Bridging the gaps: prevention, management, and future perspectives in hemolytic disease of the fetus and newborn

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How I use noninvasive prenatal testing for red blood cell and platelet antigens

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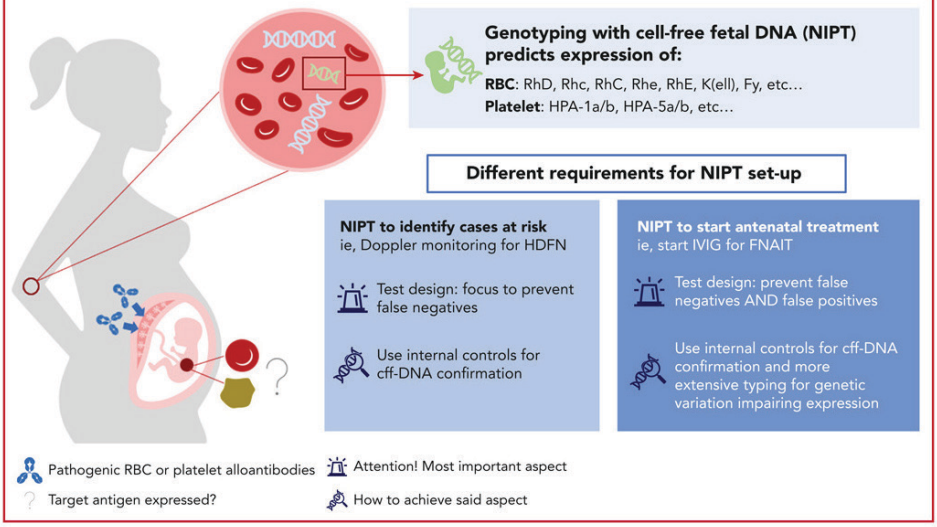
How I Use Noninvasive Prenatal Testing for Red Blood Cell and Platelet Antigens



Conclusions: **Noninvasive prenatal testing (NIPT) with cell-free fetal DNA (cff-DNA) enables personalized treatment for pregnant women with alloantibodies against red blood cells or platelets. While early genotyping platforms like RQ-PCR were reliable for fetal *RHD* screening, newer technologies now provide accurate fetal blood group antigen typing as early as 11 weeks.**

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blood Visual Abstract

Abstract

Alloimmunization during pregnancy occurs when a mother produces antibodies against fetal antigens, leading to complications like hemolytic disease of the fetus and newborn (HDFN) and fetal and neonatal alloimmune thrombocytopenia (FNAIT). HDFN involves destruction of fetal red blood cells, potentially causing severe anemia, hydrops fetalis, and fetal death. FNAIT affects fetal platelets and possibly endothelial cells, resulting in risk of intracranial hemorrhage and brain damage. Traditional invasive methods for fetal antigen genotyping, like amniocentesis, carried miscarriage risks. The discovery of cell-free fetal DNA (cff-DNA) in maternal plasma enabled safe, non-invasive prenatal testing (NIPT). Initially used for Rhesus antigen D blood group typing, NIPT now covers various blood group antigens. Advances in technology have further enhanced NIPT's accuracy. Despite challenges such as low cff-DNA fractions and complex genetic variations, NIPT has become essential in managing alloimmunized pregnancies. In NIPT it is important to prevent both false positive results and false negative results. Particularly in the coming decades, more possibilities for personalized antenatal treatment for HDFN and FNAIT cases will become apparent and accurate NIPT blood group antigen typing results are crucial for guiding clinical decisions. In this paper we describe this journey and provide practical tools for the clinic.

Introduction

Alloimmunization during pregnancy is a condition where a pregnant woman produces alloantibodies against antigens on fetal blood cells which may lead to fetal blood cell destruction necessitating perinatal treatment. Alloimmunization can happen during pregnancy, as well as in cases of incompatible blood transfusions or other scenarios involving direct exposure to allogeneic blood cells, such as organ transplants or sharing needles during drug use.[1]

