NON-INVASIVE PRENATAL TESTING FOR SUB-SAHARAN AFRICA: TAILORING APPROACHES FOR FOETAL RHD GENOTYPING IN RHD-NEGATIVE PREGNANT WOMEN TO MANAGE AFRICAN-ASSOCIATED RHD ALLELES

TESTS PRÉNATAUX NON-INVASIFS POUR L’AFRIQUE SUB-SAHARIENNE: APPROCHES SUR MESURE POUR LE GÉNOTYPAGE RHD FŒTAL CHEZ LES FEMMES ENCEINTES RHD-NÉGATIVES ET POUR LA GESTION DES ALLÈLES RHD ASSOCIÉS À L’AFRIQUE

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ABSTRACT
BACKGROUND
Non-invasive prenatal testing (NIPT) for cell-free foetal (cff) RHD genotyping has clinical value to guide pregnancy management for alloimmunised RhD-negative pregnant women and guide antenatal anti-D prophylaxis needs for all D-negative women to prevent alloimmunisation. This assay assumes there is a maternal RHD gene deletion and genotyping is challenged where the mother carries RHD alleles such as RHD*Ψ and RHD-CE-D which are frequent in Sub-Saharan Africa.

AIM AND OBJECTIVE
This paper reviews the range of RHD alleles reported in sub-Saharan African populations and strategies in managing African-associated RHD alleles to ensure the accuracy of cffRHD genotyping.

RESUME
CONTTEXTE
Le test prénatal non invasif (RNP) pour le génotypage RHD fœtal libre (CFC) a une valeur clinique pour guider la gestion de la grossesse chez les femmes enceintes RhD-négatives allo-immunisées et pour guider les femmes D-négatives alloimmunisation. Ce test suppose qu’il existe une délétion du gène RHD maternel et le génotypage est contesté lorsque la mère porte des allèles RHD tels que RHD *Ψ et RHD-CE-D qui sont fréquents en Afrique subsaharienne.

BUTS ET OBJECTIFS
Cet article passe en revue la gamme d’allèles RHD rapportés dans les populations d’Afrique subsaharienne et les stratégies de gestion des allèles RHD associés à l’Afrique pour assurer l’exactitude du génotypage cffRHD.
CONCEPTION DE L’ÉTUDE / MATÉRIELS ET MÉTHODES


RÉSULTATS

Un modèle d’essai NIPT pour cffRHD, adapté à l’approche des exons 5 et 7 de RHD recommandée par SAFE, peut fournir des prédictions fœtales D-positives ou D-négatives lorsqu’un RHD *Ψ et RHD-CE-D* matériel est présent. L’inclusion de RHD exon 4 dans NIPT est un outil pour augmenter la confiance dans la prédiction D- phénotype.

CONCLUSIONS

Une approche stratégique du génotypage cffRHD NIPT peut surmonter les défis avec l’interférence maternelle RHD des allèles RHD *Ψ et RHD-CE-D* chez les femmes enceintes D négatif. La prise en charge de ces allèles RHD fournit des connaissances pour la prise en charge clinique afin de minimiser l'allo-immunisation maternelle anti-D et la maladie hémolytique du foetus et du nouveau-né. L’étude continue des allèles RHD, y compris ceux qui sont nouveaux et à basse fréquence, est importante dans les futures approches de génotypage cffRHD NIPT dans les populations de l’Afrique subsaharienne.

INTRODUCTION

An estimated 3-5% of sub-Saharan African populations are RhD-negative. In up to 82% of this population, the D-negative phenotype results from an inactive RHD gene sequence such as the RHD*pseudogene (RHD*Ψ) and RHD-CE-D* hybrid. In marked contrast, 15-17% of the Caucasian population are D-negative, most commonly arising from a complete RHD gene deletion. In D-negative pregnant women, anti-D alloimmunisation can occur when the maternal immune system is exposed to foetal D-positive red blood cells (RBCs). Allo-anti-D is the most common clinically relevant alloantibody in African populations.

Strategies to prevent maternal alloimmunisation include routine postnatal and antenatal anti-D prophylaxis. The postnatal strategy, alone, reduces the risk of immunisation from 17% to 0.8-1.5%. Antenatal anti-D prophylaxis for all D-negative pregnant women reduces the immunisation rate to 0.2% and, therefore, also the rate of haemolytic disease of the foetus and newborn. Continuing study of RHD alleles, including those that are novel and low-frequency, is important in future approaches to NIPT cffRHD genotyping in sub-Saharan African populations.

The success of NIPT testing depends on the tailoring of an assay design for predominant RHD alleles in the population. In the large majority of Caucasian populations, the presence of cffRHD in maternal plasma is clearly differentiated as there is no maternal RHD background (RHD gene deletion). A foetal D-positive phenotype prediction can be provided with a high level of specificity and sensitivity. For sub-Saharan African populations, however, the presence of a maternal RHD allele in a D-negative mother, such as RHD*Ψ, complicates differentiation of foetal and maternal cell-free (cf) DNA and prediction of a foetal D-phenotype. It is necessary to devise a cffRHD genotyping approach to overcome the challenges presented when an African-associated RHD allele that results in a D-negative phenotype is present. The purpose of this paper is to:

• discuss the role for foetal RHD genotyping in pregnancy management for D-negative women
• describe cf DNA RHD genotyping
• review the range of RHD alleles reported in sub-Saharan African populations and
• review strategies in managing African-associated RHD alleles to ensure the accuracy of NIPT foetal RHD genotyping.

