The use of free DNA for fetal *RHD* genotyping in the Rh negative pregnant patient—the time has come

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Cell-free DNA to determine the fetal *RHD* genotype from the maternal circulation was first described in 1993. High throughput assays using polymerase chain reaction technology were introduced in Europe and gained widespread acceptance in the management of the Rhesus alloimmunized pregnancy. The specificity and sensitivity of these assays approached 99%. As confidence was gained with these results, Scandinavian countries began to employ cell-free DNA for fetal *RHD* typing as an integral component of their introduction of antenatal Rhesus immune globulin in non-alloimmunized pregnancies. Since 40% of RhD-negative pregnant women will carry an RhD-negative fetus, doses of Rhesus immune globulin were conserved. Recently 2 U.S. companies have introduced cell-free DNA assays for *RHD* as part of their noninvasive prenatal testing assays. Both utilize next generation sequencing and have developed methodologies to detect the aberrant *RHD* pseudogene and the hybrid *RHD-CE-D*^{s} genotype. In addition, excellent correlation studies with either neonatal genotyping or serology have been reported. The manufacturer of RhoGAM has recently announced a national shortage. Given the current availability of reliable cell-free DNA assays for determining the *RHD* status of the fetus, the time has come to implement this strategy to triage the antenatal use of Rhesus immune globulin in the U.S.

Key words: cell-free DNA, NIPT, *RHD*, red cell alloimmunization, RhD, *RHD-CE-D*^{s} hybrid gene, *RHD* pseudogene, Rhesus immune globulin, RhoGAM

Introduction

Cell-free DNA (cfDNA) DNA-based noninvasive prenatal testing (NIPT) for fetal trisomies and sex chromosome abnormalities became commercially available in the United States in 2011.[1] Initially, it enjoyed limited use in pregnancies at high risk from chromosomal abnormalities. However in 2020, both the American College of Obstetricians and Gynecologists (ACOG) and the Society for Maternal-Fetal Medicine recommended that prenatal genetic screening be offered to all pregnant women.[2] Given the increasing coverage by state Medicaid carriers, it is estimated that 30% to 50% of pregnancies in the U.S. undergo testing with NIPT.[3]

NIPT technology continues to improve with many companies now offering screening for microdeletions such as DiGeorge syndrome and single gene abnormalities such as cystic fibrosis. The use of NIPT to determine the fetal *RHD* status is the latest addition to NIPT assay in the U.S.

The Rh genes

The location of the Rh genes on the short arm of chromosome 1 was first described in 1991.[4] Since there are 3 types of Rh antigens expressed on the red blood cell—D or absence of D, E or e, and C or c, the original concept was that there must be 3 genes present. However we now know that there are only 2 genes present—the *RHD* gene and the *RHCE* gene (see Figure 1).[5] The *RHCE* genes encodes for both the C/c and E/e red cell antigens through altered transcription of the gene into protein antigens.[5]

As advances in genetics have entered the clinical arena, one must understand that genotype and phenotype are not always synonymous. Various *RHD* genotypes may not result in the predicted phenotype—the expression of the RhD antigen on the surface of the red cell as determined by serologic testing. Two important examples of this phenomenon are the *RHD* pseudogene (*RHDΨ*) and the hybrid *RHD-CE-D*^{s} genotype (Figure 1). In the first case, all 10 exons of the *RHD* gene are present but a stop codon in exon 4 and a nonsense mutation in exon 6 result in the lack of transcription and a RhD-negative phenotype. Another important example of the genotype/phenotype mismatch in RhD typing is the *RHD-CE-D*^{s} genotype. In this case, the *RHD* gene is a hybrid of the usual *RHD* gene and the *RHCE* gene. As a result, the patient's phenotype is RhD-negative by serology. cfDNA assays that are not specifically designed to detect these genes would inadvertently result in a false positive fDNA result.

