Clinical Utility of Red Cell and Platelet Antigen Genotyping In Transfusion Medicine

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Speaker Disclosure Information

• I have no conflicts of interest to disclose.
Learning Objectives

- Clinical utility of molecular testing in hemolytic disease of the fetus and newborn (HDFN), neonatal alloimmune thrombocytopenia (NAIT) and transfusion medicine.
- Genetics of the Rh blood group system and how allelic variants can confound molecular typing.
- Potential uses of fetal DNA in maternal plasma.
Hemolytic Disease of the Fetus and Newborn (HDFN or HDN)  
Neonatal Alloimmune Thrombocytopenia (NAIT, NATP, NAT, AIT)

- HDFN and NAIT result from the alloimmunization of a mother to a paternal alloantigen inherited by a fetus.
- Alloantibodies cross the placenta and cause destruction of red blood cells or platelets of an antigen-positive fetus leading to clinical disease.
- HDFN typically occurs in the second pregnancy.
- NAIT can occur in the first pregnancy.
- Severity increases in subsequent pregnancies.
NAIT - Clinical Features

- Incidence: 1 in 1000 to 1 in 1500 live births
- Accounts for 3% of all thrombocytopenias and for 27% of severe cases (plt ct < 50K)
- ~50% of NAIT cases present in first pregnancies
- Isolated neonatal thrombocytopenia at birth
- Petechiae, GU/GI hemorrhage, intracranial hemorrhage
- Mother has circulating anti-platelet Abs directed at a platelet specific antigen.
- Mother has normal platelet count.
- Self-limited, resolves by 2-4 weeks
NAIT - Outcome

• Mortality 1-5%*
• Intracranial hemorrhage (ICH) 10-20%
• In utero ICH up to 75% of all ICH
• Late neurologic effects possible due to “silent” ICH

HDFN and NAIT Diagnosis

Criteria For Diagnosis

• Verifying a maternal antibody to a specific antigen found on paternal but not maternal platelets or red cells
• Demonstrate maternal/paternal antigen discrepancy consistent with maternal Ab with serological and/or molecular testing
• Molecular testing of fetus or newborn

Genotyping plays a key role in the management of HDFN and NAIT
NAIT - Managing A Subsequent Pregnancy

• Prediction of subsequent affected fetus
  – Paternal zygosity
  – Fetal platelet antigen genotyping

• Determination of severity of thrombocytopenia
  – Previous affected child in family
  – Fetal platelet counts
  – Ultrasound
  – Maternal anti-platelet antibody titer?

• Effective antenatal treatment strategies are available
  – IVIG with or without steroids to the mother during pregnancy
  – Repeated antigen negative platelet transfusions with early elective delivery
Platelet Membrane Glycoprotein Polymorphisms
Alloimmune Thrombocytopenia

HPA5(Br) (Glu505Lys)
Sit (Thr799Met)
HPA9 (Max) Bak^b variant (Val837Met)
HPA3 (Bak) (Ile843Ser)
HPA1 (PI^A) (Leu33Pro)
HPA6 (Ca/Tu) (Arg489Gln)
Gro (Arg633His)
Sr (Arg636Cys)

GPIa-IIa (α2β1)
GPIIb-IIIa (αIibβ3)

Glanzmann thrombasthenia

© PJ Newman 2001
Adapted from Humphries and Mould
Science 294:316, 2001, with permission
Platelet Alloantigens and Risk of NAIT

<table>
<thead>
<tr>
<th>Ag System</th>
<th>Common Names</th>
<th>Allelic forms</th>
<th>Phenotypic frequency</th>
<th>Gene</th>
<th>Risk of incompatibility (normal population)</th>
<th>% of NAIT cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPA-1</td>
<td>Pla</td>
<td>HPA-1a, HPA-1b</td>
<td>72% a/a 26% a/b 2% b/b</td>
<td>GPIIaL33P</td>
<td>1.96% (1a) 20.16% (1b)</td>
<td>79% 4%</td>
</tr>
<tr>
<td>HPA-2</td>
<td>Ko</td>
<td>HPA-2a, HPA-2b</td>
<td>85% a/a 14% a/b 1% b/b</td>
<td>GPIbT145M</td>
<td>12.75% (2a) 0.99 (2b)</td>
<td>&lt;&lt;1% &lt; 1%</td>
</tr>
<tr>
<td>HPA-3</td>
<td>Bak, Lek</td>
<td>HPA-3a, HPA-3b</td>
<td>37% a/a 48% a/b 15% b/b</td>
<td>GpIlbI843S</td>
<td>12.75% (3a) 23.31 (3b)</td>
<td>2% &lt;1%</td>
</tr>
<tr>
<td>HPA-4</td>
<td>Pen, Yuk</td>
<td>HPA-4a, HPA-4b</td>
<td>99% a/a &lt;0.1% a/b &lt;0.1% b/b</td>
<td>GPIIaR143Q</td>
<td>&lt;0.1% (4a) &lt;0.2% (4b)</td>
<td>&lt;1% &lt;1%</td>
</tr>
<tr>
<td>HPA-5</td>
<td>Br, Hc, Zav</td>
<td>HPA-5a, HPA-5b</td>
<td>80% a/a 19% a/b 1% b/b</td>
<td>GPlaE605K</td>
<td>16.0% (5a) 0.99% (5b)</td>
<td>1% 9%</td>
</tr>
<tr>
<td>HPA-6</td>
<td>Ca, Tu</td>
<td>HPA-6a, HPA-6b</td>
<td>99% a/a &lt;1% a/b &lt;0.1% b/b</td>
<td>GPIIaR489Q</td>
<td>&lt;0.1% (6a) &lt;0.2% (6b)</td>
<td>&lt;1%</td>
</tr>
</tbody>
</table>

