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-Dˢ* 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 cff*RHD* genotyping.

RESUME

CONTEXTE

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*.

STUDY DESIGN/ MATERIALS AND METHODS

Online literature searches using Google, Google Scholar and PubMed, textbooks and International Society of Blood Transfusion Blood Group Tables.

RESULTS

A NIPT assay design for cff*RHD,* tailored with the SAFE recommended *RHD* exon 5 and 7 approach, can provide foetal D-positive or D-negative predictions when a maternal *RHD*Ψ* and *RHD-CE-Dˢ* is present. Inclusion of *RHD* exon 4 in NIPT is a tool for increasing confidence in D-phenotype prediction.

CONCLUSIONS

A strategic approach to NIPT cff*RHD* genotyping can overcome challenges with maternal *RHD* interference from *RHD*Ψ* and *RHD-CE-Dˢ* alleles in D-negative pregnant women. Accommodating for these *RHD* alleles provides knowledge for clinical management to minimise maternal anti-D alloimmunisation and 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 cff*RHD* genotyping in sub-Saharan African populations.

CONCEPTION DE L'ÉTUDE / MATÉRIELS ET MÉTHODES

Recherche de littérature en ligne à l'aide de Google, Google Scholar et PubMed, des manuels et des tables de groupes sanguins de la Société Internationale de Yransfusion Sanguine.

RÉSULTATS

Un modèle d'essai NIPT pour cff*RHD*, adapté à l'approche des exon 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-Dˢ* maternel 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-Dˢ* 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 fœtus 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[1-4]. 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-Dˢ* hybrid[5]. In marked contrast, 15-17% of the Caucasian population are D-negative, most commonly arising from a complete *RHD* gene deletion[67]. 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 pregnancies from African populations[8].

Strategies to prevent maternal alloimmunisation include routine postnatal and antenatal anti-D prophylaxis[9]. The postnatal strategy, alone, reduces the risk of immunisation from 17% to 0.8-1.5%[10]. Antenatal anti-D prophylaxis for all D-negative pregnancies further reduces the immunisation rate to 0.2% and, therefore, also the rate of haemolytic disease of the foetus and newborn (HDFN)[11]. The issue with universal anti-D prophylaxis programs is that anti-D prophylaxis is not necessary when both mother and foetus are D-negative. In pregnancies with allo-anti-D, a D-positive foetus is at high risk of HDFN .*RHD* genotyping of foetal DNA provides knowledge of the foetal D status to guide clinical management[12]. First-generation foetal *RHD* genotyping in the 1990s required invasive procedures such as chorionic villus sampling or amniocentesis[13]. Although important, these invasive procedures pose a risk, firstly of increasing maternal anti-D levels from a further sensitisation event and more significantly up to a 1% risk of pregnancy loss[14, 15]. In 1997, the discovery of cell-free foetal (cff) DNA in maternal plasma by Lo and his colleagues enabled non-invasive prenatal testing (NIPT)[16].NIPT can predict the foetal D phenotype and shows where anti-D prophylaxis is unnecessary in non-immunised pregnancies. In alloimmunised pregnancies, a non-invasive foetal *RHD* genotyping approach removes the risks associated with invasive procedures. For cff*RHD* genotyping, the detection of *RHD* sequences in maternal plasma forms a basis for prediction of a D-positive or D-negative foetal phenotype[17, 18].

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 cff*RHD* 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 cff*RHD* 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 [19]. In Africa, the most common clinically significant alloantibody reported in pregnant women is anti-D[8]. 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[20]. 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%[10]. This rate can be further reduced to 0.2% by providing routine antenatal anti-D prophylaxis in late second and early third trimester[11]. 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[20-25]. 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 *RHD* 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 cff*RHD* 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' alloimmunistion have been reported[26].

- **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 lRBCs[27]. This can lead to foetal anaemia and in severe cases, erythroblastosis fetalis, hydrops fetalis and intrauterine foetal death when left untreated[12, 28]. Hydrops fetalis has been shown to be minimised with intrauterine transfusion in 65% of cases[29]. It is important to monitor at-risk pregnancies during antenatal care to prevent HDFN[12,27]. 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[12]. If the foetus is known to be D-negative, this monitoring can be avoided as the foetus is not at risk. First-generation foetal *RHD* genotyping using invasive procedures such as amniocentesis was introduced in the early 1990s[13]. However, invasive procedures such as chorionic villus sampling and amniocentesis pose a risk of increasing maternal anti-D levels and an increased likelihood of miscarriage[15, 30]. 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 cffRHD genotyping