The review will utilise data obtained from textbooks, online literature searches using Google, Google Scholar and PubMed, and International Society of Blood Transfusion (ISBT) blood group allele tables.

1. CLINICAL ASPECTS: Role for foetal RHD genotyping in pregnancy management for D-negative women

• Strategies for preventing maternal anti-D alloimmunisation in non-immunised cohorts

During pregnancy, a D-negative mother is at risk of producing allo-anti-D when the foetus is D-positive. Allo-anti-D is produced following a sensitisation event where foetal D-positive RBCs enter the maternal circulation to expose the ‘foreign’ D antigen to the maternal immune system. Sensitising events can occur during the pregnancy or, more commonly, on delivery of a D-positive infant.
The likelihood of an RhD sensitisation event is highest in the third trimester. In Africa, the most common clinically significant alloantibody reported in pregnant women is anti-D. During postnatal care, anti-D alloimmunisation can be prevented in non-immunised mothers through prophylactic intramuscular injection of anti-D immunoglobulin within 72 hours of delivering a D-positive baby. Postnatal anti-D prophylaxis is administered when neonatal cord blood is confirmed as D-positive and has been found to decrease the rate of alloimmunisation from 17% to 0.8-1.5%. This rate can be further reduced to 0.2% by providing routine antenatal anti-D prophylaxis in late second and early third trimester. Most anti-D prophylaxis programs incorporate both postnatal and antenatal anti-D prophylaxis which has become standard of care to reduce the rate of maternal anti-D alloimmunisation.

In universal routine antenatal anti-D programs, anti-D prophylaxis is administered to all D-negative pregnant women, without determining whether the foetus is D-positive or D-negative. This means that women who carry a D-negative foetus are receiving anti-D prophylaxis unnecessarily. Foetal RH D genotyping can avoid this problem by targeting antenatal anti-D prophylaxis only when the baby is assessed as D-positive. Targeted antenatal anti-D prophylaxis programs utilise foetal D-phenotype predictions from non-invasive cfRHD genotyping assays to indicate whether anti-D prophylaxis is required or can be avoided. Despite the success of both postnatal and antenatal prophylaxis in reduction of maternal alloimmunisation rates cases of ‘breakthrough’ alloimmunisation have been reported.

**• Strategies for managing alloimmunised cohorts with anti-D**

When allo-anti-D has previously been produced in a D-negative mother from a blood transfusion or sensitising event in a previous pregnancy, there is a high risk of HDFN in the ongoing pregnancy when the foetus is D-positive. Incompatible feto-maternal D status can result in maternal anti-D IgG antibodies crossing the placenta and haemolysing feta RBCs. This can lead to foetal anaemia and in severe cases, erythroblastosis fetalis, hydrops fetalis and intraterine foetal death when left untreated. Hydrops fetalis has been shown to be minimised with intraterine transfusion in 65% of cases. It is important to monitor at-risk pregnancies during antenatal care to prevent HDFN. For obstetric management, women with anti-D are managed as high risk and receive intensive monitoring throughout pregnancy in tertiary Foetal Maternal Medicine units. This monitoring includes serial measurement of maternal antibody levels and, when required, serial middle cerebral artery Doppler assessment to measure foetal anaemia. A late sign of foetal anaemia (<7g/dL below normal for gestational age) presenting as hydrops fetalis can be detected by ultrasonography and, if left unchecked, is associated with a higher rate of foetal mortality. If the foetus is known to be D-negative, this monitoring can be avoided as the foetus is not at risk. First-generation foetal RH D genotyping using invasive procedures such as amnioncentesis was introduced in the early 1990s. However, invasive procedures such as chorionic villus sampling and amnioncentesis pose a risk of increasing maternal anti-D levels and an increased likelihood of miscarriage. Non-invasive prenatal testing methods have now been developed to predict the foetal D status and avoid unnecessary clinical monitoring.

