Frequency and characterization of known and novel RHD variant alleles in 37 782 Dutch D-negative pregnant women

Tamara C. Stegmann,1, * Barbera Veldhuisen,1, 2, * Renate Bijman,1 Florentine F. Thurik,1 Bernadette Bossers,2 Goedele Cheroutre,2 Remco Jonkers,2 Peter Ligthart,2 Masja de Haas,1, 2 Lonneke Haer-Wigman1, * and C. Ellen van der Schoot1, *

1 Sanquin Research and Landsteiner Laboratory, Academic Medical Centre, University of Amsterdam, and 2 Sanquin Diagnostic Services, Amsterdam, The Netherlands

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Correspondence: C. E. van der Schoot, Plesmanlaan 125, 1066CX Amsterdam, The Netherlands
E-mail: e.vanderschoot@sanquin.nl
*These authors contributed equally.

The D antigen of the Rh blood group system is one of the most immunogenic and complex blood group antigens (Westhoff, 2007a; Daniels & Reid, 2010). Most D- individuals lack the complete RhD protein (Colin et al, 1991; Wagner & Flegel, 2000), which underlies its high immunogenicity. Anti-D can cause severe haemolytic transfusion reactions and/or severe haemolytic disease of the fetus and newborn. To prevent anti-D formation in D- individuals, compatible red blood cells (RBCs) are transfused and anti-D prophylaxis is administrated to D- pregnant women (de Haas et al, 2015).

The Rh locus is highly polymorphic and many RHD variant alleles have been described (Flegel, 2011). One group of RHD variant alleles, the RHD hybrid alleles, arose due to genetic recombination between the RhD gene and the adjacently located RHCE gene. The second group of RHD variant alleles carry one or multiple mutations in the RHD gene. The genetic variation of the RHD alleles has different effects on the level of expression of RhD protein and the number of expressed RhD epitopes. To date, more than 60 so-called D-null alleles have been described that cause the D- phenotype due to nonsense mutations, frame shift mutations, splice site mutations or to large hybrid alleles (http://www.uni-ulm.de/~fwagner/RH/RB2/P_RHDDnegative.htm). The D-null alleles RHD*03N.01 and RHD*P occur frequently in the D- African population (Flegel, 2011). RHD positive haplotypes are rare in D- Caucasians (Wagner et al, 2001; Flegel et al, 2005; Chou & Westhoff, 2010). Individuals with a weak D phenotype express the RhD protein in low quantities, which is most often caused by mutations in the transmembrane regions of the RhD protein (Daniels, 2013a). Individuals with DEL-allele expression have an even lower amount of the RhD protein on their RBC membrane, which can only be detected with the very sensitive adsorption-elution technique (Okubo et al, 1984). DEL expression is most often caused by missense mutations causing aberrant splice sites (Reid et al, 2012). Partial D expression, in which one or more D epitopes are lacking, is most often caused by hybrid alleles or due to mutations in the extracellular parts of the RhD

Summary
To guide anti-D prophylaxis, Dutch D- pregnant women are offered a quantitative fetal-RHD-genotyping assay to determine the RhD status of their fetus. This allowed us to determine the frequency of different maternal RHD variants in 37 782 serologically D- pregnant women. A variant allele is present in at least 0.96% of Dutch D- pregnant women. The D-serology could be confirmed after further serological testing in only 54% of these women, which emphasizes the potential relevance of genotyping of blood donors. 43 different RHD variant alleles were detected, including 15 novel alleles (11 null-, 2 partial D- and 2 DEL-alleles). Of those novel null alleles, one allele contained a single missense mutation (RHD*443C>G) and one allele had a single amino acid deletion (RHD*424_426del). The D- phenotype was confirmed by transduction of human D- erythroblasts, confirming that, for the first time, a single amino acid change or deletion causes the D- phenotype. Transduction also confirmed the phenotypes for the two new variant DEL-alleles (RHD*721A>C and RHD*884T>C) and the novel partial RHD*492C>A allele. Notably, in three additional cases the DEL phenotype was observed but sequencing of the coding sequence, flanking introns and promoter region revealed an apparently wild-type RHD allele without mutations.