In 1997, Lo and colleagues discovered that cffDNA can be detected in maternal plasma during pregnancy, initiating the field of Non-Invasive Prenatal Testing (NIPT)[16]. In maternal plasma, cff 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[31, 32]. The level of cffDNA, 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[33, 34]. Early studies used RT-PCR to detect foetal-specific gene sequences such as the male sex determining Y-chromosome gene, *SRY*[31, 32, 35].NIPT application has since expanded to detect feta lchromosomal abnormalities and has been performed in South Africa[36]. 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 cff*RHD* gene sequences in maternal plasma was first used to show the clinical utility for NIPT technologies using RT-PCR.RT-PCR for *RHD* exons based on TaqMan technology detected cff*RHD* by targeting and amplifying *RHD* exons using the 5' to 3' nuclease activity of TaqMan DNA polymerase[37,38, 39]. As the large majority of D-negative Caucasians have a complete deletion of the *RHD* gene, there is usually no background maternal *RHD* signals to compete with detection of cff*RHD* fragments[40]. To perform foetal *RHD* genotyping optimally, cfDNA is isolated from maternal plasma as promptly as possible. CfDNA 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[41]. A complementary DNA strand is synthesised using TaqMan DNA polymerase from deoxynucleotides (dNTPs) in the reaction mixture[41]. When the complementary strand extends toward the probe, the reporter fluorescent dye emits a fluorescent signal after it is cleaved from the quenching dye[41]. Fluorescence indicates that cff*RHD* gene sequences are present in maternal plasma. As cff*RHD* 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 (C_t). Foetal *RHD* signals in RT-PCR typically range between 30 to 38 Ct, which indicate low DNA levels[26]. The presence of foetal *RHD* predicts the foetus is D-positive. The foetus is predicted to be D-negative when there is no detectable signal from foetal *RHD* and is consistent with an *RHD* gene deletion. For foetal *RHD* genotyping assays, which assume that the maternal *RHD* sequences are absent, the presence of a maternal *RHD* variant gene has potential to hinder interpretation of the assay. On this basis, it is important to define the range of *RHD* alleles in local populations.

3. RH GENETICS *AND* RHD ALLELES
associated with African populations

The Rh blood group system is comprised of two closely-linked and highly homologous genes on chromosome 1, *RHD* and *RHCE* [7, 42]. Both genes have ten exons, with *RHCE* encoding for C/c and E/e antigens and *RHD* for the D antigen[7]. 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%)[2,7,43]. In *RHD* genotyping, there is a diverse array of *RHD* alleles that have been described[44, 45]. *RHD* alleles have been described to possess qualitative and/or quantitative differences to the regular D antigen resulting in unusual serological findings[7, 46].

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**
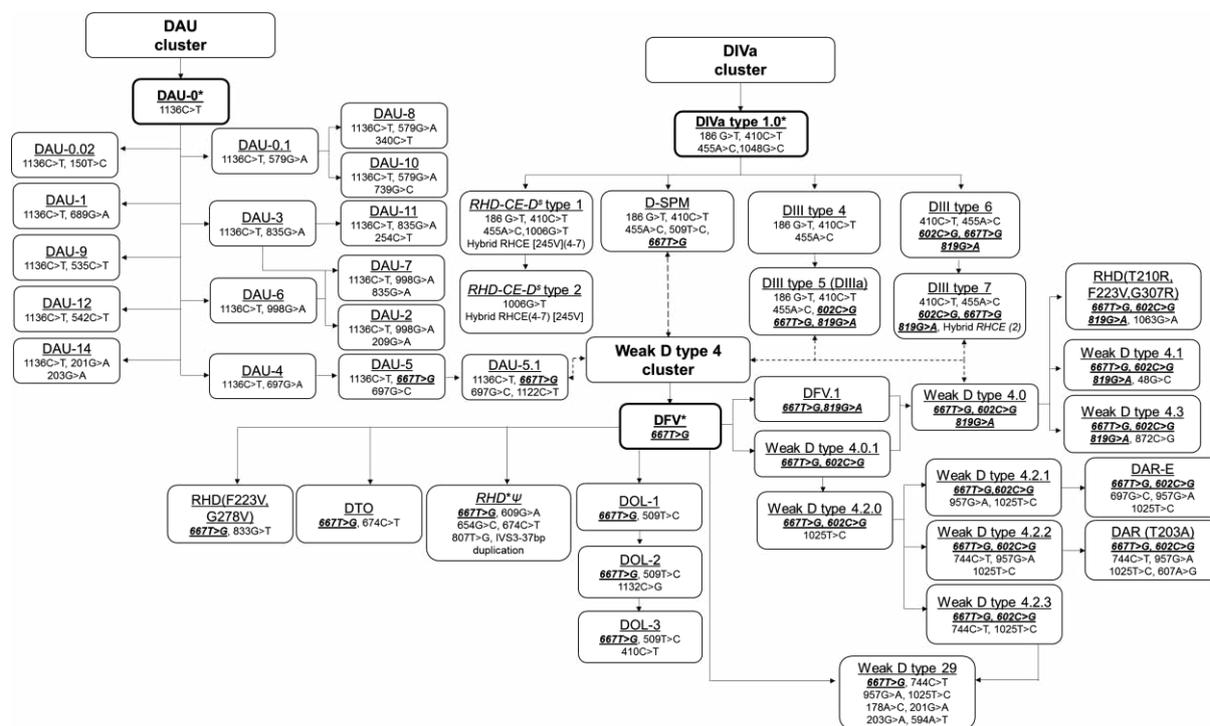

