Noninvasive Prenatal Testing in Immunohematology

Subjects: Hematology

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Hemolytic disease of the fetus and newborn (HDFN), as well as fetal and neonatal alloimmune thrombocytopenia (FNAIT), represent two important disease entities that are caused by maternal IgG antibodies directed against nonmaternally inherited antigens on the fetal blood cells. These antibodies are most frequently directed against the RhD antigen on red blood cells (RBCs) or the human platelet antigen 1a (HPA-1a) on platelets. For optimal management of pregnancies where HDFN or FNAIT is suspected, it is essential to determine the RhD or the HPA-1a type of the fetus. Noninvasive fetal RhD typing is also relevant for identifying which RhD-negative pregnant women should receive antenatal RhD prophylaxis.

Keywords: hemolytic disease of the fetus and newborn ; fetal and neonatal alloimmune thrombocytopenia ; noninvasive prenatal testing ; polymerase chain reaction

1. Hemolytic Disease of the Fetus and Newborn

Clinical Considerations

Around five decades ago, HFDN was associated with significant mortality and morbidity. Hence, HDFN affected 150 per 100,000 births and was responsible for 10% of all perinatal deaths [1]. Within a few years after the implementation of RhD prophylaxis, there was a dramatic reduction both in the number of RhD-immunized women and in the number of HDFN cases in North America and Western Europe. Thus, RhD prophylaxis became one of the most effective immunological interventions in clinical medicine.

The RhD prophylaxis was based on the administration of a one single dose of hyperimmune anti-D IgG to all RhD-negative women within 72 h after delivery of an RhD-positive child and in relation to any event that is known to be associated with the risk of fetal–maternal bleeding. Despite this initial success, it became clear that a small proportion of RhD-negative women carrying an RhD-positive fetus still became RhD-immunized despite the administration of hyperimmune anti-D IgG after delivery. Most of these cases were due to fetal–maternal bleeding in the third trimester. This led to a modification of the RhD prophylaxis, which involved the administration of one or two doses of hyperimmune anti-D IgG in the third trimester in addition to a dose after delivery of an RhD-positive child. Although this routine was adopted by several countries such as the USA, Canada and the UK, there was one drawback: Since the fetus's RhD type is not known, it is necessary to administer hyperimmune anti-D IgG to all RhD-negative pregnant women despite the fact that around 35–40% of these women will be carrying an RhD-negative fetus, and these women can of course not be RhD-immunized in the current pregnancy or after delivery of their RhD-negative child.

With the discovery of cell-free fetal DNA (cffDNA) in the plasma of pregnant women [2], it has become possible to do noninvasive RhD typing of the fetus [3]. This technique is now used in several countries in Europe to identify which RhD-negative women are carrying an RhD-positive fetus [4][5][6], and these countries have changed their routine to targeted antenatal RhD prophylaxis, i.e., hyperimmune anti-D IgG is only administered to RhD-negative women carrying an RhD-positive fetus. This is reflected in several national guidelines for the care of pregnant women in many European countries [7][8][9].

Knowledge of the fetal RhD type is also of major importance for clinicians to manage the treatment of RhD-negative pregnant women who have become RhD-immunized in relation to a previous delivery of an RhD-positive child. If the mother's anti-D levels are high, there is a need for close clinical follow-up during pregnancy, which will involve multiple ultrasonographic assessments of the fetal blood flow as a surrogate marker for fetal anemia [10]. Further, if these examinations indicate fetal anemia, the woman will need to be referred to a specialized center that will perform an intrauterine transfusion of RhD-negative blood.

2. Technical Considerations Related to Prediction of Fetal HPA-1 Type

The technical challenges associated with prenatal HPA-1a typing are considerably larger than prenatal RhD typing: In contrast to fetal RhD typing, which implies detection of sequences from the *RHD* gene in the mother's plasma, there is only a single nucleotide difference between genes encoding HPA-1a and HPA-1b. Hence, the number of the fetal DNA strands in the plasma encoding HPA-1a is very low compared with the number of the maternal DNA strands encoding HPA-1b. This small amount of cffDNA in 'a large ocean' of cell-free maternal DNA represents an analytical challenge, particularly early in the pregnancy when the proportion of cffDNA is at its lowest. To address this challenge, different methods have been used to try to make fetal HPA1a typing both cost-effective and sensitive.

The first assay for fetal HPA-1 typing used a similar method with real-time PCR as for detecting the *RHD* gene. However, this was not successful without adding a pre-PCR step utilizing the restriction enzyme MspI, which recognizes and cleaves the DNA sequences encoding the maternal HPA-1b allele, hence reducing the risk of unspecific amplification [11]. The disadvantages associated with this method are the risk of incomplete enzyme digestion and the lack of internal controls for the presence of cffDNA, which could lead to a false negative result.