Keywords: Rh blood group, RHD variant alleles, D- phenotype, blood group genotyping.
protein. Some partial RHD variant alleles cause partial and weakened expression of the D antigen (Westhoff, 2007b). The distinction between the different variant alleles is of importance, given that it is unlikely that individuals with weak D or DEL expression produce allo-anti-D, in contrast to individuals with partial D expression who are at risk of D immunization (Daniels, 2013a; Sandler et al, 2015).

The aim of our study was to determine the frequency of (known and novel) RHD variant alleles in the serologically D- Dutch population. Since July 2011, Dutch D- pregnant women have been offered a quantitative fetal-RHD-genotyping assay to guide anti-D prophylaxis. This quantitative fetal-RHD-genotyping assay is performed with cell-free DNA isolated from maternal plasma, which contains DNA of the fetus (Scheffer et al, 2011; van der Schoot et al, 2013). However, the large majority of cell free DNA is of maternal origin and, therefore, if a maternal RHD allele is present it will be recognized because it results in much stronger signals in the quantitative polymerase chain reaction (PCR) assay than expected to arise from fetal DNA. In this paper we present the frequency of RHD variant alleles and serological and genetic follow up of cases identified among 37 782 screened Dutch D- pregnant women.

Material and methods

Samples and fetal-RHD-genotyping assay

Between July 2011 and December 2012, 37 782 Dutch serologically D- pregnant women (determined using two anti-D reagents) were tested in the 27th week of pregnancy for the presence of a D+ fetus using a quantitative fetal-RHD-genotyping assay. DNA was isolated from 1 ml of maternal plasma using a DNA isolation kit (DNA and Viral NA Large Volume Kit; Roche Holding AG, Basel, Switzerland) on a MagNa Pure 96 Instrument (Roche) according to the manufacturer’s protocol. The quantitative fetal-RHD-genotyping assay has been described previously (Scheffer et al, 2011) and consists of a multiplexed TaqMan test, one targeting RHD exon 5 and one targeting RHD exon 7, performed in triplicate. When at least two of the three Ct values of both assays were below 32, a maternal variant allele was suspected. When at least two of the three Ct values of exon 7 were below 32, but Ct values of exon 5 were either negative or above 32, a RHD*Ψ or RHD*06 maternal variant allele was suspected. In these cases additional genotyping and extended serology was performed to determine whether and which variant allele was present. Of note, the relatively frequently occurring null allele RHD*03N.01 (Daniels et al, 1998) is not amplified and thus not detected in this fetal-RHD-genotyping assay.

Serology

All samples were subjected to column testing on either the Ortho Biovue Inova system (Ortho, Raritan, NJ, USA) with an ABO D card containing anti-D monoclonal antibodies (MoAbs) D7B8, or the Biorad/Diamed Diana system (Bio-Rad Laboratories, Venendaal, The Netherlands) with an ABO DVI- card, containing anti-D MoAbs LHM59/20 (LDM3) and 175-2. Plasma of all women with a negative result in this serological assay were tested in the fetal-RHD-genotyping assay. A second comprehensive serological assay was performed when a maternal variant allele was suspected. The samples were tested with three monoclonal blend reagents (IgM clone TH28 and IgG clone MS26) (Sanquin Reagents, Amsterdam, The Netherlands and Immucor, Norcross, GA, USA) and IgM clone D7B8, IgG clone H1112169 and IgG clone LORIFA (Ortho), and one IgG clone 5C8, a polyclonal IgG reagent (Bio-Rad Laboratories) in an immediate spin at room temperature, in a spin proceeded by 15 min incubation at 37°C and/or by indirect antiglobulin test. If, following this second test, the D- phenotype was suspected and the presence of the RHD*Ψ allele was excluded, absorption-elution was performed using the Gamma® ELU-KIT® II (Immucor) as per manufacturer’s protocol using a polyclonal anti-D (Bio-Rad Laboratories) to detect a DEL allele. If the second serological test detected a partial D variant other than the DVI variant, the D-epitope expression of the variant allele was determined using an in-house RhD typing kit consisting of eleven monoclonal IgG antibodies and an additional six MoAbs from the ALBAclone Advanced Partial RhD typing kit (ALBA Bioscience, Edinburgh, UK). These MoAbs were tested in anti IgG + anti C3d gel columns (Bio-Rad laboratories).

