragments have to be detected from multiplex reactions, gel electrophoresis is known to be less sensitive when only one PCR is performed and is prone to contamination when semi-nested or nested two-step protocols are used. At the moment real-time PCR has to be regarded as the method of choice for testing clinical samples; other technologies need validation in large cohorts first before they can be recommended for diagnostic laboratories.

Control Reactions to Avoid False-Negative Results

Ideally, NIPD RhD assays should include a control for the detection of fetal DNA in all samples, especially in those samples obtained from women with RhD-negative fetus. Minon et al. [28], Kimura et al. [37], and Al-Yatama et al. [38] used detection systems for Y chromosome in order to provide a control PCR which indicates the presence of cff-DNA when no RHD-specific sequences can be detected. However, testing for Y chromosome sequences is only applicable in approximately 50% of pregnancies where a male baby is born. In cases negative for both RHD- and Y chromosome-specific sequences, a set of polymorphic markers can be used to confirm the presence of fetal cff-DNA. Sequenom Ltd. (San Diego, CA, USA) is developing a specific set of over 60 markers for use with its mass spectrometry device for this purpose [5]. For the real-time PCR approach biallelic insertion deletion polymorphisms can be applied [39]. Several sex-independent, robust universal fetal markers and their feasibility of being included in diagnostic tests are currently intensively examined, e.g. fetal epigenetic markers such as hyper- or hypo-methylated placental-derived sequences in maternal plasma or mapping for single-nucleotide differences [40–42]. Up to now, a large clinical trial evaluating the effect of such universal marker on sensitivity and specificity has not been performed. As an interim solution heterologous or homologous spike controls can be used. Rouillac-Le Sciellour et al. [33] spiked plasma samples with exogenous DNA (maize) which is used as extraction/amplification control. The advantage of this approach is that common primer binding sites can be designed and that the concentration of the control DNA can be very low. Thus, inhibition of the target reaction is minimized. The addition of exogenous control DNA as an internal control is already routine when blood samples are screened for viral RNA or DNA. Many laboratories test as an internal control is already routine when blood samples are screened for viral RNA or DNA. Many laboratories test

Multicenter Studies Performed on Fetal RhD Testing from Maternal Plasma

An international external quality assurance scheme has been established by the International Society of Blood Transfusion (ISBT). Currently workshops are performed every 2 years starting from 2004 [43, 44]. Rouillac-Le Sciellour et al. [33] evaluated the first commercial assay which allows NIPD RhD (Free DNA Fetal Kit RhD, Institut de Biotechnologies Jacques-Boy, Reims, France) using 300 samples. Based on this evaluation, the kit obtained a CE mark. The availability of CE-labelled reagents improved the diagnostic accuracy in other diagnostic applications, however, the Free DNA Fetal Kit RhD currently does not fulfil the criteria for reagents used for the determination of the serological RhD status.

To evaluate different DNA extraction methods for the enrichment of cff-DNA from maternal plasma, SAFE partners performed an international Workshop using NIPD RhD testing as a model [34]. Three plasma pools from RhD-negative pregnant women, a DNA standard, real-time-PCR protocol as well as primers and probes for RHD were sent to 12 laboratories and also to one company. In pre-tests, pool 3 showed a low cff-DNA concentration, pool 1 showed a higher concentration, and pool 2 an intermediate concentration.

Various manual DNA extraction methods were suitable to reliably enrich cff-DNA for the detection of fetal RHD. However, substantial differences in yield of cff-DNA between different DNA extraction methods were observed even if different input volumes and elution volumes were taken into account. It was unlikely that these differences were caused by different real-time PCR machines because 11 of the 12 laboratories used machines from Applied Biosystems (ABI 7000, ABI 7300, ABI 7500, ABI 7700; Foster City, CA, USA), and some laboratories directly compared extraction methods by splitting samples and using the same real-time PCR machine. The best results were obtained with the QIAamp DSP Virus Kit. The QIAamp® DNA Blood Mini Kit (QIAGEN) showed very comparable results in laboratories that followed the manufacturer’s protocol and started with ≥500 µl plasma. One participant using the QIAamp® DNA Blood Midi Kit (QIAGEN) failed to detect reliably RHD in pool 3.

When automated methods were compared, the BioRobot MDx (QIAGEN) showed the highest DNA recovery in pools 1 and 2, however in pool 3 ΔCt of replicates (3 out of 3 were positive) was > 1.5, a finding which indicates that the cff-DNA concentration was below the limit of quantification (outside the quantitative range) and above the limit of detection. Therefore, in this Workshop, only the MagNA Pure LC system and the robotic Magnetic Tip System reliably detected
cff-DNA in low concentrations. However, QIAGEN has developed a new pipetting procedure for the BioRobot M48 with higher sample input volume and smaller elution volume, and this new option has been evaluated in a large-scale single center study [27]. Furthermore, 2nd-generation protocols have been released for the BioRobot M48 (MagAttract Virus IC v2.0) and EZ1 (EZ1 Virus v2.0) which should also be evaluated in future studies.