In hemolytic disease of the fetus and newborn (HDFN) alloantibodies cause clinically significant destruction of red blood cells (RBC) whereas in fetal and neonatal alloimmune thrombocytopenia (FNAIT) the alloantibodies lead to destruction of platelets, and probably also affect the endothelial cells. In the White population, severe antenatal disease may occur if the alloantibodies target the Rh (Rhesus) antigens: RhD, RhC, Rhc, RhE and very infrequently Rhe or the K (Kell) antigen. However, the distribution of RBC antigens varies among different ethnic populations. For example, in non-White populations, alloimmunization against D or K is much less frequent, whereas in Asian populations, antibodies against blood group antigens expressed by glycophorin A, such as anti-GPMur or anti-M, and anti-Di(a) cause clinically relevant problems.[1-4] In FNAIT the alloantibodies target Human Platelet Antigens (HPA) expressed by fetal platelets, but which may also be present on leukocytes, endothelial cells and even placental tissue.[5] In FNAIT, the implicated antigens are most often HPA-1a (80% of cases) or HPA-5b in the White population, but again, occurrence of disease and implicated antigens differ among populations with different ethnic backgrounds. For example, anti-HPA-4b in the Asian population and anti-CD36 in Asian and African populations can be involved.[5-7]

If the fetus carries the implicated antigen, this can lead to severe complications. In HDFN, these include severe fetal anemia, leading to immune hydrops fetalis and even fetal death. After birth, the newborn may need treatment for both anemia and hyperbilirubinemia to prevent occurrence of kernicterus.[1,3,4,8] In FNAIT, due to very severe thrombocytopenia and possible damage to endothelial cells, antenatal bleeding can occur with intracranial hemorrhage (ICH) being the most severe outcome, potentially resulting in irreversible brain damage or death.[5] Postnatally, FNAIT can cause bruising, petechiae and brain- or organ bleeds, although most severe bleeding occurs before birth.[5]

Depending on the father's zygosity, the paternally-derived antigens on fetal blood cells may be present or absent in a pregnancy. If the implicated antigen is absent, further

laboratory testing and clinical follow-up can be omitted and future parents can be reassured.[1] If the father is heterozygous for a certain blood group antigen, there is a 50% chance the fetus will be antigen-positive. Before the turn of the century, clinicians relied on invasive techniques such as chorionic villous sampling or amniocentesis to collect fetal genetic material to perform genotyping. These invasive procedures carried a miscarriage risk of about 1% and the risk of additional alloimmunization and boostering.[9,10] In 1997, Lo and his colleagues discovered that during pregnancy, from the end of first trimester, there is sufficient cell-free fetal DNA (cff-DNA) present in maternal plasma for genotyping.[11] The amount of cff-DNA increases during pregnancy,[12] but the fraction of cff-DNA compared to maternal circulating DNA remains low - usually a few percent up to 10-20 %.[13] The source of cff-DNA is trophoblast cells undergoing apoptosis, releasing in general fragments of a small size of less than 150 bp, although recently it was found that larger fragments of cff-DNA also circulate.[14-16] For a comprehensive overview of cff-DNA characteristics, including its potential applications in various testing methods and test design considerations using cff-DNA, we refer to the works of Chiu et al.[17], Kjeldsen-Kragh and Hellberg[18] and Hyland et al..[19]

The first example of non-invasive prenatal testing (NIPT) for blood group antigen genotyping was the determination of the fetal RhD blood group.[11,20] Following this example, numerous other tests and assays were developed, allowing identification of fetal blood group genotype for most common antigens involved in HDFN.[21-33] Similar advancements have occurred for fetal HPA typing.[27,29-31,34-37] However, NIPT for fetal RBC or HPA antigens has not yet been adopted by all clinical centers worldwide providing care to alloimmunized women. As suggested by Clausen and van der Schoot, this may be related to the advanced equipment necessary to perform the tests.[38] At the start of this century, NIPT for alloimmunized women was primarily developed within reference laboratories of academic or blood institutes[18,19] in Europe and Australia, which offered the tests internationally. It has not yet been widely included in guidelines for the management of alloimmunized pregnant women.[39-42] In Asia, fetal genotyping for clinically relevant populations is expanding.[42,43]The latest technology now allows laboratories specializing in NIPT testing for chromosomal abnormalities to offer this type of testing[33] more widely, including in the United States of America.