RH-MLPA

Maternal DNA was isolated from white blood cells using a DNA extraction kit (QIAamp DNA Blood Mini Kit, Qiagen Benelux, Venlo, The Netherlands). To determine RHD copy number and the presence of RHD variant alleles, DNA samples were analysed with the RH-Multiplex Ligation-dependent Probe Amplification (RH-MLPA) assay (mix p401-A1, p402-A1 and p403-A1, MRC-Holland, Amsterdam, The Netherlands) (Haer-Wigman et al, 2013). In some cases an RHCE MLPA was performed using RHCE-specific probes to determine the copies of RHCE exons 1, 3, 4, 5, 6, 7, 9 and 10 (probes listed in Table SI). One case was tested with seven new MLPA probe combinations (targeting c.-698T, c.123A, c.149-4875A, c.149-882G, c.244T, c.335 + 2838C and c.1112G of RHD and RHCE) that were developed to determine the combined RHD and RHCE copy numbers of the 5’UTR, exon 1, 2 and 8 and intron 1 and 2 (Table SI).

The MLPA reaction was performed according to the manufacturer’s protocol on a Veriti Thermocycler (Applied Biosystems, Nieuwerkerk aan de IJssel, The Netherlands). A mixture of 1.0 µl MLPA sample, 8.5 µl Hi-Di™ Formamide (Applied Biosystems) and 0.5 µl GeneScan™ 500-Liz® Size Standard (Applied Biosystems) was analysed on a 3130 Genetic Analyser (Applied Biosystems). Data analysis was
performed using Genemarker software version 1.85 (Softgenetics, State College, PA, USA).

**DNA sequencing**

When indicated, all exons and intron boundaries of \( \text{RHD} \) were sequenced and/or the promoter region of \( \text{RHD} \) was sequenced (hg19, chr.1:g.25597899\textsuperscript{-}25598887; primer sequences are shown in Supplementary Table SI or as published previously (Haer-Wigman et al., 2013). The PCR was performed on a Veriti thermocycler in a total volume of 20 µl, containing 50–100 ng DNA, 10 µl of 2× GeneAmp Fast PCR Master Mix (Applied Biosystems), 0.5 µmol/l forward and reverse primer. PCR conditions were: 10 s at 95°C, 35 cycles of 10 s at 95°C, and a specific annealing/elongation temperature and time for each primer set ranging from 62 to 70°C, followed by 1 min at 72°C. PCR products were purified using ExoSAP-IT (GE Healthcare, Eindhoven, The Netherlands), according to the manufacturer’s protocol. PCR products were sequenced with ABI BigDye Terminator v3.1 kit on an ABI 3130XL sequencer (Applied Biosystems).

**Heterologous transfection system**

The \( \text{RHD} \) coding sequence flanked by a BamH1 and Not1 digestion site was ordered from Invitrogen (Breda, The Netherlands) and cloned into a lentiviral vector containing IRES-GFP for bicistronic gene expression driven under the EF1a promoter. The c.424–426del, c.443G, c.492A, c.721C and c.1154C mutations were mutated into the wild-type \( \text{RHD} \) construct using QuickChange II XL Site-Directed Mutagenesis Kit (Agilent, Amstelveen, The Netherlands) according to the manufacturer’s protocol (Table SI). Lentivirus was produced by transfecting 293T cells with helper plasmids using the Calcium Phosphate method (Sambrook & Russell, 2001). The supernatant containing the virus particles was then harvested for 3 days and concentrated through ultracentrifugation. Erythroblast from five different D- (ccd-dee) donors were cultured from peripheral blood mononuclear cells according to the protocol described by van den Akker et al. (2010) and kept in expansion medium for 2–3 d. Erythroblasts were then lentivirally transduced with the \( \text{RHD} \) wild-type construct or the different variant constructs. After 48-h transduction, cells were transfected into StemSpan medium (Stem Cell Technologies, Grenoble, France) supplemented with stem cell factor (SCF; supernatant equivalent to 100 ng/ml), erythropoietin (10 µ/ml, ProSpec; East Brunswick, NJ, USA), holotransferrin (0.5 mg/ml; Sigma-Aldrich, Zwijndrecht, The Netherlands) and insulin (10 mg/ml; Sigma-Aldrich) in order to allow cell differentiation (van den Akker et al., 2010).