In three subsequent workshops SAFE partners optimized the protocol for DNA extraction with QIAamp DSP Virus Kit and real-time PCR for RH D exons 5 and 7. These efforts lead to improved sensitivity and specificity in some of the laboratories which provide a clinical service for NIPD RhD in Europe (E. Tait, S Urbaniak, personal communication).

Prenatal RhD Testing for Decision Making on Rh Prophylaxis

According to the German guidelines [45], every RhD-negative pregnant woman in Germany should receive a standard dose (300 μg) of anti-D immunoglobulin between week 28 to 30 of gestation. A second dose is to be administered within 72 h after delivery of a RhD-positive baby or in case of a miscarriage, extrauterine gravidity or abortion.

Today, the overall risk of anti-D alloimmunization has been substantially reduced from 1.2 to 0.28% in several countries by replacing postnatal prophylaxis only with combined ante- and postnatal prophylaxis [46]. The main disadvantage of this approach is that antenatal Rhlg is unnecessarily given to women carrying a RhD-negative fetus (approximately 38–40%), that is about 46,000 women annually in Germany. Thus, not only time but also money and, more importantly, valuable anti-D immunoglobulin is wasted. One have to keep in mind that Rhlg is produced from voluntary plasma donors who have been immunized. Therefore, it needs to be discussed if wasting Rhlg in cases where the fetus is RhD-negative is still ethical today.

In the 1990s, few incidents of the transmission of hepatitis C virus via contaminated Rhlg occurred [47–49]. Nowadays however, Rhlg is a very safe drug. But being sourced from human beings, it still remains potentially infectious material. The extent of transmissibility of the variant Creutzfeld-Jakob disease (vCJD) by blood products for example is not yet known. Therefore the UK mainly imports plasma from North America for the production of Rhlg and has stopped the production of Rhlg from UK plasma [50]. Consequently, a reliable, noninvasive technique allowing the specific administration of Rhlg only when indicated would be of great benefit for the safety of RhD-negative pregnant women and their unborn children worldwide.

False-negative determination of the newborn’s RhD status via postnatal serology from cord blood may occur: Müller et al. [24] found that serology failed to identify one normal, RhD-positive newborn (0.1% of 1,022 cases tested) correctly detected as such in real-time PCR. This observation affirms the assumption that antenatal genotyping supports postnatal serology because mix-up of samples and other human errors still occur.

Recently, difficulties were reported to reliably and consistently identify individuals carrying weak D variants with approved serologic techniques [51]. This issue was also highlighted in the study of Müller et al [24]: among 1,022 samples tested for fetal RH D and compared with serological reference obtained after birth, two weak D type 2 (0.2%), confirmed by sequencing results from the newborn’s buccal swab DNA, were correctly predicted to be RhD-positive in real-time PCR, but failed to be identified in serology. Hence, respective mothers received antenatal but no postnatal anti-D immunoglobulin. Likewise, Minon et al. [28] identified, when screening in 563 cases for fetal RH D, 3 newborns (0.5%) to be false-negative in serology, but correct positive in real-time PCR. In the study performed by Rouillac-Le Sciellour et al. [29], 2 potential cases remained unclear due to lack of follow-up, and 5 cases of potential D variants remained unresolved in the study performed by van der Schoot et al. [31] due to the lack of additional material from the newborns.

Serology may not only fail in the identification of weak D types in newborns but also in pregnant women. In the study of Müller et al. [24], all study subjects were previously typed as RhD-negative by serologic techniques in conformity with current German guidelines for the determination of the RhD status in pregnant women, albeit serology was found to fail in the identification of a weak D type 1, which was detected via the indirect antiglobulin test (IAT) performed in the reference laboratory. No event of alloanti-D immunization in carriers of weak D type 1 was reported so far, and, due to this knowledge, the application of anti-D prophylactic treatment in women of this phenotype is not considered necessary so that valuable RhIg should be saved [52]. Thus, fetal RH D genotyping could contribute to the correct handling and treatment in pre- and postnatal care of weak D individuals who account for approximately 0.44% of the RhD-positive phenotypes in the Caucasian population [53].