The majority of Caucasians that are RhD-negative will have a simple *RHD* gene deletion at both genetic loci. However, the *RHDΨ* and the *RHD-CE-D*^{s} genotypes are more common in Rh-negative individuals of African descent. One study showed the in RhD-negative black Africans, 18% had a deletion of the *RHD* gene, 67% had the *RHDΨ*

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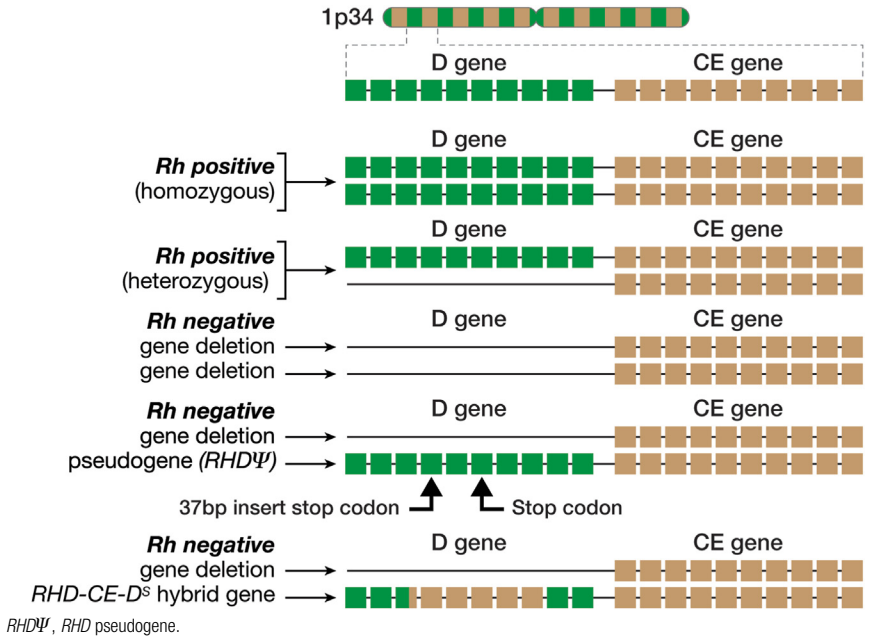
genotype, and 15% had the *RHD-CE-D*^{s} genotype.[6] The percentages in RhD-negative African-Americans were 54%, 24%, and 22%, respectively.

Cell-free DNA for *RHD* determination

With the introduction of cordocentesis into clinical practice, direct serologic testing of fetal blood for the RhD antigen was introduced in pregnancies complicated by Rh alloimmunization with an inherent risk of fetal loss of 1.6%.[7,8] After the *RHD* gene was discovered, amniocentesis for analysis of fetal DNA in amniotic fluid became standard practice to determine the fetal *RHD* status in cases of an unknown paternity or paternal heterozygosity.[5,9] This reduced the risk of fetal loss secondary to invasive testing to 0.4%.[10] Chorion villus sampling was avoided for fetal *RHD* determination due to the possibility of enhanced maternal antibody response secondary to disruption of the feto-maternal interface.[11]

Lo et al[12] was the first to report the successful use of cfDNA in the blood of a pregnant woman to detect the *RHD* genotype of the fetus. In 2001, the International Blood Group Reference Laboratory in Bristol, England began to offer prenatal cfDNA *RHD* testing using quantitative polymerase chain reaction methodology.[13] With the advent of cfDNA to determine the fetal *RHD* status, a noninvasive test entered the clinical care of the alloimmunized pregnancy in many European countries. A meta-analysis of 30 studies involving 10,290 tests found a sensitivity of 99.3% with a specificity of 98.4% for determining the fetal *RHD* status.[14] In cases of pregnancies complicated by red cell alloimmunization, cfDNA was used as the next step to see if the fetus was at risk for anemia due to a positive antigen status. In the case of a positive cfDNA, serial middle cerebral artery peak systolic velocity (MCA-PSV) Doppler measurements could be undertaken and if elevated suggesting fetal anemia, intrauterine transfusion could be performed. With the introduction of reliable cfDNA testing in the U.S. for fetal *RHD*, ACOG has recently issued a Clinical Practice Update to suggest that this assay would be reasonable to use as an alternative tool to amniocentesis for fetal red cell typing in the alloimmunized pregnancy.[15]

FIGURE 1
***RHD* and *RHCE* genes and their associated genotypes and phenotypes**



RHDΨ, *RHD* pseudogene.