Cells were harvested within 5 days of differentiation and screened for D-expression by flow cytometry using six human monoclonal IgG anti-D of the ALBAclone Advanced partial RhD typing kit (ALBA Bioscience), namely LHM169/81, LHM76/59, LHM76/55, LHM169/80, LHM57/17 and LHM76/58. Data analysis was performed with FlowJo Version 8 software (TreeStar, Ashland, OR, USA).

To measure mRNA levels of the transduced variants, RNA was isolated from \( 1 \times 10^6 \) differentiated erythroblasts using TRIzol (Life Technologies, Paisley, UK) (Chomczynski & Sacchi, 1987) and 1 µg of RNA was used to synthesize cDNA using random hexamers (Invitrogen). Real-time quantitative reverse transcription PCRs (RQ-PCRs) were performed using the reporter dye SYBR-green (Sybrgreen Mastermix, Applied Biosystems) on a StepOnePlus (Applied Biosystems) with \( \text{RHD} \)-specific primers as listed in Table SI.

**Results**

A variant \( \text{RHD} \) allele is present in 0.96% of Dutch D-pregnant women

Between 2011 and 2012, the fetal-\( \text{RHD} \)-genotyping assay, to determine whether anti-D prophylaxis is indicated, was performed on a total of 37 782 D- pregnant women. In 493 women (1.3%) a maternal variant allele was suspected based on Ct-values and genetic follow-up was performed in 309 of these cases (Table SII). Genetic follow-up was not performed in 184 cases due to (i) missing samples \((n = 31); \) (ii) Ct-values of around 31–32 \((n = 83)\) and analysis in cases with stored DNA had shown that this is virtually always due to high fetal DNA levels and (iii) PCR results pointed to the presence of either \( \text{RHD}^{*06} \) or \( \text{RHD}^{*06} \) allele, as was shown in the first series of 159 cases with similar Ct values for exon 5 and exon 7 (Table I).

In 39 (12.6%) of the 309 evaluated D-pregnant women in whom genetic follow-up was performed, the \( \text{RHD} \) negativity was based on complete deletion of the \( \text{RHD} \) gene because the homozygous presence of the \( \text{RHD}^{*01N.01} \) allele was confirmed. In these cases the obtained Ct-values arose from high fetal \( \text{RHD} \)-DNA concentrations.

In the remaining 270 women a variant allele was identified (Table I). The distribution of \( \text{RHD} \) alleles in these cases was used to calculate the distribution of \( \text{RHD} \) alleles in cases without follow-up (Table SII). We estimate that 0.96% (95% confidence interval [CI] 0.86–1.06%) of the Dutch serologically D-pregnant women had a variant allele containing \( \text{RHD} \) exon 5 and/or 7. The most frequently detected variant allele is the \( \text{RHD}^{*06} \) allele, which was present in 47% of the women carrying a variant \( \text{RHD} \) allele.

**Pregnant D-women determined with standard serology carried at least 43 different variant alleles**

In 218 of the 270 analysed cases carrying variant alleles and on whom genotyping were performed, the \( \text{RHD-MLPA} \) genotyping assay directly identified a specific known \( \text{RHD} \) variant allele (listed in Table I). In the remaining 52 cases (as indicated in Table I) additional genotyping was performed,
Table I. RHD variant alleles detected in 270 women determined D- with standard serology.