Table 1: Anti-D alloimmunisation from African populations

Cluster	RHD variant	Sex	Rh Phenotype	Anti-D (titre given, if available)	Immunisation Event	Ethnicity	Study
DAU	DAU-3	Not described	ccDee	Yes	Not described	African	48
	DAU-4	Male	ccDee	Yes	1 D+ RBC transfusion (treatment of sickle cell anaemia)	Not described	87
DIVa	DIVa type 1.0/ RHD* Ψ	Female	ccDee	256	2 pregnancies	Mulatto (Dominican Republic)	50
	DIVa type 1.0	Female	ccDee	16	2 live births 1 miscarriage	African (Togo)	
	RHD-CE-Dˢ type 1 (2 cases)	Not described	(C)cdee	Yes	Yes	African and Afro-Caribbean population	88
Weak D type 4	DOL/RHD-CE-Dˢ	Not described	(C)cDee	128	2 RBC transfusions (treatment of sickle cell/β-thalassemia)	Ewe (West Africa)	49
	DAR (weak D type 4.2.0)	Female	ccDee	Yes	Multiple RBC transfusions over years (treatment of sickle cell anaemia)	African black	89
	DOL-1	Female	ccDee	8	Pregnancy	African descent	90
	DOL-2	Female	ccDee	128	Pregnancy	African descent	
		Female	ccDee	Global titer:64	Pregnancy	African descent	
	RHD* Ψ (3 cases)	Female	ccDee (presumably)	Yes	Not described	African	5
	RHD* Ψ	Female	ccDee (presumably)	Yes	Pregnancy	Ghana	
	RHD* Ψ	Female	ccdee	Yes	Not described	Not described	26

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'[7, 46]. Interestingly, for some *RHD* variants there is RhC/c and RhE/e haplotype association[47]. 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[7]. 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>T*[48]
- weak D type 4.0 cluster :*RHD*c.667 T>G*[49]
- DIVa cluster: *RHD* c.455A>C* [750].

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 populations[51]. Interestingly, the weak D type 4 and DIVa clusters contain the *RHD* allele, *RHD* pseudogene (RHD*Ψ)* and *RHD-CE-D^s*, 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, 5]. The first gene mechanism is a homozygous *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^s* are notably associated with the D-negative phenotype in sub-Saharan African populations[5, 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* 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) (Figure2)[5]. These changes predict a completely inactive gene and indeed mRNA transcripts for this *RHD* allele have not been detected[5]. 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 mutation[7, 49, 53].Out of 5 African women with anti-D reported by Singleton and colleagues, four had the *RHD*Ψ* with one case involving a Ghanaian woman and resulting in HDFN (Table 2).

• RHD-CE-D^s hybrid
The *RHD-CE-D^s* 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^s* because of the differences in the gene sequence of exon 3. The type 1 hybrid, *RHD-DIIIa-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* exon3 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*DIIIa* 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 3[7, 50] (Figure 1). The *RHD-CE-D^s* type 2 hybrid, *RHD*D-CE(4-7)-D*, resembles *RHD-CE-D^s* type 1 except that *RHD* exon 2 and 3 has remained intact and allele is also included in the DIVa cluster (Figure 1)[7]. 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 allele[7, 49]. The ISBT has recognised a range of *RHD* alleles that encode a D-negative phenotype (Figure 2)[44]. The D-negative population in Brazil resemble African populations with the presence of *RHD*Ψ* and *RHD-CE-D^s* but at a lower frequency[54, 55].In the Tunisian D-negative population with C+ or E+, *RHD-CE-D* hybrids (Figure 2) were observed [56]. Among East Asian and European D-negative populations, the most frequently observed *RHD-CE-D* hybrid was *RHD-CE(2-9)-D_2*[57-59].In the Australian D-negative population with C+ and/or E+ haplotypes, this was the *RHD-CE(3-8/9)-D* hybrid [47].