A short-lived American assay

In 2010, a California-based company (Sequenom, Inc; LabCorp, California) developed a cfDNA assay for fetal *RHD* and branded it SensiGene *RHD* genotyping laboratory developed test.[16] A prospective trial using this assay was undertaken in 120 non-alloimmunized RhD-negative patients undergoing samplings in all 3 trimesters.[17] When comparing the cfDNA results to cord serology at birth, only 1 false negative result occurred in the 349 total samples that were deemed reportable (6.3% of samples were reported as inconclusive). An investigation of the 1 error indicated that the sample was mislabeled with the incorrect patient's name. Hawk et al[18] studied the projected costs of using cfDNA tor triaging antenatal Rhesus immune globulin (RhIG) in the U.S. and noted that the break-even reimbursement for the assay was $119. A subsequent analysis using the outcomes of the Sequenom assay revealed that when the costs of the first and a second alloimmunization pregnancy were combined, a strategy of universal use of RhIG without paternity testing was the least expensive to the U.S. health system.[19] These costs were lower than a strategy employing cfDNA for the triage of antenatal RhIG. The Sequenom assay was discontinued several years later.

With no available assay in the U.S., most centers continued to use amniocentesis to decide if the fetus was at risk for the development of anemia in the Rh alloimmunized pregnancy. The introduction of NIPT for chromosomal abnormalities has resulted in a 30% reduction in the uptake of amniocentesis to detect fetal chromosomal abnormalities.[20] Even the alloimmunized patient often refused "invasive" testing to determine the fetal *RHD* status. The clinician's only recourse was then to monitor these pregnancies with serial MCA-PSV Doppler assessments even though 40% of cases involved an RhD-negative fetus. More importantly, a false positive rate of 12% to 18% has been reported for an elevated MCA-PSV of >1.5 MoM.[21,22] Thus an elevated MCA-PSV

in these cases has the potential to result in an unnecessary cordocentesis with its associated complications.

New assays enter the U.S. market

In September 2022, a new assay for *RHD* fetal testing was introduced in conjunction with NIPT screening for chromosomal abnormalities. In addition, fetal testing for C, c, E, Kell, and Fy[a] was also introduced. A study of 1061 preclinical samples reported a sensitivity of 100% (confidence interval [CI]: 99%−100%), a specificity of 100% (CI: 99%−100%) and a no call rate of 0.1%.[23] A subset of this data involving *RHD* revealed 100% concordance (Table). A subsequent study was reported comparing NIPT results with neonatal buccal genotypes in 41 Rh-negative patients with 100% concordance between NIPT results and neonatal testing.[24] Finally, a study of 401 nonalloimmunized RhD-negative women was undertaken comparing the cfDNA result to neonatal serology. Two hundred sixty-one of the cases involved a RhD-positive fetus and 140 involved a RhD-negative fetus.[25] The authors reported 100% concordance between the cfDNA result and neonatal serology (sensitivity: 100%; 95% CI: 98.6−100) and (specificity: 100%, 95% CI: 97.4−100). Of interest, 5 cases involved the *RHDΨ* and 5 cases involved the *RHD-CE-D*^{s} genotype.

In over 20,000 clinical samples for *RHD* determination, the "no-call" rate was 0.03% (personal communication: Julia Wynn, BillionToOne, Inc). These are usually due to low levels of cfDNA molecules and are resolved with a repeat sample from the patient.