<table>
<thead>
<tr>
<th>RhD phenotype</th>
<th>RHD allele 1</th>
<th>RHD allele 2</th>
<th>MLPA</th>
<th>Number of cases positive for genotype</th>
</tr>
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<td>D-</td>
<td>RHD*Ψ</td>
<td>RHD*Ψ</td>
<td>c</td>
<td>98 (2)</td>
</tr>
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<td>RHD*Ψ</td>
<td>RHD*Ψ</td>
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<td>13</td>
</tr>
<tr>
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<td>RHD*Ψ</td>
<td>c</td>
<td>6</td>
</tr>
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<td>RHD*01N.01</td>
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<tr>
<td></td>
<td>RHD*[361T&gt;A; 383A&gt;G; 455A&gt;C; 602C&gt;G; 667T&gt;G; 819G&gt;A]§</td>
<td>RHD*01N.01</td>
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<td>RHD*01N.01</td>
<td>c</td>
<td>6</td>
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<td>RHD*06.02</td>
<td>RHD*01N.01</td>
<td>c</td>
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</tr>
<tr>
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<td>RHD*06.02</td>
<td>RHD*03N.01</td>
<td>c</td>
<td>1</td>
</tr>
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<td>RHD*10.02</td>
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<td>i</td>
<td>2</td>
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<tr>
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<td>RHD*11</td>
<td>RHD*01N.01</td>
<td>c</td>
<td>9 (1)</td>
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<td></td>
<td>RHD*15</td>
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<td>RHD*01N.01</td>
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<td>RHD*01EL.01</td>
<td>RHD*01N.01</td>
<td>c</td>
<td>9</td>
</tr>
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<td>RHD*01N.22‡</td>
<td>RHD*01N.01</td>
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<td>1</td>
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<td>i</td>
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<td>i</td>
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<td>n</td>
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<td>RHD*01N.01</td>
<td>i</td>
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</tr>
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<tr>
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<td>RHD*01</td>
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<tr>
<td>Weak RhD</td>
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<td>RHD*01N.01</td>
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<td>RHD*01W.38</td>
<td>RHD*01N.01</td>
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</table>

Multiplex Ligation-dependent Probe Amplification (MLPA) result: c, conclusive; i, inconclusive; n, seemingly normal RHD gene. All RHD exons were sequenced if MLPA i or n. (. ) Cases where no extended serology could be performed.

†The literature has described that the RHD*DEL5, RHD*DEL8 and RHD*DEL9 alleles cause the DEL phenotype (Singleton et al, 2001; Flegel et al, 2009; Reid et al, 2012); however, we detected a D- phenotype.

‡The literature indicates that RHD*01N.22 allele causes D- phenotype (Reid et al, 2012); however, we detected a DEL phenotype.

§Novel variant allele.
Red blood cells from 259 cases (including all cases with novel serology) were analyzed. Phenotype analysis of the fifteen novel variants using adsorption-elution. The initial D- phenotype was confirmed in 139 (54%) cases. In 33 (13%) cases a DEL phenotype, in 26 cases an apparently normal wild-type allele, detected in seven cases, was available for additional serological analysis, including adsorption-elution. The initial D- phenotype was confirmed in erythroblast expression system, even at lower expression levels when compared to the control, and as a control for our expression system, the RHD*01W.02 allele was transduced. RHD transcript levels, demonstrated that RHD mRNA levels did not significantly differ between RHD*01 and the RHD variants, indicating that the loss of expression was caused by the mutation and not by differences in transcription efficiency or RNA stability (Fig 2).

Phenotype analysis of the fifteen novel variants using serology

Red blood cells from 259 cases (including all cases with novel variant alleles) out of the 270 cases with a variant RHD allele were available for additional serological analysis, including adsorption-elution. The initial D- phenotype was confirmed in 139 (54%) cases. In 33 (13%) cases a DEL phenotype, in 77 (30%) cases a partial D and in ten (4%) cases a weak D phenotype was determined (Table I). The D- phenotype was confirmed for the novel alleles: RHD*1084C>T, RHD*124_125del and RHD*1174del (RHD*(2-10)) (RHD*335G>T, RHD*[634+1G>T, 1136C>T], RHD*1073+1G>T and RHD*1074-1G>A) and the novel variant composed of the known RHD*03.03 and RHD*09.03.01 (Table III).