The limitations of fetal RH D genotyping from maternal plasma are that there might be a small number of false-negative test results (0.2–0.5% in large-scale studies; table 1) if not multiple replicates are tested from different exons and/or follow-up samples [28, 33]. At best these false-negative test results will not occur as frequent as in the published studies because in order to study the effect of transportation time those samples which were accepted in the studies would have been rejected in a routine laboratory. Similarly samples in early gestation were tested for study purposes, but we recommend not to draw final conclusions when blood samples have been collected before week 12 of gestation. Additionally, there is a certain percentage among the apparent RhD-negative pregnant women, who are carriers of RH D. NIPD RhD could potentially contribute to the identification of these cases, but will not give accurate results as far as the child’s RhD sta-

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tus is concerned. Furthermore, it was recently reported that donor DNA present in the plasma of a solid-organ recipient confounded fetal RHD genotyping results [54], pointing out another group where difficulties concerning the application of fetal RHD genotyping might be encountered.

In summary, NIPD RhD for targeted antenatal Rh prophylaxis would not only contribute to a better resource management by making prenatal anti-D prophylactic treatment more efficient but may also play an important role in the identification of false RhD-negative determinations in postnatal serology. In cases where a positive real-time PCR but a negative serologic result is obtained, the performance of an IAT (if not performed routinely) will elucidate if the presence of a weak or partial D variant in the newborn is the reason for the observed discrepancy. A second blood sample requested in this case will even reveal mix-up of samples. Furthermore, identification of RHD-positive mothers allows identifying women with common weak D types (i.e. weak D type 1–3) through specific additional PCR on maternal buffy coat. These women are not at risk for anti-D alloimmunization and thus do not need to receive Rh prophylaxis.

Cost-Effectiveness of Prenatal RhD Testing for Decision Making on Rh Prophylaxis

An economic decision model was developed to compare benefits, risks, and costs of routine antenatal anti-D prophylaxis (RAADP) and NIPD RhD in combination with targeted antenatal RhD prophylaxis (C. Krauth, A. Haverkamp, personal communication). A bottom-up approach to cost estimating was applied. Testing cost estimates were based on microeconomic data collected from a regional laboratory in Germany. Test cost components included blood sample collection, consumables, sample transport, equipment, labor, retests, result reporting, and overheads. NIPD RhD costs were determined at EUR 26.71 per pregnancy in RhD-negative women. Lower costs can be anticipated by utilizing economies of scale as rationalized workflows or higher throughputs in centralized diagnostic centers. Because savings can be made by NIPD RhD and targeted antenatal prophylaxis through more efficient use of RhIg, a break-even price of of EUR 26.50 for NIPD RhD could be determined. The analysis demonstrates economic benefits for NIPD RhD implementation and with higher throughputs the technology becomes a cost-efficient alternative to RAADP.

Conclusions

The availability of NIPD RhD is an obvious benefit for alloimmunized pregnant women. This test should be offered in all cases to avoid unnecessary invasive procedures and expensive ultrasound screening in combination with antibody titrations in short intervals. The benefit of NIPD RhD is less obvious for the counseling of women without alloimmunization who currently receive antenatal Rh prophylaxis routinely. On the one hand medical treatment always requires a clear indication (which is not available if the fetus is RhD-negative), on the other hand none of the published large-scale studies tested the influence of controls testing the presence of fetal DNA. It might be that these new controls reduce the sensitivity of current assays. In our opinion there are three options for policy makers to manage the future of NIPD RhD in RhD-negative women without alloimmunization. First, continue with routine antenatal Rh prophylaxis without NIPD RhD. The advantage of this strategy is that the safety of this policy has been determined within almost 20 years. The disadvantage is that side effects of RhIg such as severe anaphylactic reactions can be life-threatening for some women and outbreaks of pathogens which escape current quality control can never be excluded. Such a disaster usually affects hundreds of women and their unborn children. Second, continue with routine antenatal Rh prophylaxis without NIPD RhD and support the development of next-generation assays which include a control for the presence of fetal DNA. This policy requires collaboration with patent holders on a nationwide level because otherwise there is a high risk that such an assay will not be implemented due to the lack of cost-effectiveness. However, in our opinion this policy will result in NIPD RhD with better prices for German-speaking countries compared with the policy of waiting for the first commercial test coming to our market. Third, allow gynecologists and women to choose between general Rh prophylaxis and targeted Rh prophylaxis in combination with current NIPD RhD assays. The advantage of this policy is that reliable data of the tests and costs are available; however, in contrast to the experience we have with general antenatal Rh prophylaxis, only research data have been published, and there are no data from any country available where this policy has been implemented and afterwards alloimmunization rates were determined. Since there are countries who have not implemented general antenatal Rh prophylaxis yet, we assume that those countries will move from postnatal prophylaxis only to targeted Rh prophylaxis in the near future. Due to the data collected in those countries we will probably be forced to implement targeted Rh prophylaxis by using patented expensive assays. Thus following the second policy discussed here is in our opinion the most wise option policy makers can choose.

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