~90% of NAIT cases involve HPA-1a, HPA-5b and HPA-3a
HPA1: Higher risk of response associated with HLA- DRB3*0101 positive mothers.

Iso-Bases Increase Specificity of Molecular Assays

- Novel Base Pairs that do not Cross React with Natural Bases:
  - Pair with Novel Complement Only
  - Recognized by common Enzymes
  - Licensed to EraGen Biosciences
MULTI-CODE™
Step 1 – Gated Multiplexed PCR

What is the Genotype of this "R" SNP?

Multiplex Gated PCR
(-)iGTP

[A] iCT
[T]
MULTI-CODE™

Step 2 – Addition of Multiplexed ASPE

Bead mixture and Strepavidin-phycoerythrin added after ASPE
All steps performed in one tube with no transfers, washes, or specialized separations
Unique microsphere sets are color-coded using a blend of different fluorescent intensities of two dyes. >100 addresses can be created.
Platelet Antigen Genotyping

HPA1
HPA2
HPA3
HPA4
HPA5
HPA6
HPA9
HPA15
Hemolytic Disease: Fetus and Newborn

Pathology

- Hemolytic anemia - Mild
- Kernicterus – Moderate
- Fetal hydrops - Severe

Treatment

- Phototherapy
- Exchange transfusion at birth
- Intrauterine transfusion
Severe HDN most commonly caused by RhD, Rhc, Kell and Fya

Bowman, J (1998) In Hematology of Infancy and Childhood, ed Nathan and Oski
## Red Cell Antigens

<table>
<thead>
<tr>
<th>Ag System</th>
<th>Antigen Names</th>
<th>Gene</th>
<th>Alleles</th>
<th>Phenotype frequency*</th>
</tr>
</thead>
<tbody>
<tr>
<td>RhD</td>
<td>D, d</td>
<td>RHD</td>
<td>RHD+, RHD-, RHDψ many variants</td>
<td>85% D+ 15% D- 92% D+ 8% D-</td>
</tr>
<tr>
<td>RhCc</td>
<td>C, c</td>
<td>RHCE</td>
<td>RHC, RHc, RHD-CE(3-7)-D</td>
<td>19% CC 49%Cc 32% cc 2% CC 25%Cc 73% cc</td>
</tr>
<tr>
<td>RhEe</td>
<td>E, e</td>
<td>RHCE</td>
<td>RHE, RHe</td>
<td>3% EE 26%Ee 71% ee 1% EE 21%Ee 78% ee</td>
</tr>
<tr>
<td>Kell</td>
<td>K, k (Kell, Cellano)</td>
<td>KEL</td>
<td>K1, K2 KEL1, KEL2</td>
<td>&lt;1% KK 9% Kk 91% kk Rare KK 2% Kk 98% kk</td>
</tr>
<tr>
<td>Kidd</td>
<td>Jka, Jkb</td>
<td>JK</td>
<td>JKA, JKB</td>
<td>26% JkAA 50%JkB 24% JkB 51% JkAA 41%JkB 8% JkB</td>
</tr>
<tr>
<td>Duffy</td>
<td>Fya, Fyb</td>
<td>FY</td>
<td>FYA, FYB, FYGATA</td>
<td>17% FyAA 49%FyAB 34% FyBB Rare Fynull 9% FyAA 1%FyAB 22% FyBB 68% Fynull</td>
</tr>
<tr>
<td>MN</td>
<td>M, N</td>
<td>GYP A</td>
<td>M, N</td>
<td>28% MM 50% MN 22% NN 24% MM 50% MN 26% NN</td>
</tr>
</tbody>
</table>

*Calculated from: The Blood Group Antigen Facts Book, Reid and Lomas-Francis, 2004
RhD Immunization

• Incidence of anti-D alloimmunization reduced from 2% to 0.1% by rhesus immune globulin

• Administer at 28 wks and within 72 hours of birth if the fetus is RhD-positive.