In May of this year, a second company reported the addition of fetal *RHD* testing in conjunction with its NIPT assay. The results of the assay were compared to a polymerase chain reaction (PCR)-based assay from Europe with 100% concordance (Table).[26] A second study in 655 RhD-negative patients compared cfDNA results to neonatal serology and revealed an overall sensitivity of 100% (95% CI: 96.9% −100%) and a specificity of 99.3% (95% CI: 97.6%−99.8%) (Table).[27]

Both currently available cfDNA U.S. assays use next generation sequencing (NGS). The process consists of an initial extraction of cfDNA (containing both maternal and fetal DNA) from the maternal blood sample. This is followed by a PCR using primers to amplify specific regions of the target gene to create amplicons or multiple replications of these target gene regions (see Figure 2). The NGS is then used to simultaneous sequence the amplicons. The sequenced amplicons are analyzed to determine the region of the gene and quantify the number of molecules of specific regions to determine the genotype of the fetus. Algorithms and bioinformatics are then used to create an interpretation of the results.

These assays differ from the current PCR technology used in European countries for the detection of fetal red cell antigens. The 2 U.S. based assays include fetal red cell antigen typing as part of NIPT for chromosomal screening and quantifying the proportion of cfDNA fetal fraction is standard practice. The European assays may not quantify the cfDNA. In addition, the PCR technology they use relies on the assumption that the mother's genotype is the *RHD* gene deletion and the fetus has either the *RHD* gene deletion or the usual *RHD* gene. This assumption leads to incorrect or inconclusive calls for non-*RHD* gene deletions (*RHDΨ* and *RHD-CE-D*^{s} hybrid gene), more common in individuals of non-European ancestry. Additionally, because these assays are qualitative and not quantitative and in cases of a low fetal fraction a false negative result may occur.

The 2 U.S. based assays may differ in their capability to detect *RHD* gene variants especially if these are inherited from the mother of the fetus. As an example, an *RHDΨ* in the mother (who is serologically RhD-negative) may be difficult to detect in the fetus if this gene has been inherited from her.

These assays are performed as part of the standard NIPT testing for

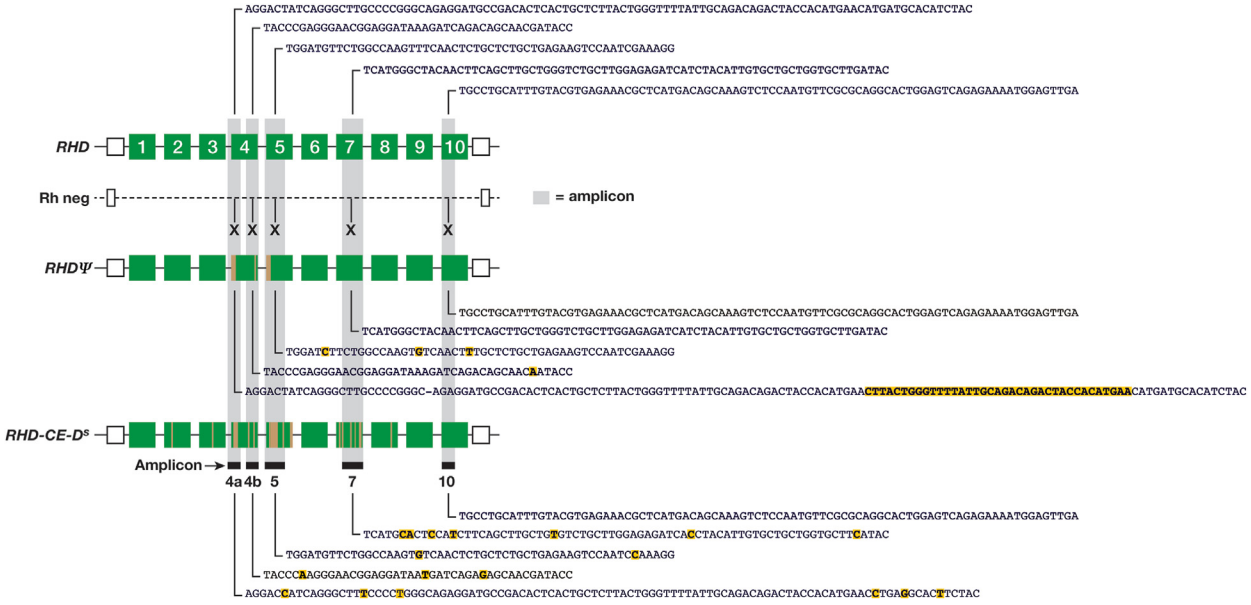
TABLE
Correlation studies of 2 U.S. based free DNA assays for fetal *RHD*