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

For sub-Saharan African populations, design of a non-invasive cff*RHD* genotyping assay requires a strategy to minimise false-positive results caused by *RHD* alleles encoding a D-negative phenotype[60,61]. 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 *RHD*exons that are informative (specific for the population).

• Preanalytical factors – sample collection and transport
Cff*RHD* 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-D[39, 62, 63]. 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 cff*RHD* in maternal plasma[64]. 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 days[65, 66]. Finning and colleagues have found an increase in false-negative and inconclusive results when the sample was older than 14 days[67]. The role of a housekeeper gene, such as *CCR5*, that is mutual to the foetus and mother, is incorporated into cff*RHD* genotyping as a control[39, 68]. Quantification of a housekeeper gene shows whether DNA extraction was successful and quantifies the total cfDNA present in the plasma[39]. A high level of *CCR5* indicates that there is an excess of maternal cfDNA that may interfere with the assay[68]. High cfDNA levels have a minimal negative effect on cff*RHD* genotyping where a maternal homozygous *RHD* deletion is present[65]. 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 times[69]. 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 cff*RHD* genotyping [65].

- **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[39]. Most published assays for cff*RHD* 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_t value for one or more *RHD* exons to represent a high level of cfDNA (Table 3). Maternal *RHD* gene signals typically range between 25-30 C_t instead of a typical foetal *RHD* signal at 30-38 C_t[26, 39] (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 cff*RHD* 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*[39, 47, 68]. 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[18, 26, 63, 67, 68, 70-79]. 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[77]. 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*[s]. A false-negative rate of approximately 1.1% was reported for samples after 8 weeks gestation in the study[80]. Accuracy of phenotype predictions for a cff*RHD* 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[64]. 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[70]. Cff*RHD* 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[18, 63, 67, 70-76]. 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*DBT1* (*RHD-CE5-7-D*) and *RHD*DBT2* (*RHD*D-CE (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 [78, 81]. With this approach, false-negative predictions from fetal*RHD*DBT1* and *RHD*DBT2* 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 10would have high specificity and be advantageous in detecting *RHD* alleles in Caucasian populations [26, 68, 79, 82]. This strategy has been adapted by our own group and successfully flags for Caucasian-associated maternal *RHD* alleles, such as *RHD*DVI,* in both immunised and non-immunised D-negative obstetric groups[26, 68, 69]. 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*[s] 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[2-4]. 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*[s] assists in providing accurate foetal D-negative/positive phenotype predictions[39] .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 [39]. An advantage in targeting *RHD* exons 5 and 7, as recommended by SAFE, is that this approach provides foetal D-positive phenotype prediction when a maternal *RHD-CE-D*[s]hybrid is present (Table 4). Maternal *RHCE* gene sequences in exon 5 and 7 from the *RHD-CE-D* [s] 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 7but 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 4and 5 may help in overcoming the challenge of relying on one exon to show whether there are foetal *RHD* signals (Table 4) [5, 68, 76].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 encode a D-negative phenotype and typically found in the Eurasian cluster.
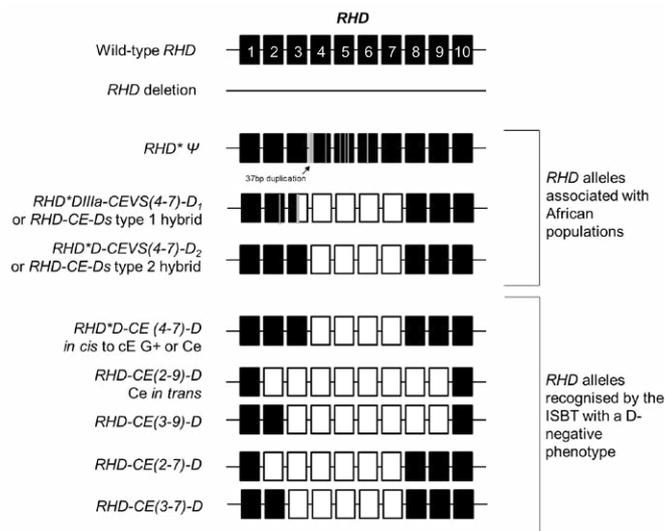