In 2022, a group of experts representing the cfDNA subgroup from the International Society of Blood Transfusion (ISBT) Working Party on Red Cell Immunogenetics and Blood Group Terminology published comprehensive recommendations for validation and quality assurance of NIPT for blood fetal groups, including for fetal *RHD* typing in

alloimmunized women.[44] For further information on requirements for validation assays for routine fetal *RHD* typing in the screening setting (beyond the scope of this current review) the reader is referred to a number of recent publications.[38,39,45]

In this article, we describe 3 representative case stories; 2 related to HDFN and 1 to FNAIT, to highlight important factors to consider when using NIPT in alloimmunized pregnancies. In addition, some key learning points on technical issues are addressed separately. The clinical monitoring and treatment of HDFN are discussed by Savoia et al.[8] in this How I Treat Series.

Case 1

A gravida 2 para 1 (G2P1) RhD-negative woman presented with a positive RBC antibody screen in the first trimester. This routine antibody screening was performed as part of the free-of-charge, nation-wide Dutch population screening program. Her first pregnancy was uncomplicated and no antibodies were detected in the first trimester or at 27 weeks of gestation. She had also received anti-D prophylaxis (RhIg, 1000 IU, 200 µg) at 30 weeks gestation and immediately after birth, based on the routine screening *RHD* genotyping results obtained with the sample taken at gestational week 27.[46] There were no complications during or after birth. In the current pregnancy, antibody specification showed anti-D antibodies with a titer of 32 and a 30% antibody-dependent cell-mediated cytotoxicity (ADCC) test result. The ADCC test is used in the Netherlands to establish the hemolytic activity of the antibodies.[47] Both values were above the critical cut-off value used to identify a risk of severe HDFN, making further testing imperative.[1,3,8] Even though there were no recognized high-risk events in the previous pregnancy, such as cesarean section or manual removal of the placenta, sometimes alloantibodies can still develop.[48,49]

An EDTA-anticoagulated blood sample was drawn from the mother and sent in for NIPT using cff-DNA to test for fetal *RHD*. The *RHD* NIPT test results showed no amplification of *RHD* sequences, whereas amplification of the fetal control marker hypermethylated *RASSF1a* (*mRASSF1a*) was above the test acceptance criteria to confirm that sufficient cff-DNA was present.[50] These two results combined, predicted an RhD-negative blood group of the fetus with no further need for laboratory or clinical monitoring. As an example, in Figure 1 the interpretation for the current diagnostic NIPT used in our country is visualized. This approach is in line with the recommendations of Clausen et al. who strongly advocate the use of fetal DNA controls to confirm the presence of fetal DNA in cases of negative blood group genotyping result. The authors also mention the possibility to use two independent negative fetal typing results before concluding the fetal blood group status.[44]

In NGS-based platforms, controls for so-called individual identification single nucleotide polymorphisms (IISNPs) can be combined with the fetal RBC or HPA typing in one single test, as reviewed by McGowan et al.[31] The pregnant woman had an uncomplicated course for the remainder of her pregnancy and delivered a healthy newborn. A cord blood sample was used to confirm RhD negativity of the newborn.

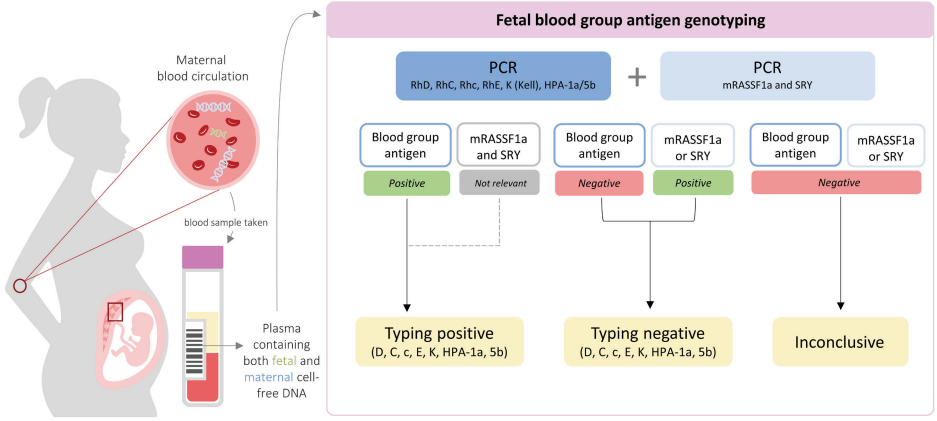

Figure 1. Test interpretation of our current set-up for fetal blood group antigen typing. PCR: polymerase chain reaction; HPA: human platelet antigen; SRY: sex determining region of the Y chromosome.