• Sensitization to RhD still occurs due to lack of anti-D administration, unrecognized miscarriage and fetomaternal hemorrhage.
HDFN Predictive Parameters

- Past Pregnancy History
- Maternal Alloantibody Titers
- Amniotic Fluid Spectrophotometry ($\Delta OD_{450}$)
- Perinatal Ultrasound
- Middle Cerebral Artery (MCA) Peak Systolic Blood Flow
- Percutaneous Umbilical Blood Sampling (PUBS)
- Paternal Zygosity testing
- Molecular Analysis of Fetal DNA
Monitoring HDFN by MCA Doppler

- More sensitive than $\Delta OD_{450}$
- Equal or better specificity than $\Delta OD_{450}$
- More accurate than $\Delta OD_{450}$

Clinical Management: First Affected Pregnancy

- Determine paternal zygosity
- If paternal sample heterozygous, draw sample for fetal genotyping.
- Maternal titers repeated every month until ~24 weeks then every 2 weeks.
- At critical titer, serial MCA Doppler at 24 weeks then every 1-2 weeks

Clinical Management
Previously Affected Fetus

- Patient referred to center experienced in the care of severely alloimmunized pregnancy.
- Titers are not predictive of the degree of fetal anemia.
- If paternal sample heterozygous, draw sample for fetal genotyping.
- Serial MCA Doppler at 18 weeks and every 1-2 weeks.

Genetics of Rh System

Chromosome 1

- 90% homology at nucleotide and amino acid level resulting from gene duplication event. \textit{RHCE} is the ancestral gene.
- \textit{RHD} gene encodes the D antigen.
- D-negative is generally caused by absence of \textit{RHD} gene.
- \textit{RHCE} gene encodes RhC/c and RhE/e antigens.
- \textit{RHD} and \textit{RHCE} inherited as a haplotype.
Proposed Mechanism For The Formation of RhD- Negative Haplotype in Caucasians

Hybrid Rhesus Box is a potential molecular marker for a RHD-Negative allele

Gene Conversion in *RH* Genes

RHCE polymorphisms are transferred to the *RHD* gene.

Variant *RHD*-positive and -negative alleles

**RHD-Positive Alleles**

1. 2 3 4 5 6 7 8 9 10
   - RHD
   - DAR
   - DIIIa
   - DIVa
   - DIVb
   - DVI
   - DHAR

**RHD-Negative Alleles**

- 37 bp insert
- Stop codon
- RHD Deletion

- 1 2 3 4 5 6 7 8 9 10
  - RHD$_{\psi}$
  - RHD-CE(3-7)-D
  - RHD-CE(2-9)-D
  - RHD-CE(8-9)-D
  - RHD-CE(4-7)-D

*RHD$_{\psi}$* observed in 24% RhD-negative African Americans

Partial D individuals can make anti-D
**RHD Zygosity Testing**

- Paternal RhD zygosity:
  - DD: All fetuses will be RhD-positive
  - Dd: 50% of fetuses will be RhD-positive.
- 30-50% are heterozygous for RhD
- Historically, zygosity prediction was performed using serology and Rh haplotype frequencies. Not accurate in all ethnic groups.
- Hybrid box assay detects the common RHD deletion but not other RHD-negative alleles.
**RHD Zygosity Assay**

- Quantitative fluorescent PCR of *RHD* exons 5 and 7 using RHCE Exon 7 as a two copy internal control.

- Ratio of exon 5 or 7 to RHCE exon 7 using to determine zygosity. (~0.5 for heterozygotes, ~1.0 for homozygotes)

- Exon 5 specifically detects RHD, not RHDψ

- Gene variants identified by copy number discrepancies between exon 5 and 7.
Molecular Testing for *RHD* Zygosity

Exon ratios distinguish heterozygous and homozygous samples.

Variants recognized by differences in zygosity between exon 5 and 7.

Fetus is RHD-positive and at risk for HDFN
Pregnancy monitored with Doppler
Transfusion as required.