Another method, the coamplification at lower denaturation temperature PCR (COLD-PCR), utilizes melting temperature differences between variant and wild-type sequences, which will favor the amplification of the less abundant allele [12]. This method has been included in a workflow where first the HPA-1 status of the mother is determined by high-resolution melting (HRM) PCR, and if the mother is HPA-1-negative, COLD-PCR is applied on the same extracted DNA to determine the fetal genotype [13]. The set up shows accurate results as early as gestational week 12, but suffers from the same weakness as the previous methods since no internal control is included [13].

Novel technologies such as NGS and digital PCR are promising alternatives but involve expensive equipment not available everywhere. NGS, a technology in which millions of nucleotides are sequenced at the same time, consists of several steps: (1) DNA fragmentation, (2) adapter ligations, (3) sequencing and (4) alignment and data analysis. Currently, two main platforms using different ways of nucleotide detection are on the market. Ion Torrent™ (Themo Fischer, Waltham, MA, USA) uses a semiconductor chip where the pH change is detected when a nucleotide is incorporated, whereas the Illumina technology use fluorescence. Both whole genome sequencing and exome sequencing as well as targeted sequencing can be performed. Target enrichment of only selected parts of the genome is suitable for fetal genotyping because it increases the proportion of the targeted region [14]. Targeting can be done with an additional step using PCR (amplicon-based target enrichment) or probes (hybridization-based target enrichment) to capture target sequences.

The successful detection of fetal HPA-1a-positive sequences has been demonstrated in several studies using NGS [15][16]. The use of single-nucleotide variants (SNVs) for estimation of the fetal fraction works as an internal control, which makes NGS a reliable method [15]. To conclude, NGS is highly specific but time-consuming, and requires the analysis of several samples at the same time to be cost-effective.

Digital PCR (dPCR) is a sensitive technology based on PCR performed on partitions, i.e., the sample is separated in units, containing either zero, one or a few copies of the targeted DNA before PCR. After amplification, partitions containing target DNA are detected by fluorescence, and the positive and negative reactions are counted. To account for partitioning errors, a Poisson correction model is applied by the software. There are a number of different methods for separating the partitions such as microchambers, channels, printing-based sample dispersion and microfluidic technology, where the DNA is divided into water-in-oil droplets [17]. dPCR performs with high accuracy even when only a small volume of sample is available, which makes the method suitable for accurate noninvasive prenatal typing early in pregnancy [18]. Moreover, both *RASSF1a* and autosomal SNPs from the SNPforID panel have been used as internal controls with good results [18]. However, this method still has some disadvantages such as contamination risk, limited possibility for high-throughput, cost and lack of commercial assay kits. **Table 1** summarizes some characteristics of the mentioned methods for HPA-1a typing.

Table 1. Inhouse assays for fetal HPA-1 typing.

Realtime PCR with Digestion of Maternal Allele	Cold PCR	NGS (Targeted Massive Parallel Sequencing)	Digital PCR	
Use from gestational week	**18**	**12**	**13**	**8**
Control for cffDNA	**no**	**no**	**yes**	**yes ***
Cost	**low**	**low**	**high**	**high**

Realtime PCR with Digestion of Maternal Allele	Cold PCR	NGS (Targeted Massive Parallel Sequencing)	Digital PCR	
Turnaround time	**Medium**	**Medium**	**Long**	**Medium**
References	[11]	[13]	[15][16]	[18]

* can be included.

Today, routine maternal HPA-1a typing is not used for identifying pregnant women at risk of having a child with FNAIT. Antenatal HPA-1a typing is only used for HPA-1a-immunized women at some centers in Europe. However, a hyperimmune anti-HPA-1a IgG is under clinical development for the prevention of HPA-1a immunization and FNAIT [19]. If the pivotal phase 3 clinical trial demonstrates that this drug can prevent HPA-1a immunization and FNAIT, there are reasons to believe that national health authorities will recommend that maternal HPA-1a typing be included in their antenatal health care program. In that case, there will also be a need for targeted HPA-1a prophylaxis. As HPA-1a immunization is primarily restricted to women who are HLA-DRB3*01:01-positive, it will only be necessary to administer the hyperimmune anti-HPA-1a IgG to those women who are HPA-1a-negative and HLA-DRB3*01:01-positive carrying an HPA-1a-positive fetus. NGS could be used to determine if a pregnant woman, who initially has been typed as HPA-1a negative, is at risk of having the pregnancy complicated with FNAIT. It is feasible to design one NGS assay that determines the maternal HPA-1 type (as a control of the initial screening), the maternal HLA-DRB3*01:01 status and fetal HPA-1 type. Since NGS is a very sensitive assay (**Table 1**), it could be applied late in the first trimester, and as explained below, an early answer regarding the risk of FNAIT is essential for the expecting mother. Thus, future inclusion of FNAIT screening in the antenatal health care program will hopefully go hand in hand with the development of tests that can determine the FNAIT risk status early in pregnancy.