Table 2: Frequency of three *RHD* alleles in a sub-Saharan African D-negative population

RhD-negative population	Frequency (%)				
	RHD deletion	*RHD*Ψ*	*RHD-CE-Dˢ*	Other	
Africa, blacks (South Africa, Zimbabwe, Ghana) [5]	18.5	66.7	14.8		
African Americans [5]	53.7	22.0	24.0		
Mali [52]	56.5	26.2	17.3		
France, (African origin) [91]	66.7	19.4	8.3	2.8	*RHD*Ψ/ RHD-CE-Dˢ*
				2.8	Novel – *RHD* c.952C>T*
Democratic Republic of the Congo [84]	53.6	31.8	5.5	2.7	*RHD*Ψ/RHD-CE-Dˢ*
				6.4	Weak D type 4.2.2
					Weak D type 4.0.1
					Weak D type 4.2.3
					Weak D type 4.0.1/ *RHD-CE-Dˢ*
					DAR/RHD-CE-Dˢ
	Gene frequency				
South Africa, blacks [5]	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).
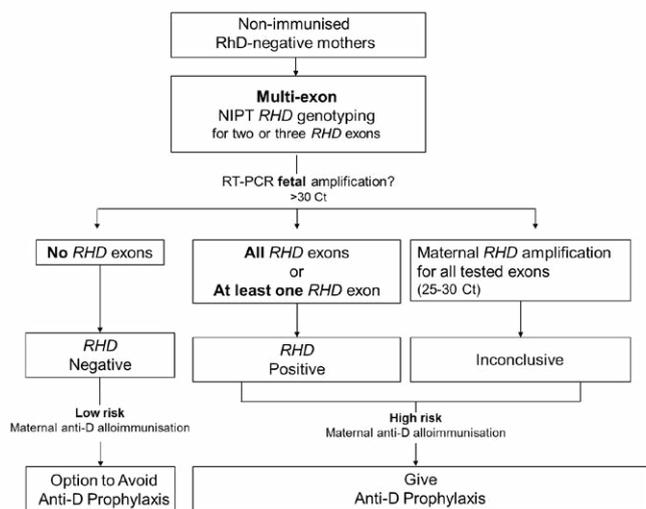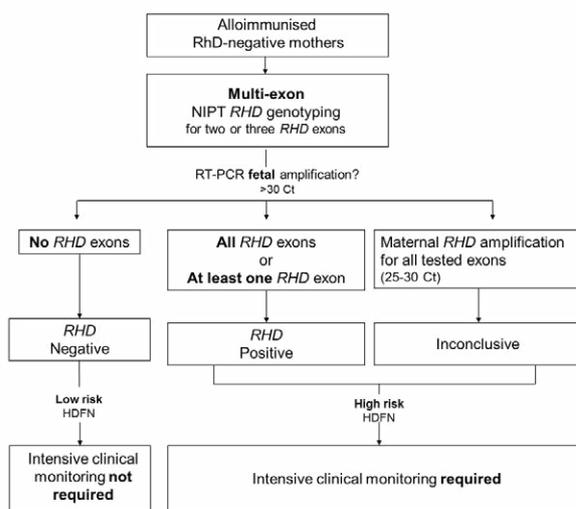
Table 3: Summary of foetal RhD phenotype predictions from cell-free foetal RHD genotyping where a RHD exon discrepancy was exhibited