Adapted from Wagner et al (2001) BMC Genetics 2:10
Prenatal Testing: Variant RHD Alleles

<table>
<thead>
<tr>
<th>Exon</th>
<th>NC</th>
<th>D+</th>
<th>D-</th>
<th>Dψ</th>
<th>F1</th>
<th>M1</th>
<th>M2</th>
<th>F2</th>
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<tr>
<td>4</td>
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<tr>
<td>7</td>
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RHD-negative mother

RHD-Positive mother
# Prenatal Samples for HDFN Testing

<table>
<thead>
<tr>
<th>Sample</th>
<th>Risks and Concerns</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amniotic Fluid</td>
<td>Risk of fetal loss (&lt;0.5%) and hemorrhage (17%)</td>
</tr>
<tr>
<td>CVS</td>
<td>Risk of fetal loss (1-2%)</td>
</tr>
<tr>
<td>PUBS</td>
<td>Risk of fetal loss and hemorrhage, more difficult to perform.</td>
</tr>
<tr>
<td>Fetal Cells</td>
<td>Insufficient quantity</td>
</tr>
</tbody>
</table>
Properties of Cell Free DNA in Plasma

• Plasma DNA are mainly short fragments
• Fetal fragments are shorter than maternal fragments (<0.3 kb).
• Mean half-life 16.3 minutes (range 4-30)
• Rapid turnover suggests fetal DNA is liberated at 22,400 copies per minute.
• Less susceptible to false-positives results as caused by persistence of fetal cells.
Clinical Utility of Non-Invasive Prenatal Diagnosis (NIPD): HDFN and NAIT

- Avoids amniocentesis and the risk of further alloimmunization while identifying fetuses at risk.
- Avoid IgG prophylaxis in patients with a negative fetus. (Cost and human blood product)
- Clinical studies in Europe and US demonstrate the accuracy of testing (2002-2011).
- In the US, intellectual property has limited the use of NIPD. RHD testing became available in 2008. One laboratory currently offers the test. First clinical study published in 2011.
## Estimated Quantity of Fetal DNA in Maternal Plasma

(Finning et al. Transfusion 42: 1079)

<table>
<thead>
<tr>
<th>Gestation (weeks)</th>
<th>Fetal DNA Copy number/ml plasma</th>
<th>Median</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;14</td>
<td>Mean 28 ± 17</td>
<td>25 (12-61)</td>
<td>10</td>
</tr>
<tr>
<td>15-28</td>
<td>Mean 44 ± 28</td>
<td>35 (6-130)</td>
<td>45</td>
</tr>
<tr>
<td>29-42</td>
<td>Mean 200 ± 329</td>
<td>80 (23-1324)</td>
<td>16</td>
</tr>
<tr>
<td>Unknown</td>
<td>Mean 96 ± 65</td>
<td>73 (26-228)</td>
<td>10</td>
</tr>
</tbody>
</table>
### Diagnostic Accuracy of RHD NIPD Assays

<table>
<thead>
<tr>
<th>Reference</th>
<th>Wk Gest</th>
<th># Patients</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Accuracy*</th>
<th>False Pos</th>
<th>False Neg</th>
<th>Inconclusive # (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Finning et al 2008</td>
<td>≤28</td>
<td>1869</td>
<td>96.7</td>
<td>98</td>
<td>95.7 99.1</td>
<td>14</td>
<td>3</td>
<td>64 (3.4)</td>
</tr>
<tr>
<td>Clausen et al 2011</td>
<td>25</td>
<td>2312</td>
<td>99.9</td>
<td>99.3</td>
<td>96.5 99.6</td>
<td>6</td>
<td>2</td>
<td>74 (3.2)</td>
</tr>
<tr>
<td>Akolekar et al 2011</td>
<td>11-13</td>
<td>586</td>
<td>98.2</td>
<td>96.4</td>
<td>84.6 97.6</td>
<td>6</td>
<td>6</td>
<td>84 (14.3)</td>
</tr>
<tr>
<td>Bombard et al 2011</td>
<td>11-13</td>
<td>234</td>
<td>97.2</td>
<td>96.9</td>
<td>85.6 97.1</td>
<td>2</td>
<td>4</td>
<td>27+ (11.5)</td>
</tr>
<tr>
<td>Bombard et al 2011</td>
<td>6-30</td>
<td>205</td>
<td>100</td>
<td>98.3</td>
<td>95.6 99.5</td>
<td>1</td>
<td>0</td>
<td>6 (2.9)</td>
</tr>
<tr>
<td>Tyman et al 2011</td>
<td>≤32</td>
<td>148</td>
<td>100</td>
<td>100</td>
<td>98.7 100</td>
<td>0</td>
<td>0</td>
<td>2++ (1.4)</td>
</tr>
</tbody>
</table>

*Accuracy with and without inconclusive samples
*11/27 were RHD variants/RHD pseudogene / 19% African Americans
**4% cases fetal sex assignment was incorrect.