Use of fetal *RHD* NIPT: role of paternal typing

In Western settings, anti-D alloimmunization mostly occurs due to a pregnancy with an RhD-positive fetus and not because of RhD-incompatible transfusions or organ transplantation. Before NIPT for fetal *RHD* typing was possible, serological testing of the biological father's Rh phenotype was used to predict fetal RhD status when the mother had anti-D antibodies. The father's RhDCcEe phenotype could suggest whether he was likely homozygous for RhD expression, allowing clinicians to assess fetal risk without invasive testing. However, variations in CE-D haplotype distribution across populations made this approach unreliable in non-White or mixed-ethnicity groups.[51,52] Currently, genomic methods to test for the presence of one or two copies of *RHD* are possible and could be used. However, one might now rely more on NIPT for fetal RhD typing, which may be the only possibility in cases involving assisted reproductive technology with a donor or if the biological father is not known, certain or available. [53]

As illustrated by Figures 1 and 2, NIPT with cff-DNA isolated from the mother's plasma can be utilized in cases of RhD alloimmunization and is currently already used in many centers[18,38,41] However, not all centers using NIPT for fetal *RHD* testing in alloimmunized

women base their antenatal management decisions on the fetal typing result. For example, in the Netherlands, if the test predicts that the fetus is RhD-negative, no further laboratory testing or clinical monitoring is conducted.[24] When NIPT shows a positive fetal blood group, repeated anti-D titers are performed, and after the critical cut-off is reached, monitoring occurs via Doppler ultrasound at a specialized referral center.[54]

Case 2

A G2P1 woman with an uncomplicated first pregnancy presented in her second pregnancy in gestational week 10 with anti-K (Kell) antibodies at first screen. The anti-K titer was 4, which was above the critical cut-off for detection of high-risk anti-K complicated pregnancies.[1,3,8] Priority was given to provide an additional blood sample from the mother for NIPT fetal K genotyping with cff-DNA. The test result indicated fetal K-positivity, and the immunohematology consultant of the laboratory immediately contacted the obstetric care provider to explain the implications of these results. When anti-K antibodies are found and the fetus carries the K antigen, there is a 50% chance that severe HDFN will occur.[55] The pregnant woman was thus examined as soon as possible at a highly specialized tertiary facility and arrangements for weekly close monitoring were made with the referring hospital, Figure 2. It is important that care is provided in centers where pregnancies complicated by alloimmunization are seen regularly to ensure adequate and timely treatment of severe anemia of the fetus, as this yields the best outcome.[8,56] The woman was seen weekly for monitoring, and at 28 weeks of gestation, she required her first intrauterine transfusion (IUT) . Thanks to a prompt referral, she was well-prepared for the procedure. After the initial IUT, two additional IUTs were necessary. A healthy newborn was delivered at 37 weeks of gestation. During the first three months of life, the newborn required one additional top-up transfusion due to the prolonged suppression of erythropoiesis associated with K-mediated HDFN.[1] Fortunately, children who experience HDFN typically have excellent long-term outcomes when treatment is initiated in a timely manner.[57]

Prevention of alloimmunization, and use of NIPT for fetal K typing

For blood group incompatibilities other than RhD, the decision to use cff-DNA testing upon identification of an RBC alloantibody depends on specificity of the alloantibody and whether the occurrence of RBC alloantibodies in women of childbearing age is prevented by matching RBC units. This approach aims not only to prevent the development of anti-D but also to prevent the formation of other types of RBC alloantibodies. In the Netherlands, RBC units have been matched for D for many years, for K since 2004 and for cE since 2011 for women of childbearing potential.[58] In other countries, it

is common to match for D and, in some cases, for c and K.[59] Especially in cases of K immunization, it is crucial to determine if the mother has ever received a non-matched K blood transfusion. K-negative women who receive K-positive blood have a relatively high risk of developing anti-K antibodies.[60,61] In the White population, the prevalence of K-negativity is over 90% and it is even much higher in other ethnic populations.[61,62] Hence, if a K-negative woman develops anti-K upon a K-positive blood transfusion, there is a very high chance she is pregnant by a K-negative partner. In low resource settings, paternal serological K typing might be a first approach to select only high-risk pregnancies. In our pregnant population the policy of K-matched transfusions for women under 45 has dramatically reduced the prevalence of anti-K and subsequently also halved the prevalence of K-mediated HDFN.[61] Since in K-alloimmunization, fetal disease can occur early in pregnancy, we currently advise to perform NIPT for fetal K typing as early as possible without the need to first type the father (Figure 1). In serologically typed K+k- individuals (apparently homozygous K-positive) still around 7% have a k-allele encoding a low or absent expression of k also making identification of the relative rare homozygous K-positive phenotype of less importance in our setting.[63]