### 2. CELL-FREE DNA TECHNOLOGY for non-invasive cfRHD genotyping

In 1997, Lo and colleagues discovered that cfDNA can be detected in maternal plasma during pregnancy, initiating the field of Non-Invasive Prenatal Testing (NIPT). In maternal plasma, cf DNA are short DNA fragments (~200 base-pairs (bp)) that constitute 3-6% of total cfDNA when measured with a real-time polymerase chain reaction (RT-PCR) method. The level of cfDNA, which is placental derived, increases as the foetus grows during the pregnancy but is rapidly cleared after delivery and, therefore, does not carry over from pregnancy to pregnancy. Early studies used RT-PCR to detect foetal-specific gene sequences such as the male sex determining Y-chromosome gene, SRY. NIPT application has since expanded to detect feta lchromosomal abnormalities and has been performed in South Africa. Knowledge of foetal D status is important in guiding clinical management of D-negative pregnancies where maternal allo-anti-D is present or when anti-D prophylaxis is standard of care (see above Section 1). Foetal D status predicted from the presence or absence of cfRHD gene sequences in maternal plasma was first used to show the clinical utility for NIPT technologies using RT-PCR. RT-PCR for RH D exons based on TaqMan technology detected cfRHD by targeting and amplifying RH D exons using the 5’ to 3’ nuclease activity of TaqMan DNA polymerase. As the large majority of D-negative Caucasians have a complete deletion of the RH D gene, there is usually no background maternal RH D signals to compete with detection of cfDNA fragments. To perform foetal RH D genotyping optimally, cfDNA is isolated from maternal plasma as promptly as possible. CF DNA is first denatured at a high temperature (e.g. 95°C) so that the sequence-specific primer (SSP) and dual-labelled fluorogenic probe can anneal to the targeted gene sequence of template DNA. A complementary DNA strand is synthesised using TaqMan DNA polymerase from deoxynucleotides (dNTPs) in the reaction mixture. When the complementary strand extends toward the probe, the reporter fluorescent dye emits a fluorescent signal after it is cleaved from the quenching dye. Fluorescence indicates that cfRHD gene sequences are present in maternal plasma. As cfRHD is present at a low percentage, more PCR cycles are required to detect a signal at a defined threshold in the assay, referred to as the cycle threshold (Ct). Foetal RH D signals in RT-PCR typically range between 30 to 38 Ct, which indicate low DNA levels. The presence of foetal RH D predicts the foetus is D-positive. The foetus is predicted to be D-negative when there is no detectable signal from foetal RH D and is consistent with an RH D gene deletion. For foetal RH D genotyping assays, which assume that the maternal RH D sequences are absent, the presence of a maternal RH D variant gene has potential to hinder interpretation of the assay. On this basis, it is important to define the range of RH D alleles in local populations.

### 3. RH GENETICS AND RH D ALLELES associated with African populations

The Rh blood group system is comprised of two closely-linked and highly homologous genes on chromosome 1, RH D and RHCE. Both genes have ten exons, with RHCE encoding for C/c and E/e antigens and RH D for the D antigen. The RhD blood group antigen is serologically and genetically diverse. In routine blood group typing, individuals can phenotype as D-positive (~95% in sub-Saharan Africa) or D-negative (3-5%). In RH D genotyping, there is a diverse array of RH D alleles that have been described. RH D alleles have been described to possess qualitative and/or quantitative differences to the regular D antigen resulting in unusual serological findings.
**Figure 1:** Primordial RHD gene mutation (*) of African-associated DAU, DIVa and weak D type 4 clusters giving rise to a range of RHD alleles. Single nucleotide polymorphisms (SNPs), RHD*c. 667T>G, c.602C>G and c.819G>A, cross over two or more clusters (bolded, underlined and italicised) 48-50, 86. RHD alleles in the African-associated DAU, DIVa and Weak D type 4 cluster

**Table 1:** Anti-D alloimmunisation from African populations

<table>
<thead>
<tr>
<th>Cluster</th>
<th>RHD variant</th>
<th>Sex</th>
<th>Rh Phenotype</th>
<th>Anti-D (titre given, if available)</th>
<th>Immunisation Event</th>
<th>Ethnicity Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAU</td>
<td>DAU-3</td>
<td>Not described</td>
<td>ccDee</td>
<td>Yes</td>
<td>Not described</td>
<td>African 48</td>
</tr>
<tr>
<td></td>
<td>DAU-4</td>
<td>Male</td>
<td>ccDee</td>
<td>Yes</td>
<td>1 D+ RBC transfusion (treatment of sickle cell anaemia)</td>
<td>Not described 87</td>
</tr>
<tr>
<td>DIVa</td>
<td>DIVa type 1.0/ RHD* Ψ</td>
<td>Female</td>
<td>ccDee</td>
<td>256</td>
<td>2 pregnancies</td>
<td>Mulatto (Dominican Republic) 50</td>
</tr>
<tr>
<td></td>
<td>DIVa type 1.0</td>
<td>Female</td>
<td>ccDee</td>
<td>16</td>
<td>2 live births 1 miscarriage</td>
<td>African (Togo)</td>
</tr>
<tr>
<td></td>
<td>RHD-CE-D* type 1 (2 cases)</td>
<td>Not described</td>
<td>(C)cdee</td>
<td>Yes</td>
<td>Yes</td>
<td>African and Afro-Caribbean population 88</td>
</tr>
<tr>
<td></td>
<td>DOL/RHD-CE-D*</td>
<td>Not described</td>
<td>(C)cDee</td>
<td>128</td>
<td>2 RBC transfusions (treatment of sickle cell/β-thalassemia)</td>
<td>Ewe (West Africa) 69</td>
</tr>
<tr>
<td></td>
<td>DAR (weak D type 4.2.0)</td>
<td>Female</td>
<td>ccDee</td>
<td>Yes</td>
<td>Multiple RBC transfusions over years (treatment of sickle cell anaemia)</td>
<td>African black 89</td>
</tr>
<tr>
<td>Weak D</td>
<td>DOL-1</td>
<td>Female</td>
<td>ccDee</td>
<td>8</td>
<td>Pregnancy</td>
<td>African descent 90</td>
</tr>
<tr>
<td>type 4</td>
<td>DOL-2</td>
<td>Female</td>
<td>ccDee</td>
<td>128</td>
<td>Pregnancy</td>
<td>African descent 90</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Female</td>
<td>ccDee</td>
<td>Global titer:64</td>
<td>Pregnancy</td>
<td>African descent 90</td>
</tr>
<tr>
<td></td>
<td>RHD* Ψ (3 cases)</td>
<td>Female</td>
<td>ccDee (presumably)</td>
<td>Yes</td>
<td>Not described</td>
<td>African 5</td>
</tr>
<tr>
<td></td>
<td>RHD* Ψ</td>
<td>Female</td>
<td>ccDee (presumably)</td>
<td>Yes</td>
<td>Pregnancy</td>
<td>Ghana</td>
</tr>
<tr>
<td></td>
<td>RHD* Ψ</td>
<td>Female</td>
<td>ccdee</td>
<td>Yes</td>
<td>Not described</td>
<td>Not described 26</td>
</tr>
</tbody>
</table>
D variants with weak agglutination (2+ or less) are termed ‘weak D’ and those that lack D-epitopes (partial D serological profile) are termed ‘partial D’44, 46. Interestingly, for some RHD variants there is RhC/c and RhE/e haplotype association47. African-associated D variant phenotypes are often in haplotype with c+ and e+ antigens and with multiple single nucleotide polymorphisms (SNPs) in the RHD gene. African-associated RHD alleles have been categorised into three main clusters based on a primordial RHD allele (Figure 1). These are:

- DAU (D of African origin) cluster: RHD*c.1136 C>T48
- weak D type 4.0 cluster :RHD*c.667 T>G49
- DIVa cluster: RHD*c.455A>C50.

These three African-associated clusters, include RHD alleles which cause a qualitative change of D antigen expression on the red cell, resulting in partial D phenotype, where some, but not all D-epitopes are lacking. Patients with an RHD allele expressing a partial D phenotype from these African-associated clusters are at risk of anti-D alloimmunisation when transfused or sensitised with D-positive RBCs (Table 1). RHD alleles from these clusters are reported as predominant in sub-Saharan populations51. Interestingly, the weak D type 4 and DIVa clusters contain the RHD allele, RHD* pseudogene (RHD*Ψ) and RHD-CE-D*, respectively, that expresses a D-negative phenotype rather than a partial D phenotype (Figure 1)49, 50.

3.1 RHD alleles expressing a D-negative phenotype

There are three main underlying RHD genetic mechanisms associated with the D-negative phenotype and these vary in frequency across African populations (Table 2)1–3. The first gene mechanism is a homoyzogous RHD deletion that is also associated with the D-negative phenotype in Caucasian populations. The second and third gene mechanisms, RHD* pseudogene (RHD*Ψ) and RHD-CE-D* are notably associated with the D-negative phenotype in sub-Saharan African populations1, 7, 52. Irrespective of the genetic mechanism, all individuals with the D-negative phenotype are at risk of being alloimmunised and making anti-D and the management strategies required are therefore the same.

- **RHD* pseudogene (RHD*Ψ)**

  The RHD*Ψ allele is an inactive RHD gene sequence that encodes for the D-negative phenotype. In this sequence, there is a frameshifting 37bp duplication in the intron 3 (19 nucleotide bases) to exon 4 (18 nucleotide bases) region and five single nucleotide polymorphisms (SNPs): RHD exon 4 c.609 G>A, RHD exon 5 c.654 G>C, c.667 T>G and c.674 C>T and exon 6 c.807 T>G encoding for a translation-termination codon (TAG) (Figure 2)1. These changes predict a completely inactive gene and indeed mRNA transcripts for this RHD allele have not been detected1. Inclusion of this RHD allele in the weak D type 4 cluster, proposed by Flegel and colleagues, is based on the presence of the primordial RHD*c.667 T>G gene mutation1, 47, 52. Out of 5 African women with anti-D reported by Singleton and colleagues, four had the RHD*Ψ allele with one case involving a Ghanaian woman and resulting in HDFN (Table 2).

- **RHD-CE-D* hybrid**

  The RHD-CE-D* hybrid involves large gene rearrangements with the RHCE gene and encodes a D-negative phenotype (Figure 2). There are two types of RHD-CE-D* because of the differences in the gene sequence of exon 3. The type 1 hybrid, RHD-Dilla-CE-(4-7)-D, has RHD exons 1, 8, 9 and 10 (Figure 2). RHD exons 4, 5, 6 and 7 have been replaced by RHCE. RHD exon 2 has RHD*c.186G>T and RHD exon 3 has RHD*c.410C>T and been partially replaced by RHCE. These mutations in the RHD-CE-D* type 1 hybrid is thought to be derived from the RHD*Dilla sequence at exon 2 for RHD*c.186G>T, and exon 3 for RHD*c.410C>T and c.455A>C (Figure 2). Inclusion of this RHD allele in the DIVa cluster is based on the presence of RHD*c.455A>C in exon 31–30 (Figure 1). The RHD-CE-D* type 2 hybrid, RHD*D-CE-(4-7)-D, resembles RHD-CE-D* type 1 except that RHD exon 2 and 3 has remained intact and allele is also included in the DIVa cluster (Figure 1). The RHD-CE-Ds allele has been involved in three cases of anti-D alloimmunisation from African populations (Table 1).