No of samples/patients	*RHD pos* NIPT	*RHD neg* NIPT	Rh *pos* result	Rh *neg* result	Sensitivity (95% CI)	Specificity (95% CI)
Initial U.S. assay[a]						
456[24]	254	191	454	192	100% (98.6−100)	100% (98.1−100)
401[25]	261	140	261	140	100% (98.6−100)	100% (97.4−100)
Second U.S. assay[b]						
110[26]	70	40	70	40	100% (94.9−100)	100% (91.2−100)
655[27]	358[c]	295	356	297	100% (98.9−100)	99.3% (97.6−99.8)

NIPT, noninvasive prenatal testing.

[a] UNITY assay (BillionToOne, Inc); [b] Panorama (Natera, Inc); [c] Two false positive results (fetal *RHD* result: *RHD* pos; neonate RhD negative by serology).

FIGURE 2
Next generation sequencing using one of the currently available U.S. cfDNA assays for *RHD*[23]



Gray vertical boxes indicate the position of the amplicons on the various exons of the *RHD*, *RHDΨ*, and the *RHD-CE-D*^{s} genes. *Yellow letters* indicate base sequence differences from the *RHD* gene detected by the specific amplicons. *cfDNA*, cell-free DNA; *RHDΨ*, *RHD* pseudogene.

aneuploidies and currently cannot be ordered as a separate test. However, if an NIPT test is ordered earlier in gestation, the companies offering these assays can be contacted later in gestation to provide the results of the *RHD* test without any additional charge to the patient.

A new paradigm shift occurs

The use of Rhesus immune globulin (RhIG) after a RhD-negative patient delivers an RhD-positive infant has been the standard of care in developed countries for more than 60 years. In the mid-1980's, North American countries implemented antenatal RhIG in the early third trimester to further reduce the incidence of Rh alloimmunization in pregnancy. Due to concerns with Creutzfeldt-Jakob disease, plasma obtained in the U.S. has been used to develop plasma-derived products including RhIG in many industrialized countries. Additionally, many European countries have banned the purposeful Rh immunization of male donors (a common source of high titer Rh plasma in the U.S.). This led to a limited supply of RhIG in most countries outside of North America. As a result, routine antenatal RhIG prophylaxis was not routinely adopted in many developed countries.