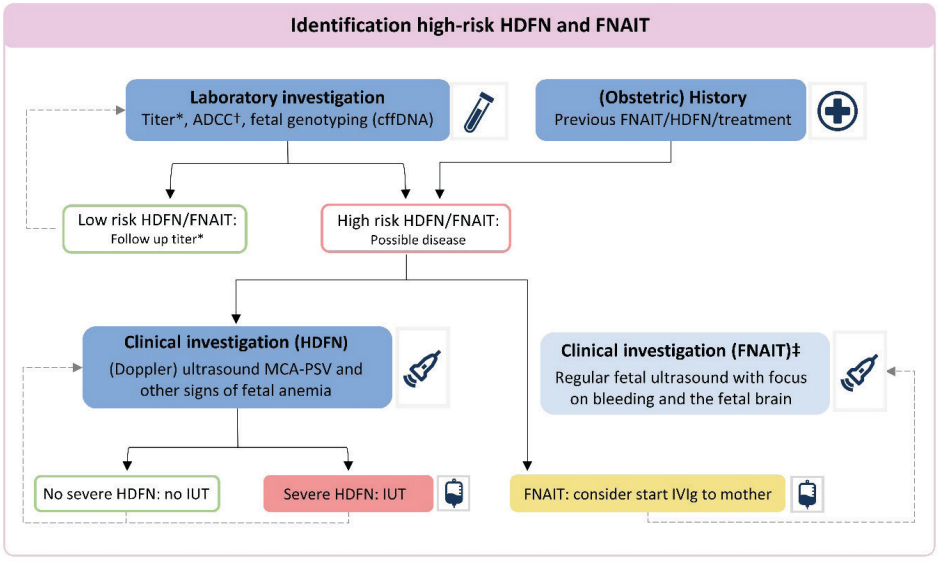

Figure 2. Flowchart of identification of high-risk HDFN and FNAIT cases. After high risk cases are identified using laboratory testing, for both HDFN and FNAIT fetal ultrasounds are made to look for signs for fetal anemia (HDFN) and bleeding, in particular in the brain (FNAIT). *In some countries, HPA-1a antibody quantification is performed for FNAIT †ADCC only for red cell antibodies in the Netherlands, ‡Fetal ultrasound does not indicate start IVIg, solely for monitoring possible bleeding.

HDFN: hemolytic disease of the fetus and newborn; FNAIT: fetal/neonatal alloimmune thrombocytopenia; ADCC: Antibody Dependent Cell-mediated Cytotoxicity; cffDNA: cell free fetal DNA; MCA-PSV: middle cerebral artery peak systolic velocity; IUT: intrauterine transfusion; IVIg: intravenous immunoglobulin.

The K/k polymorphism is caused by only one single nucleotide variation.[51] Early genotyping assays using real-time quantitative PCR struggled with specificity of the test due to background amplification of the mother's k-allele, necessitating assay modifications that reduced the sensitivity and making repeated testing at later gestational age necessary.[21,24,64] These limitations have been solved employing other technical platforms for genotyping.[16,19,21-28,32] Both droplet digital PCR (ddPCR) platforms and techniques such as massive parallel sequencing (next generation sequencing or NGS) can be employed early in pregnancy with very reliable results.[19,28,37,65] This approach further reduces the likelihood of false-negative results caused by rare instances of mispriming.[66]

Use of NIPT for fetal genotyping for other RhCE antigens

NIPT can also be employed when antibodies against other Rh antigens are present: RhC, Rhc, RhE. In our setting, for RhC and Rhc, fetal genotyping is performed without typing of the father when alloantibodies are found. However the approach differs for anti-E antibodies. It is known that anti-E can occur "naturally" during pregnancy, without prior incompatible blood transfusions or pregnancy.[67] In every population, this concerns a high number of pregnant women, since more than 60% of women will be E-negative, irrespective of their ethnic background.[52] This is why, if the routine RBC screen early in pregnancy shows anti-E, it is reasonable first to type the partner and only if he has an E+ e+ phenotype, the fetus will be genotyped. If no samples from the biological father are available, NIPT fetal RhE genotyping is also performed.