3.2 Non-African associated RHD alleles expressing a D-negative phenotype

The Eurasian cluster consists of RHD alleles that have been associated with non-African populations and have the wild-type RHD gene as the primordial allele7, 49. The ISBT has recognised a range of RHD alleles that encode a D-negative phenotype (Figure 2)49. The D-negative population in Brazil resemble African populations with the presence of RHD*Ψ and RHD-CE-D* but at a lower frequency48, 49. In the Tunisian D-negative population with C+ or E+, RHD-CE-D hybrids (Figure 2) were observed56. Among East Asian and European D-negative populations, the most frequently observed RHD-CE-D hybrid was RHD-CE(2-9)-D7, 17, 18. In the Australian D-negative population with C+ or E+ haplotypes, this was the RHD-CE(3-8/9)-D hybrid7.

4. STRATEGY TO MANAGE population-based blood group variants in cfRHD genotyping

For sub-Saharan African populations, design of a non-invasive cfRHD genotyping assay requires a strategy to minimise false-positive results caused by RHD alleles encoding a D-negative phenotype65, 66. These strategies include both quality control measures for sample management (common to all populations) and strategies in design of the RT-PCR assay to target genotyping of RHDexons that are informative (specific for the population).

- **Preanalytical factors – sample collection and transport**

  cfRHD genotyping has accurately predicted a foetal D phenotype as early as 11 weeks gestation which provides time to show whether anti-D prophylaxis is necessary and early management of pregnancies immunised with allo-anti-D50. Testing samples collected at earlier gestations, such as 9 weeks, has been shown to give false-negative results associated with an insufficient level of detectable cfRHD in maternal plasma64. With the use of ethylenediaminetetraacetic acid (EDTA) tubes, maternal DNA levels increases over time due to maternal white cell lysis releasing DNA into the sample. CffDNA has been found to be highly stable for RHD genotyping in EDTA tubes when sample transport is at ambient temperature (-10°C to 28°C) for up to 9 days60, 62. Finning and colleagues have found an increase in false-negative and inconclusive results when the sample was older than 14 days62. The role of a housekeeper gene, such as CCR5, that is mutual to the foetus and mother, is incorporated into cfRHD genotyping as a control61, 63. Quantification of a housekeeper gene shows whether DNA extraction was successful and quantifies the total cfDNA present in the plasma63. A high level of CCR5 indicates that there is an excess of maternal cfDNA that may interfere with the assay64. High cfDNA levels have a minimal negative effect on cfRHD genotyping where a maternal homozygous RHD deletion is present65. When a maternal RHD gene is present, as found in African populations, there is an increased likelihood of false-negative and inconclusive results with prolonged processing times66. Prompt processing and isolation of maternal plasma from the EDTA tube decreases this risk, by avoiding further maternal cfDNA accumulation in the plasma sample that has been shown to interfere with cfRHD genotyping65.
• Testing at the limits of detection of RT-PCR
Cff DNA concentrations are present at low levels in the maternal plasma and, therefore, cffRHD exons are amplified near the limits of detection in RT-PCR assays. Finning and colleagues first applied a multiple exon and multiple replicate strategy. Most published assays for cffRHD exons have tested in triplicate or quadruplicate (Table 3). These studies are based on an approach to interpretation to manage cases with low level cffDNA when amplification is not observed for all tested replicates. For example, if an RHD exon was tested in triplicate, amplification has to be observed in a minimum of two out of three replicates for detection of RHD gene sequences to be interpreted.

• RHD background from maternal RHD alleles
Most RHD genotyping strategies target at least two RHD exons. The presence of maternal RHD gene sequences is exhibited by a low C value for one or more RHD exons to represent a high level of cffDNA (Table 3). Maternal RHD gene signals typically range between 25-30 C instead of a typical foetal RHD signal at 30-38 C. (Table 3). When a maternal RHD exon is present, cff RHD cannot be differentiated with RT-PCR and its associated interpretative algorithm. Overcoming maternal RHD background requires a population-based strategy in choosing which RHD exons would be most suitable for testing. In these scenarios, in Caucasian populations, the foetal RHD genotyping outcome will be “inconclusive” and the foetus will be managed as though D-positive, for all safeguards to prevent alloimmunisation and/or HDFN to be in place (Figure 3). For African population groups, a higher proportion will carry the RHD*Ψ (or other alleles) and an RHD exon strategy to provide a conclusive outcome is required.