The novel variants RHD*884T>C and RHD*721A>C caused the DEL phenotype (Table II). The RHD*492C>A allele was serologically determined to lead to partial D expression, as epitope 5 (trD7C2) and epitope 8 (HIMA-36) were absent, whereas all other evaluated epitopes (including epitope 8 tested with LHM76/58) were detected (Table II). Several anti-D MoAbs were only positive in the indirect agglutination test indicating that this variant allele has next to partial D also weakened D expression (data not shown). The RBC expressing the novel RHD*[178A>G; 689G>T] allele next to the known RHD*10.01 showed loss of epitopes 1, 5 and 8. However, as this corresponds to the epitope pattern for the RHD*10.01 allele (Wagner et al., 2002), the exact phenotype of the new allele could not be determined (Table II).

Discussion

In the present study we determined that a variant RHD allele containing RHD exon 5 and/or 7 is present in ~0.96% of the Dutch D- pregnant women. Genetic follow-up determined that almost half of the women with a variant allele carried the RHD*P variant allele and about 16% carried one of the RHD*06 variants. All other detected RHD variants are rare and in total we identified 43 different variant alleles, including 15 novel RHD variant alleles. Extensive serological follow-up confirmed the D- phenotype in 54% of the women, but partial D expression was found in 29%, DEL expression in 14% and weak D expression in 4% of the women. In three cases we detected an apparently normal wild-type RHD allele.
Table II. D-epitope expression in the RHD*443G, RHD*492A, RHD*[178A:689T], RHD*10.01, RHD*721A, RHD*01W.02 and RHD*01 alleles.

<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>RhD epitope</th>
<th>RHD*01</th>
<th>RHD*01W.02</th>
<th>RHD*424_426del</th>
<th>RHD*443C&gt;G</th>
<th>RHD*492C&gt;A</th>
<th>RHD*721A&gt;C</th>
<th>RHD*[178A&gt;C; 689G&gt;T] and RHD*10.01 IAT</th>
<th>RHD*10.01 IAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>LHM70</td>
<td>1</td>
<td>3</td>
<td>++</td>
<td>−</td>
<td>−</td>
<td>4</td>
<td>++</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>LHM169/80</td>
<td>1</td>
<td>4</td>
<td>++</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>5C8</td>
<td>2</td>
<td>3</td>
<td>++</td>
<td>−</td>
<td>−</td>
<td>3</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>LHM76/59</td>
<td>3</td>
<td>4</td>
<td>++</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>3</td>
<td>+</td>
<td>−/−</td>
</tr>
<tr>
<td>LHM76/55</td>
<td>3</td>
<td>4</td>
<td>++</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>3</td>
<td>+</td>
<td>−/−</td>
</tr>
<tr>
<td>AUB-2F7/Fiss</td>
<td>5</td>
<td>2</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>RD7C2</td>
<td>5</td>
<td>5</td>
<td>++</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>LHM50/28</td>
<td>6/7</td>
<td>4</td>
<td>−</td>
<td>−</td>
<td>4</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>LHM169/80</td>
<td>6/7</td>
<td>4</td>
<td>++</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>4</td>
<td>++/−</td>
</tr>
<tr>
<td>LHM57/17</td>
<td>6/7</td>
<td>4</td>
<td>++</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>4</td>
<td>++</td>
<td>−</td>
</tr>
<tr>
<td>LOSI</td>
<td>6/7</td>
<td>4</td>
<td>−</td>
<td>−</td>
<td>4</td>
<td>+/−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>HIRI-5</td>
<td>6/7</td>
<td>4</td>
<td>−</td>
<td>−</td>
<td>4</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>LHM76/58</td>
<td>8</td>
<td>4</td>
<td>++</td>
<td>−</td>
<td>−</td>
<td>4</td>
<td>++</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>HIMA-36</td>
<td>8</td>
<td>3</td>
<td>++</td>
<td>−</td>
<td>−</td>
<td>4</td>
<td>++</td>
<td>−</td>
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<tr>
<td>LHM77/64</td>
<td>9</td>
<td>9</td>
<td>−</td>
<td>−</td>
<td>3</td>
<td>+/−</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>MS26</td>
<td>9</td>
<td>6/7,9</td>
<td>4</td>
<td>−</td>
<td>−</td>
<td>3</td>
<td>−</td>
<td>−</td>
<td>−/−</td>
</tr>
<tr>
<td>Blend anti-D</td>
<td>6/7,9</td>
<td>4</td>
<td>−</td>
<td>−</td>
<td>3</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Polyclonal anti-D</td>
<td>3</td>
<td>3</td>
<td>−</td>
<td>−</td>
<td>3</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

D-epitope expression as determined by serology and heterologous expression system for the novel variants RHD*[178A>C;689G>T], RHD*424_426del, RHD*443C>G, RHD*492C>A and RHD*721A>C. As a sensitivity control, the well-known variants RHD*01, RHD*10.01 and RHD*01W.02 were also determined.