Gestational age at testing

Recent literature, including several meta-analyses and overviews, concludes that NIPT for blood groups can be reliably performed as early as gestational week 11.[18,38,45] In-house developed assays have been designed and validated at several reference centers, but for *RHD* typing commercial kits are also available.[18] These commercial kits were initially developed for high-throughput fetal *RHD* typing to target anti-D prophylaxis in RhD-negative, non-alloimmunized women.[18] However, both in-house developed and some commercially available tests can be used for fetal *RHD* typing in alloimmunized women.[18] Most of these tests still use real time quantitative PCR (RQ-PCR), whereas more recently, assays based on ddPCR, NGS or other technical platforms have been published.[25-33,68] Since samples from alloimmunized women are relatively scarce it is a challenge to validate these assays for all blood group antigens with a range of samples, including those from early gestational age. In general, for diagnostic NIPT, experts

recommend using controls to ensure the accuracy of the test and to confirm the presence of sufficient amount of amplifiable cff-DNA in cases of negative blood group genotyping results.[44] For male fetuses, a relatively simple and robust control is testing for Y chromosome sequences (*SRY*).[24,69] Alternatively, we and others have used sets of genetic markers that differ between the mother and fetus to confirm a sufficient cff-DNA fraction for conclusive results.[24,33,69] Although challenging, one can also use the universal fetal marker *mRASSF1a*.[31,50] In our experience, when robust tests for fetal blood group typing and fetal Y-chromosome markers are combined with the universal *mRASSF1a* marker, only about four percent of cases require an additional blood sample taken later in pregnancy due to insufficient fetal DNA concentration.[70]

False negatives and false positive NIPT blood group antigen typing results

At the start of using NIPT for blood group antigens in alloimmunized pregnancies as part of clinical decision making, the primary concern was the sensitivity of the test to ascertain a 100% negative predictive value. A false negative NIPT result would imply risk of development of unnoticed severe disease or even fetal demise. Early studies showed that high levels of maternal DNA could impact the specificity of the test result, and the use of blood collection tube with a preservative to prevent decay of leukocytes is recommended.[71] In some women, the cff-DNA concentration is extremely low, making testing for cff-DNA markers in diagnostic testing a prerequisite to prevent false-negative typing results.

In time, the impact of false-positive NIPT results has evolved. In the early years of blood group typing using NIPT, the risk of false positive results was accepted. Although these would lead to unnecessary diagnostic testing and clinical follow up during pregnancy and an induced delivery, this did not significantly harm the mother or the fetus as, the peak systolic velocity of the middle cerebral artery would generally remain below 1.55 MoM (Multiple of the Mean), and no cordocentesis would be conducted.[72] However, a false positive result could affect the timing and setting of the delivery leading to iatrogenic prematurity. Moreover, with the development of new immunomodulatory agents such as FcRn blockers, there is an additional reason to prevent false positive blood group antigen typing results, as they could lead to unnecessary initiation of antenatal treatment.[8,73,74] FcRn-blocking therapy should be started early in pregnancy making it necessary to have reliable assays available at a gestational age of about 11 weeks. The reviews of test performances are reassuring, showing that false negatives are extremely rare. Moreover, if the testing laboratory accounts for variations in *RHD* genes, these instances will become even rarer.[18,38,41]

Decoding *RHD* Genotyping: mitigating false positives in ethnically diverse populations