• RHD exons for testing
A strategic approach to the RHD exons tested for cffRHD genotyping is required to accommodate the diversity of RHD alleles predominant in the population. The design of primers and probes specific for wild-type RHD exon 4, 5 and 6, by Finning and colleagues, does not amplify the RHD*Ψ or RHCE. These primers and probes for RHD exon 4 and 5 have been combined with either RHD exon 7 or exon 10 in a duplex assay. Foetal-specific RHD wild-type sequences in exon 4 and/or 5 against a background of maternal RHD signals from RHD*Ψ, which only amplify for exons 7 and/or 10, can be detected. Different RHD exon combinations have been targeted in automated screening assays to determine whether anti-D prophylaxis is required in non-immunised women and in diagnostic assays to guide clinical management in alloimmunised pregnancies. In Sweden, a single-exon screening approach for non-immunised mothers used primers and probes, designed by Finning and colleagues, to target RHD exon 4. This assay was specifically designed to avoid amplification of maternal and fetal RHD*Ψ gene sequences, as the clinical management route from such phenotype predictions would be the same for mothers with a complete RHD deletion. This design also gives an accurate foetal D phenotype prediction for the RHD-CE-D+. A false-negative rate of approximately 1.1% was reported for samples after 8 weeks gestation in the study. Accuracy of phenotype predictions for a cffRHD genotyping assay may be improved by testing for two or more RHD-specific exons. A multi-exon approach using multiplexed RT-PCR assays is used most widely and has been tailored to detect a range of RHD alleles. Primer and probe design for RHD exon 5, by Finning and colleagues, combined with RHD exon 7, by Faas and colleagues, has been recommended by Special Non-Invasive Advances in Foetal and Neonatal Evaluation Network (SAFE) European Community (EC)-funded network of excellence. CffRHD genotyping using RHD exon 5 and 7 has been used for populations in Europe (The Netherlands, Denmark, Germany and Italy), United Kingdom (UK) and Brazil. With the most common cause of the D-negative phenotype being a complete RHD deletion in this population, detection of RHD exons 5 and 7 sequences in maternal plasma shows the foetus has RHD gene sequences and is predicted to be D-positive. If RHD exons 5 and 7 are not detected, it is consistent with an RHD gene deletion and the foetus is predicted to be D-negative. The discrepancy when RHD exon 5 is negative and RHD exon 7 is positive flags for most RHD-CE-D hybrids, including the various Caucasian-associated partial DVI types that require clinical management of the foetus as D-positive. This approach, however, would give a false-negative result if the foetus had RHD*DSBT1 (RHD-CE5-7-D) and RHD*DSBT2 (RHD*DSBT2 (RHD-CE-D (5-9)-D) gene variants expressing a partial D phenotype. An RHD exon 7 and 10 approach has been reported with 100% concordance between foetal D phenotype predictions and cord blood phenotype in 45 samples. With this approach, false-negative predictions from fetal RHD*DSBT1 and RHD*DSBT2 would be addressed and foetal RHD-CE-D hybrids encoding partial D phenotypes can still be detected for management as D-positive in the D-negative mother. However, inclusion of RHD exon 10 may give rise to false-positive results from other RHD alleles that encode the D-negative phenotype (Figure 2). Extended RHD genotyping of maternal genomic DNA is required to identify the alloimmunisation risk associated with the D-phenotype encoded from the RHD allele. An approach targeting RHD exons 4, 5 and 10 would have high specificity and be advantageous in detecting RHD alleles in Caucasian populations. This strategy has been adapted by our own group and successfully flags for Caucasian-associated maternal RHD alleles, such as RHD*DSVI, in both immunised and non-immunised D-negative obstetric groups. In our studies, extended RHD genotyping has been used successfully to identify maternal RHD alleles arising from SNPs and gene rearrangements.

• Strategies for cffRHD genotyping in sub-Saharan Africa
For sub-Saharan African populations, design of a protocol to provide correct foetal D phenotype predictions needs to accommodate a higher frequency of RHD*Ψ and RHD-CE-D that may be found in maternal D-negative populations. With approximately 95% of the sub-Saharan African population as D-positive, there is a high likelihood the D-negative mother is at risk of maternal anti-D alloimmunisation. Managing the array of African-associated RHD alleles requires a multi-exon approach. Utilising RHD exon 4 to 6 primers and probes, designed by Finning and colleagues, to avoid amplification of maternal RHD*Ψ and the RHCE gene from RHD-CE-D assists in providing accurate foetal D-negative/positive phenotype predictions. Finning and colleagues have recommended the use of an RHD exon 4 and RHD exon 5 assay with an alternative exon such as RHD exon 7 or 10 to detect RHD alleles. An advantage in targeting RHD exons 5 and 7, as recommended by SAFE, is that this approach provides foetal D-negative phenotype prediction when a maternal RHD-CE-D hybrid is present (Table 4). Maternal RHCE gene sequences in exon 5 and 7 from the RHD-CE-D are not amplified with the SAFE recommended approach, allowing for foetal RHD exons 5 and 7 to be detected. Using a two-exon approach such as with the SAFE RHD exon 5 and 7 maternal RHD*Ψ sequence interferes with foetal D phenotype predictions for RHD exon 7 but foetal D-phenotype predictions can be made based on detection of the one RHD exon 5 (Table 4). Utilising a three-exon approach with RHD-specific primers for RHD exon 4 and 5 may help in overcoming the challenge of relying on one exon to show whether there are foetal RHD signals (Table 4) in D-negative cases. The foetus should be managed as D-positive if foetal RHD exon 4 and 5 amplification was detected.
Figure 2: Diagrammatic representation of the ten RHD exons adapted from previous studies 7, 44, 57, 92, 93. RHD exons are shown in black, RHCE exons are shown in white and gene variation from wild-type RHD sequence are shown in grey boxes. Regular D-positive phenotype is encoded by wild-type RHD gene. The RHD gene deletion represents complete absence of the RHD gene and is the main molecular basis for the D-negative phenotype in Caucasian populations. In contrast to the African D-negative population, the RHD*Ψ and RHD-CE-Ds hybrids are more frequently occurring. The International Society of Blood Transfusion (ISBT) has recognised additional RHD alleles that also encodes a D-negative phenotype and typically found in the Eurasian cluster.