- Despite the high sensitivity and specificity of these assays, there is still a significant level of false positive, false negative and inconclusive results compared to conventional testing.
Causes of Analytic Problems in NIFD

- Quality of DNA preparation
- Difficulty controlling appropriate input of fetal genomes
- Lack of adequate control fetal genetic markers
- Variant alleles (ethnic-specific variants)
- “Vanishing” twin
- General limitations of current technology.

NIPD brings its own unique set of quality assurance challenges for the clinical laboratory.
Fetal Quantifier Assay

Identify differentially-methylated targets in placenta

Digest maternal DNA

Amplify and detect fetal and competitor DNA

Quantify fetal target in relation to competitor copy number

Nygren et al 2010 ClinChem 56::1627-365
Serological reagents are not available for many red cell and platelet antigen systems
Applications of Genotyping for Red Cell Antigens

- Predicting phenotype in chronically transfused patients who has developed an antibody.
- Screening of donors for antigen-negative blood.
- Better antigen matching prior to transfusion.
- Distinguishing RhD-negative, weak D or DEL.
- Typing for antigen systems where serological reagents are rare or nonexistent.
- Characterization of serological variants.

Providing rare and antigen-matched blood requires screening of thousands of donors.
Alloimmunized Transfusion Recipients

- There are +15MM transfusions/yr in the US
  - 1st transfusion/pregnancy → ~4% alloimmunized
  - Chronic transfusion? up to 50% of alloimmunized
- Increased demand for expanded patient typing and antigen-matched blood to avoid transfusion reactions.
  - +100,000 patients: SCD, MDS, AA
- Increasing demand for antigen typed blood
  - increase the number of antigen-typed donors (multiple antigens)
  - Increase number of uncommon and rare requests for donors

There is a need for mass-screening of blood donors
The OpenArray Plate

- A unique method to run ~3000 low-volume solution phase assays in parallel
- Equivalent to eight 384-well or thirty-two 96-well plates
OpenArray Anatomy

- Hydrophilic and hydrophobic coatings
- Fluid retention by capillary action
- Accurate and precise fluidics

33 nL volume
**Platform Flexibility**

Analyze different combinations of samples per plate & assays per sample

<table>
<thead>
<tr>
<th>Samples/plate</th>
<th>Assays/sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>144</td>
<td>16</td>
</tr>
<tr>
<td>96</td>
<td>32</td>
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<tr>
<td>48</td>
<td>64</td>
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<tr>
<td>24</td>
<td>128</td>
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<tr>
<td>12</td>
<td>256</td>
</tr>
<tr>
<td>6</td>
<td>512</td>
</tr>
<tr>
<td>3</td>
<td>1024</td>
</tr>
<tr>
<td>1</td>
<td>3072</td>
</tr>
</tbody>
</table>
# Red Cell Genotyping Panel

## Common Panel (16) (Tx/WAIHA)

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
</table>
| Rh | C/c  
  C (+190ins)  
  E/e  
  Rhce nt48C |
| Kell | K/k  
  Kp(a/b)  
  Js(a/b) |
| Duffy | Fy(a/b)  
 **FYnull -67t/c** |
| Kidd | Jk(a/b) |
| MNS | M/N  
  S/s  
  U (nt230C>T)  
  U (i5+5g>t) |
| Lu | Lua/b |
| Do | Doa/b |

## Rh Variant Panel (Sickle Cell Disease)

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
</table>
|  | 85  
  137 'Altered C'  
  223  
  233  
  238  
  245  
  336  
  342 |

Altered C, c, e antigens  
V, VS, Crawford  
hrB/hrS: ceMO, ceEK, ceTI
Multiple assays are performed in "parallel simplex" on the same OpenArray.
Summary

• NAIT and HDFN are caused by alloimmunization to a paternally-inherited form of an antigen.
• Molecular testing is useful for identifying antigen discrepancies, paternal zygosity, prenatal testing. Beware of variants!
• Prenatal testing using fetal DNA in maternal plasma may help avoid invasive procedures.
• Red cell genotyping can be used to provide an expanded antigen profile on patients and for identifying antigen-negative donors.
Self-Assessment Questions

• How is molecular testing used in the management of NAIT and HDFN cases?
• Is it possible for an Rh-positive women to make anti-D?
• A fetus was tested for RHD from maternal plasma and found to be an RHD-negative female. What controls are needed to confirm this result?
• Explain how molecular testing can be used to determine blood type in a transfused patient.