False positives in *RHD* genotyping often result from the presence of *RHD* variant alleles. While most White individuals with an RhD-negative phenotype have a complete deletion of the *RHD* gene, *RHD* variants exist that result in a complete absence of RhD expression on RBCs while retaining large parts of the *RHD* gene.[51,52] Therefore, analyzing multiple *RHD* exons is essential. Some of these variants are more prevalent in different ethnic groups, leading to differences in the genetic background of the *RHD* genotype across the world.[52] For instance, the *RHD* pseudogene (*RHD*08N.01*), common in the Black African population, retains most of the gene but because of a 37-bp insertion and a missense mutation in exon 6, it does not express the RhD-protein on RBCs, making the mother susceptible to immunization.[51,52] To accurately determine the fetal *RHD* type, amplification of genetic sequences absent in the *RHD* pseudogene but present in the wild-type, particularly in exons 4 and 5, is necessary.[51] Additionally, distinguishing between a fetus with an *RHD* pseudogene (who is RhD-negative and will not suffer from HDFN due to maternal anti-D) and a DVI variant fetus (for example *RHD*06.01*, who will express fewer RhD molecules on their RBCs, but may theoretically suffer from HDFN) is critical to avoid false positives and unnecessary treatment. Similarly, it is important to detect fetal variants that lead to extremely low levels of RhD expression, such as variants present in people from Asian descent like *RHD*01EL.01,* when designing test algorithms to personalize the start of IVIg or FcRn-targeting therapies. Until comprehensive NGS-based typing becomes available, in the most genetically complex cases, we currently still rely on genotyping the biological father for *RHD* variants to collect additional information for correct prediction of the fetal RhD type.[51,75]

The variation in *RHD* null alleles makes it very challenging to mitigate all risks of false positive results.[76] Furthermore, in women carrying *RHD* null alleles of which the molecular background is still not yet determined, it remains difficult to have assays available for routine typing other than NGS based assays.

Overall, evidence is increasing that in various populations, NIPT for *RHD* typing is possible, especially if additional paternal testing is incorporated in the typing algorithm, as indicated by reports from Argentina, China and Japan.[42,75,77]

Case 3

A neonate was born following an uncomplicated pregnancy of a first-time mother (G1P0). The nurse noticed that the neonate showed several petechiae and significant bruising on the trunk and limbs. A full blood count was requested, revealing an isolated

and severe thrombocytopenia of $10x10^9$/L, prompting further investigation. Cerebral ultrasonography revealed an ICH. Blood samples were taken from the mother, the biological father and the neonate to assess the possibility of FNAIT. The mother was found to be HPA-1a negative, with the presence of anti-HPA-1a antibodies, while the father and neonate were both HPA-1a positive, showing an HPA-1ab genotype. The parents were informed that in future pregnancies, there is a 50% chance that the fetus will again be HPA-1a positive. Therefore, NIPT with cff-DNA to determine the HPA status of the fetus was recommended in any subsequent pregnancy.

One month later, the sister of this HPA-1a-negative mother attended a pre-conception counseling appointment to inquire about her own risk of having a child with FNAIT. In accordance with our protocol in such cases, HPA-1a typing was requested and she was also found to be HPA-1a negative. Additionally, since the HLA-DRB3*0101 type is correlated with an increased risk of alloimmunization, this typing was also determined with a positive test result.[78] Given these findings, she is considered to have an elevated risk of developing FNAIT and was also advised to pursue non-invasive genotyping in any future pregnancies to determine the fetus's HPA status.

FNAIT

FNAIT is a very rare condition and many aspects remain unclear. Different countries have varying guidelines on whether to treat suspected cases of FNAIT during pregnancy. Unlike HDFN, where Doppler ultrasound can non-invasively assess whether the fetus suffers from anemia, there is no similar method for detecting fetal thrombocytopenia. The only direct approach, cordocentesis, is not recommended as a routine diagnostic tool due to the high risk of bleeding complications in the fetus with low platelet counts.[5,79] Currently, the primary treatment option for managing potential FNAIT cases is administering intravenous immunoglobulins (IVIg) to the mother (with or without steroids), aiming to reduce antibody levels in the fetus and thereby preventing bleeding complications.[5,79,80] While IVIg generally has mild side effects, usually only headaches, it may rarely lead to thrombotic complications, aseptic meningitis, renal failure and hemolytic anemia.[79] Despite a calculated 98.7% success rate in preventing ICH in the systematic review from Winkelhorst et al., the quality of evidence is limited by inadequate control groups and the heterogeneity of the trials performed.[79,81] Currently IVIg is thus still being used off-label. In some countries it is combined with corticosteroids.[79] To start timely antenatal treatment, NIPT for HPA genotyping of the fetus plays a crucial role in guiding management decisions, Figure 2.

In general, HPA antigens are encoded by single nucleotide variation change. Similar to the RBC antigens, the first developed genotyping assays by RQ-PCR showed limitations in achieving conclusive test results at early gestational ages. However, ddPCR and NGS techniques make reliable typing from gestational week 11 onwards possible.[21,24,26-35,37,71] A recent review discusses the various platforms used for HPA-1a genotyping early in pregnancy.[35,82]

All key learning points from this paper are summarized in Table 1 outlining challenges and solutions encountered along the way.