Table 2: Frequency of three RHD alleles in a sub-Saharan African D-negative population

<table>
<thead>
<tr>
<th>RHD-negative population</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RHD deletion</td>
</tr>
<tr>
<td>Africa, blacks (South Africa, Zimbabwe, Ghana)</td>
<td>18.5</td>
</tr>
<tr>
<td>African Americans</td>
<td>53.7</td>
</tr>
<tr>
<td>Mali</td>
<td>56.5</td>
</tr>
<tr>
<td>France, (African origin)</td>
<td>66.7</td>
</tr>
<tr>
<td>Democratic Republic of the Congo</td>
<td>53.6</td>
</tr>
</tbody>
</table>

Gene frequency

| South Africa, blacks | 0.10 | 0.07 | 0.04 |

Figure 3: Role of non-invasive prenatal testing (NIPT) for cell-free foetal (cff) RHD genotyping using a multi-exon approach in two clinical pathways. One (1) pathway demonstrates use of cffRHD genotyping in the administration of antenatal anti-D prophylaxis in non-immunised D-negative mothers. The second pathway (2) shows clinical management of pregnancies with anti-D at risk of haemolytic disease of the foetus and newborn (HDFN).

1. ANTENATAL ANTI-D PROPHYLAXIS FOR NON-IMMUNISED PREGNANCIES PATHWAY

2. CLINICAL MANAGEMENT FOR ALL IMMUNISED PREGNANCIES PATHWAY
Table 3: Summary of foetal RhD phenotype predictions from cell-free foetal RHD genotyping where a RHD exon discrepancy was exhibited

<table>
<thead>
<tr>
<th>Population</th>
<th>Real Time-PCR for cell-free foetal RHD</th>
<th>No. of samples with RHD exon Ct discrepancy</th>
<th>RHD exons positive</th>
<th>Maternal RHD Variant</th>
<th>Foetal phenotype prediction</th>
<th>Additional testing</th>
</tr>
</thead>
<tbody>
<tr>
<td>South Eastern Brazil (antenatal)</td>
<td>CCRS 5 and 7 (not RHD*Ψ)</td>
<td>At least 2/3 replicates</td>
<td>3 out of 88</td>
<td>10</td>
<td>No additional genotyping - RHD*Ψ or RHD-CE-D suspected</td>
<td>Neg</td>
</tr>
<tr>
<td>Säo Paulo, South East, Brazil (antenatal)</td>
<td>CCRS 5 and 7</td>
<td>At least 2/3 replicates</td>
<td>7 out of 220</td>
<td>7</td>
<td>RHD*Ψ</td>
<td>5 = D-negative</td>
</tr>
<tr>
<td>The Netherlands (antenatal)</td>
<td>Albumin and CCRS 5 and 7</td>
<td>At least 2/3 replicates</td>
<td>6 out of 168</td>
<td>5 (foetal below Ct 34)</td>
<td>RHD*Ψ</td>
<td>D-Positive</td>
</tr>
<tr>
<td>Portugal</td>
<td>CCRS 5 and 7</td>
<td>Triplet or quadruplet testing approach</td>
<td>8 out of 189</td>
<td>7 only</td>
<td>RHD variant</td>
<td>Possibly by foetal RHD*Ψ or RHDV1 gene. Four were serologically RHD negative and remaining four were RHD positive</td>
</tr>
<tr>
<td>United Kingdom (non-immunised population)</td>
<td>CCRS 5 and 7</td>
<td>Various: 6 out of 8 replicates, 3 out of 4 replicates, 3 out of 3 replicates and 4 out of 6 replicates.</td>
<td>Data not shown</td>
<td>Data not shown</td>
<td>RHD variant</td>
<td>Inconclusive</td>
</tr>
<tr>
<td>United Kingdom (immunised population)</td>
<td>CCRS, GADPH or SOD 5, 7, 10, 11</td>
<td>Various: 6 out of 8 replicates, 5 out of 4 replicates, 4 out of 3 replicates and 3 out of 2 replicates.</td>
<td>Data not shown</td>
<td>Data not shown</td>
<td>RHD variant</td>
<td>Inconclusive</td>
</tr>
<tr>
<td>Denmark (non-immunised population)</td>
<td>CCRS, SRY 4 and 5</td>
<td>Quadruplet (at least 2/4 replicates)</td>
<td>7 out of 713</td>
<td>4 and 5</td>
<td>RHD*Ψ</td>
<td>D-Positive</td>
</tr>
<tr>
<td>Australia (immunised population)</td>
<td>CCRS, SRY 4 and 5</td>
<td>Quadruplet (at least 2/4 replicates)</td>
<td>7 out of 713</td>
<td>4 and 5</td>
<td>RHD*Ψ</td>
<td>D-Positive</td>
</tr>
<tr>
<td>Australia (non-immunised and immunised population)</td>
<td>CCRS, SRY 4 and 5</td>
<td>Quadruplet (at least 2/4 replicates)</td>
<td>7 out of 713</td>
<td>4 and 5</td>
<td>RHD*Ψ</td>
<td>D-Positive</td>
</tr>
</tbody>
</table>