Population	Real Time-PCR for cell-free foetal *RHD*			No. of samples with *RHD* exon Ct discrepancy	*RHD* exons positive	Maternal *RHD* Variant	Foetal phenotype prediction	Additional testing
	Internal Controls	*RHD* Exons Tested	Replicates for *RHD* positive call in an exon					
South Eastern Brazil [82] (antenatal)	*SRY* and *CCR5*	4, 5 and 10 (not *RHD*Ψ*)	At least 2/3 replicates	3 out of 88	10	No additional genotyping - *RHD*Ψ* or *RHD-CE-D^s* suspected	NEG	
São Paulo, South East, Brazil [76] (antenatal)	*CCR5*	5 and 7	At least 2/3 replicates	7 out of 220	7	*RHD*Ψ*	5 = D-negative	
						Multiplex PCR for *RHD* exon 3 to 7 and 9 → No amplicon obtained	2 = D-positive	BeadChip and gene sequencing for *RHD* exon 5 and 8 → Foetal DAU-5
The Netherlands [92] (antenatal)	*Albumin* and *CCR5*	5 and 7	At least 2/3 replicates	6 out of 168	7 (Ct above 34) 5 (foetal below Ct 34)	*RHD*Ψ*	D-Positive	
				1 out of 168	5 only (Ct 38)	*RHD* deletion	D-Positive	Paternal DVa/d
				1 out of 168	7 only	*RHD* deletion	D-positive	Paternal *RHD/DAU5*
				1 out of 168		*RHD*Ψ /d*	D-negative	Paternal *RHD*Ψ/DAU*
				1 out of 168	(Ct above 36)	*RHD*Ψ/RHD*CE-D^s*	D-negative	N/A
United Kingdom [39, 67]	*CCR5*	5 and 7	Triplicate or quadruplicate testing approach	8 out of 1869	7 only		*RHD* variant	Possibly by foetal *RHD*Ψ* or *RHDVI* gene. Four were serologically RhD negative and remaining four were RhD positive
				3 out of 1869	5 and/or 7	*RHD* deletion	Inconclusive	
				10 out of 1869		*RHD*Ψ*		
				1 out of 1869		*RHD*DVI*		
				1 out of 1869		*RHD*722C>T* (Weak D type 67)		
Denmark [72, 94]	*CCR5*, *GADPH* or *SOD*	5,7; 5,10; 7;10	Various: 6 out of 8 replicates, 3 out of 4 replicates, 3 out of 3 replicates and 4 out of 6 replicates.	Data not shown	Data not shown	*RHD*Ψ* (8 out of 12 668)	Inconclusive	
						*RHD*CE-D^s* (9 out of 12 668)		
						Weak D types (93 out of 12 668)		
						*RHD*DVI* (14 out of 12 668)		
						DAU2/Ccdes-1 (1 out of 12 668)	False D-positive	
						IVS3+1G>A (1 out of 12 668)		
						DIII (1 out of 12 668)		
						Maternal/foetal *RHD*Ψ* (1 out of 12 668)		
								Foetal *RHD* variants detected were: *RHD*Ψ, RHD-CE (3-7)-D, RHD-CE (2-9)-D, 952C>T* (D-negative), c.351delC.
Australia [68] (immunised population)	*CCR5, SRY*	4,5 and 10	Quadruplicate (at least 2/4 replicates)	2 out of 140	10	*RHD*Ψ*	Inconclusive	Confirmed *RHD*Ψ* with *RHD*Ψ* Exon 4 and Exon 5 specific primers
						RHD exon 4, 5 and 7 negative. Inactive *RHD* gene suspected		
Australia [26] (non-immunised and immunised population)				7 out of 713	4,5 and 10	*RHD*1227G>A, RHD*113G RHD*IVS1+G>A, RHD*94insT, RHD*361del11nt(F,155X)* and *RHD*del329-330*	Inconclusive	
				5 out of 713	10	*RHD*(D-CE(4-6)-D), RHD*D-CE(3-9)-D, RHD*DIIIa-CE(3-7)-D, RHD* Ψ*	4 = D-positive 1 = D-negative	

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).

	RHD signal from a NIPT cff*RHD* genotyping RT-PCR assay					
		FETUS with wild-type *RHD*				Foetal D phenotype prediction
	Maternal *RHD* allele	4	5	7	10	
1	*RHD* deletion	F	F	F	F	D-positive
2	*RHD*Ψ*	F	F	M	M	D-positive
3	*RHD-CE-D^s*	F	F	F	M	D-positive
4	*RHD*DAR1* (weak D type 4.2.0)	F	F	F	M	D-positive
5	*RHD*DAU3*	M	M	M	M	Inconclusive
6	*RHD*DAU4*	M	F	M	M	D-positive
7	*RHD*DOL1* or *RHD*DOL2*	F	F	M	M	D-positive

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[76] .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-Ds* allele. On the other hand, in cases where a maternal *RHD*Ψ* and paternally-derived foetal *RHD*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[83-85]. Both maternal *RHD* alleles, *RHD*Ψ* and *RHD-CE-Ds* 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 D-positive RBCs (Table 2). Cff*RHD* genotyping will be interpreted as inconclusive due to high maternal *RH*D 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 cff*RHD* genotyping in D-negative women, including those with *RHD*Ψ* and *RHD-CE-Ds*, 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 NIPT, 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 NIPT cff*RHD* 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 NIPT cff*RHD* 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.

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