Table 1. Challenges and possible solutions in development of NIPT for RBC and platelet antigens.

	Challenges	***Risks***	***Risk mitigations***
General	High amount of maternal DNA; leukocyte derived, post sampling	False negatives in NGS, due to too few fetal reads False negatives in RQ-PCR have been mentioned[71] False positive signals in RQ-PCR, due to amplification from maternal sequences Inconclusive result in ddPCR due to saturation of PCR reaction	Use of blood sampling tubes with preservation
	Too low cff-DNA concentration	False negative result	Use a threshold for acceptable cff-DNA concentration (RQ PCR, ddPCR) or cff-DNA fraction (NGS). If cff-DNA too low, repeat at later GA Isolate DNA from at least 500 µl, but preferably 1 ml of maternal plasma
RHD	Genetic variation	False negatives False positives	Assay design detecting more than one fetal *RHD* exon increases sensitivity. Consider assays for determination of fetal *RHD* pseudogene or other type of *RHD* variants leading to absent or very low expression making HDFN not likely
	Paternal *RHD* type	Serological determination of Rh phenotype is inaccurate	Paternal genomic zygosity determination possible, however NIPT fetal *RHD* is preferred. In complex cases, paternal genotyping can be informative.
K	Specificity of fetal K RQ-PCR based typing by use of Locked Nucleic Acids or Peptide Nucleic Acid probes	False negatives and necessity for repeat typing at later GA	Change in platform from RQ-PCR to mass-based, ddPCR or NGS-based typing[21,24,26-33,37]
HPA-1a	Specificity of fetal HPA-1a RQ-PCR based typing by use of Peptide Nucleic Acid probes or enzymatic pre digestion	False negatives and necessity for repeat typing at later GA	Change in platform from RQ-PCR to ddPCR or NGS-based typing[27,29-31,34,35,37]

Challenges in NIPT for fetal blood group typing and their possible solutions. ddPCR: digital droplet PCR; cff-DNA: cell free fetal DNA; GA: gestational age; HDFN: hemolytic disease of the fetus and newborn; NIPT: non-invasive prenatal testing; RQ-PCR: real-time quantitative PCR; NGS-based: next generation sequencing based; HPA: human platelet antigen.

Conclusion

Introduction of routine fetal *RHD* typing to target anti-D prophylaxis in RhD-negative women was at the start of this century an opportunity to obtain experience with NIPT and served as a driver to develop assays for fetal blood group antigen typing in alloimmunized women. The first available technology, RQ-PCR, proved to be a useful and a robust technical platform for reliable fetal *RHD* screening in non-immunized women, but had drawbacks in typing alloimmunized women for a broader panel of blood group antigens.[18,21,24,38,41,45] However, new technologies such as ddPCR, and as most promising platform, NGS-based typing, now allow for accurate diagnostic fetal typing as early as 11 weeks of gestation. These technologies incorporate sufficient controls to ensure a qualitatively correct test result based on sufficient amounts of cff-DNA, reducing the risk of false-negative results. The use of NGS-based typing is particularly valuable, as it allows for the analysis of multiple gene sequences that encode specific blood group antigens and iiSNPs.[31] Some countries continue monitoring pregnancies after negative cff-DNA results with titers and Doppler measurements. However with the current accuracy it has been proven that this is redundant.[83,84] Currently, some laboratories use assays without information on the cff-DNA concentration and if the test predicts an antigen-negative fetus, they opt to repeat testing at a later gestational age, extending the period of uncertainty and still carrying a risk of false negatives.[83] To use NIPT for RBC and HPA antigens in a clinical setting, we would like to reiterate the recommendations from Clausen et al., that clinicians should be aware of the usually low number of cases used to validate the assays and test performance related to false negative and false positive results.[44] Furthermore, clinicians should be informed about the laboratory's strategy for confirming the isolation of sufficient cff-DNA. Finally, it is important that clinicians are informed about the detection of *RHD* variants and how the laboratory translates this information into predictions of fetal RhD-antigen positivity. When fetal genotyping is the qualifier to start therapy, such as IVIg in FNAIT, and in rare cases in HDFN, but also for FcRn blockers in future, preventing false-positive results is essential.

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