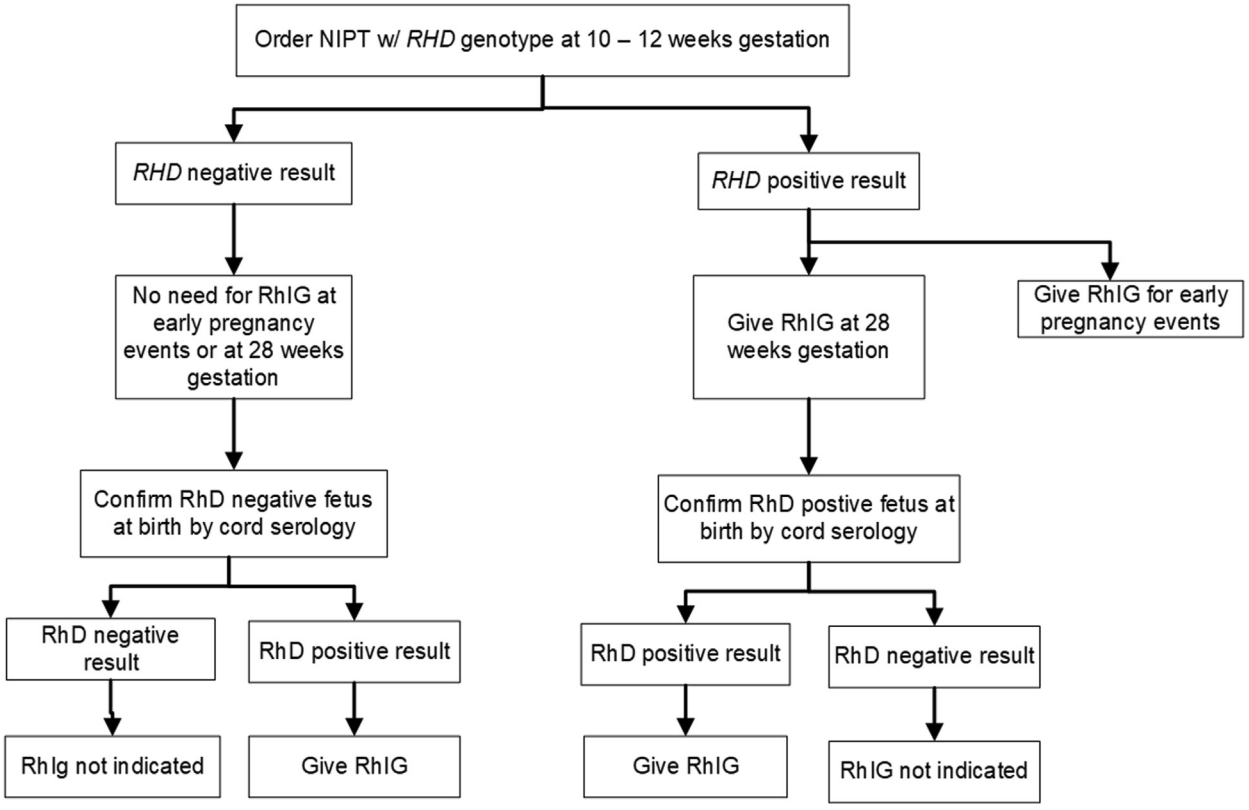
As confidence was gained with the use of cfDNA for fetal *RHD* typing in alloimmunized pregnancies, several countries proposed the use of this assay to limit the use of antenatal RhIG to the 60% of pregnancies involving an RhD-positive fetus. In 2010, Denmark was the first country to implement this program.[28] Finland followed soon thereafter with additional implementation in Sweden and the Netherlands.[29–31] Other countries around the world including England, Australia and New Zealand have now implemented this strategy.

On July 1, 2023, Kedrion Biopharma, Inc announced that RhoGAM was temporarily out of stock.[32] A later letter was released by the company in January, 2024 stating that a shortage would continue through 2024.[33] This led ACOG to release a practice advisory in March 2024 suggesting that other RhIG products be considered as an alternative to RhoGAM. However due to dwindling supplies, an updated ACOG advisory on April 24, 2024 suggested that cfDNA to determine the fetal *RHD* status could be used to prioritize use of RhIG and conserve the supply.[34]

Conclusion

The recent limited supply of one of the major RhIG products in the U.S. has allowed for consideration of the use of cfDNA for *RHD* typing in an effort to triage antenatal RhIG prophylaxis. A suggested clinical algorithm is noted in Figure 3. In the case of a RhD-negative patient undergoing NIPT where a *RHD*-negative cfDNA result is noted, the clinician should consider foregoing antenatal RhIG for second trimester events, such as suspected abruption, as well as routine administration at 28 weeks gestation. The practice of serologic typing of cord blood at the time of delivery should continue as a checks and balance on this new practice. Currently both U.S. companies only provide the fetal *RHD* assay as part of their standard NIPT test for chromosomal abnormalities and single gene defects. If a patient has already undergone NIPT testing with another

FIGURE 3
Suggested algorithm for incorporation of cell-free DNA *RHD* typing to triage the use of antenatal Rhesus immune globulin



NIPT, noninvasive prenatal testing; *RhIG*, Rhesus immune globulin.

company, repeat NIPT testing is often denied by insurance carriers. A separate Current Procedural Terminology, CPT code 81403 exists for cfDNA fetal *RHD* typing. As clinicians, we would encourage industry to undertaken a cost analysis to be able to order this cfDNA test as a stand-alone test in an effort to effectively triage the antenatal administration of RhIG. ■

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