Table 4: Delineating wild-type foetal RHD signals from a maternal RHD signal in the non-invasive prenatal testing (NIPT) cell-free foetal (cff) RHD genotyping assay to provide foetal D-positive phenotype predictions (dependent on primer and probe combinations).

<table>
<thead>
<tr>
<th>RHD signal from a NIPT cffRHD genotyping RT-PCR assay</th>
<th>FETUS with wild-type RHD</th>
<th>Foetal D phenotype prediction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal RHD allele</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>1</td>
<td>RHD deletion</td>
<td>F</td>
</tr>
<tr>
<td>2</td>
<td>RHD*Ψ</td>
<td>F</td>
</tr>
<tr>
<td>3</td>
<td>RHD-CE-D</td>
<td>F</td>
</tr>
<tr>
<td>5</td>
<td>RHD*DAU3</td>
<td>M</td>
</tr>
<tr>
<td>6</td>
<td>RHD*DAU4</td>
<td>M</td>
</tr>
<tr>
<td>7</td>
<td>RHD<em>DOL1 or RHD</em>DOL2</td>
<td>F</td>
</tr>
</tbody>
</table>

F(white) = Foetal RHD signal detected
M(grey) = Maternal RHD gene signal detected
“Inconclusive” (orange) = Maternal RHD background present in all exons tested. Foetal RHD signals cannot be differentiated.
RT-PCR = Real-Time Polymerase Chain Reaction
In summary, an approach incorporating RHD-specific exon 4, 5 and 7 approach can overcome challenges with a maternal RHD*Ψ. The issue of the foetus inheriting a paternal African-associated RHD variant allele also needs consideration. Paternally-derived foetal RHD variant alleles, such as RHD*DAU-5 (RHD*c.667T>G and RHD*c.697G>C in exon 5), can be detected and interpreted as correctly as D-positive when there is a maternal RHD gene deletion. With the SAFE RHD exon 5 and 7 approach, paternally-derived foetal RHD variant alleles can also be detected despite the presence of a maternal RHD-CE-D⁺ allele. On the other hand, in cases where a maternal RHD-Ψ and paternally-derived foetal RH-D variant allele associated with a partial or weak D phenotype with changes RHD exon 5 sequences, a false-negative foetal D-phenotype prediction is theoretically possible but not reported to our knowledge. The most predominantly occurring weak D RHD allele in African populations, weak D type 4.0 (DAR 3.1), has RHD gene variation in RHD exons 4 to 6. Both maternal RHD alleles, RHD-Ψ and RHD-CE-D⁺ also have gene variations in RHD exons 4 to 6. A maternal RHD*DAU-3 allele has wild-type RHD exons 4, 5, 7 and 10. These mothers can produce allo-anti-D if exposed to foetal RHD-Ψ and RH-D variant allele associated with African populations where complications from inactive maternal RHD signals with this approach (Table 4). In such cases, the foetus will be managed as for an RhD positive outcome which is conservative but safe management.

In summary, tailoring an approach for cfRHD genotyping in D-negative women, including those with RHD-Ψ and RHD-CE-D⁺, is possible. There have been various approaches in Caucasian populations where complications from inactive maternal RHD genes have been overcome. With careful design and interpretation of genotyping outcomes, these approaches can be tailored to accommodate the array of variant RHD alleles in the African population. Capturing foetal RHD alleles in NPT, including RHD alleles associated with African populations, when a maternal inactive RHD gene is present, while possible, requires further confirmatory studies.

CONCLUSIONS AND FUTURE WORK

The application of NPT cfRHD genotyping in the clinical management of immunised and non-immunised D-negative pregnant women provides an opportunity of improved antenatal care in sub-Saharan Africa. Mothers with African-associated RHD alleles, including the frequently occurring inactive RHD genes, encoding a D-negative phenotype, are at risk of developing allo-anti-D. In Africa, a tailored design of a NPT cfRHD genotyping assay, as outlined in this review, can overcome challenges associated with African-associated RHD alleles. In addition, the continuing study of RHD alleles to identify novel gene variants associated with D-negative phenotypes in African populations, including those of low-frequency, is important for future assay design, clinical management and prevention of maternal anti-D alloimmunisation in Africa.

REFERENCES