3. Necessity of Noninvasive Prenatal Blood and Platelet Typing—Ethical Considerations

With the introduction of noninvasive prenatal testing in immunohematology, it has become possible to circumvent many ethical challenges: Many countries that have implemented antenatal RhD prophylaxis, administer hyperimmune anti-D IgG to all pregnant RhD-negative women. For 35–40% of these women, there is no risk of HDFN in the current pregnancy because they will be carrying an RhD-negative fetus. Hence, these women receive a drug for which there is no indication, which basically violates the generally accepted ethical position that a patient should not receive an unnecessary drug. Furthermore, hyperimmune anti-D IgG is a special drug as it is manufactured from plasma collected from RhD-immunized individuals. Most of these RhD-negative individuals are frequently transfused with RhD-positive RBCs in order to boost their antibody response to maximize the amount of anti-D in the plasma that is harvested from these special donors. These individuals are exposed both to the risks associated with the transfusion of RhD-positive RBCs (such as transfusion-associated infections) and the risks associated with plasmapheresis. Although both these risks are small, it is highly questionable if it can be justified to use a significant amount of the drug manufactured from plasma harvested from these special donors to patients who do not need the drug.

It also worth mentioning that while parts of the world, mainly high-income countries, are using RhD prophylaxis for women who do not need it, approximately 50% of the women around the world who require this type of immunoprophylaxis have no access to the drug. A nonprofit organization, Worldwide Initiative Rh Disease Eradication (WIRhE), has been launched to spread awareness about Rh disease and the lack of access to both blood typing and prophylaxis [20][21].

The screening programs of pregnant RhD-negative women that include noninvasive fetal RhD typing, which have been implemented in many European countries [4][5][6], have avoided the above-mentioned ethical challenges by identifying those RhD-negative women who should receive antenatal RhD prophylaxis to prevent RhD immunization and HDFN in subsequent pregnancies.

For an HPA-1a-immunized pregnant woman with FNAIT in her obstetric history, it is essential to know the HPA-1 type of the fetus. As a first step, it is common practice at many centers to do HPA-1a genotyping of the father, and if he is HPA-1a/b to continue with amniocentesis of the mother. Hence, the platelet type of the (presumed) father determines if the mother should undergo amniocentesis or not. This becomes an ethical challenge if the mother knows that her spouse is not the father. Furthermore, during the couple's consultation with the physician, she will be put into a very difficult situation when the physician asks for a sample from her spouse! Furthermore, the increased use of assisted reproductive technology (ART) reinforces limited use of testing the spouse [22][23].

Another ethical aspect is related to the invasive nature of amniocentesis. This procedure is associated with a risk of fetal death, which should be weighed against the risk of having a severely thrombocytopenic child. Moreover, amniocentesis may also set off fetal–maternal bleeding that could boost the mother's production of anti-HPA-1a, which in turn could worse fetal thrombocytopenia and increase the risk of severe intrauterine bleeding such as ICH.

A procedure for fetal HPA-1 determination that involves paternal HPA-1a typing, amniocentesis, expansion of fetal amniotic cells, extraction of DNA from fetal amniotic cells and finally HPA-1a typing by PCR takes a long time. This waiting time is very stressful for the expecting mother: Will the fetus be HPA-1a-positive and then at risk of FNAIT or would it be HPA-1a-negative? The latter result would be a relief for both the mother and the maternal–fetal medicine specialist, because no further follow-up during pregnancy would be necessary. Hence, it is preferable to know the fetal HPA-1 type as soon as possible after the woman has discovered she is pregnant. As explained above, some of the techniques that are used for noninvasive prenatal HPA-1 typing can be performed late in the first trimester and will minimize the waiting time for the pregnant woman. In addition, noninvasive prenatal HPA-1 typing will also avoid the other ethical challenges discussed above.

Although the results obtained from the (presumed) father should not be used for making clinical decisions for the mother, it is essential for the treating physician to know if the pregnancy is the result of ART, and if this is the case, if the conceived oocyte comes from the mother or from another woman. In the latter case, there is a risk that the fetus is homozygous for the RBC or platelet antigen to which maternal antibodies have been produced, which may increase the severity of the affected fetus. Due to the large economic and emotional costs related to these pregnancies, Curtis et al. has recommended that surrogate mothers and women who are donating oocytes for ART should be HPA-1 typed [22].

The use of noninvasive prenatal fetal RhD and HPA-1a is still limited. The reasons are both due to the technical challenges and the costs associated with these analyses. It has been argued that it is cheaper to administer anti-D to all RhD-negative women irrespective of whether or not they are carrying an RhD-positive fetus, as opposed to a program that includes noninvasive prenatal fetal RhD typing of all RhD-negative women and the administration of anti-D to only those women who need the prophylaxis.

There are reasons to believe that the use of noninvasive preclinical testing in immunohematology will increase in the near future because this will eliminate a number of ethical challenges and also because technical developments and decreasing prices will make this technology accessible to more laboratories.

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