IAT, indirect antiglobulin test; FACS, fluorescence-activated cell sorting.
<table>
<thead>
<tr>
<th>Allele</th>
<th>Nucleotide Changes‡</th>
<th>Exon (Intron)</th>
<th>Protein change(s)‡</th>
<th>Initial serology</th>
<th>Extended serology</th>
<th>Adsorption-Elution</th>
<th>RhD phenotype</th>
<th>Linked RHCE genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RHD*1084C&gt;T</strong></td>
<td>c.1084C&gt;T</td>
<td>8</td>
<td>p.Gln362Ter</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>D-</td>
<td>RHCE*02</td>
</tr>
<tr>
<td><strong>RHD*124_125del</strong></td>
<td>c.124_125delAA</td>
<td>1</td>
<td>p.Lys42 fs</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>D-</td>
<td>RHCE*02</td>
</tr>
<tr>
<td><strong>RHD*1174del</strong></td>
<td>c.1174delA</td>
<td>9</td>
<td>p.Ile392 fs</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>D-</td>
<td>RHCE*02</td>
</tr>
<tr>
<td><em><em>RHD</em>(2-10)</em>*</td>
<td>c.1-?_148 + ?del</td>
<td>1</td>
<td>p.?</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>D-</td>
<td>RHCE*01</td>
</tr>
<tr>
<td><strong>RHD*335G&gt;T</strong></td>
<td>c.335G&gt;T</td>
<td>2</td>
<td>p.Ser112Ile r.spl?</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>D-</td>
<td>RHCE*02</td>
</tr>
<tr>
<td>*<em>RHD</em>[634 + 1G&gt;T; 1136C&gt;T]**</td>
<td>c.[634 + 1G&gt;T; 1136C&gt;T]</td>
<td>(4), 8</td>
<td>r.Spl</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>D-</td>
<td>RHCE*01</td>
</tr>
<tr>
<td><strong>RHD*1073 + 1G&gt;T</strong></td>
<td>c.1073 + 1G&gt;T</td>
<td>7</td>
<td>r.Spl</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>D-</td>
<td>RHCE*01</td>
</tr>
<tr>
<td><strong>RHD*1074-1G&gt;A</strong></td>
<td>c.1074-1G&gt;A</td>
<td>7</td>
<td>r.Spl</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>D-</td>
<td>RHCE*02</td>
</tr>
<tr>
<td><em><em>RHD</em>[361T&gt;A; 380T&gt;C; 383A&gt;G; 455A&gt;G; 602C&gt;G; 667T&gt;G; 819G&gt;A]</em>*</td>
<td>c.[361T&gt;A; 380T&gt;C; 383A&gt;G; 455A&gt;G; 602C&gt;G; 667T&gt;G; 819G&gt;A]</td>
<td>3, 4, 5, 6</td>
<td>p.[Leu121Met; Val127Ala; Asp128Gly; Asn152Thr; Thr201Arg, Phe223Leu; Ala273Ala]</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>D-</td>
<td>RHCE*01</td>
</tr>
<tr>
<td><strong>RHD*443C&gt;G</strong></td>
<td>c.443C&gt;G</td>
<td>3</td>
<td>p.Thr48Arg</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>D-</td>
<td>RHCE*02</td>
</tr>
<tr>
<td><strong>RHD*424_426del</strong></td>
<td>c.424_426delATG</td>
<td>3</td>
<td>p.Met142del</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>D-</td>
<td>RHCE*02</td>
</tr>
<tr>
<td><strong>RHD*721A&gt;C</strong></td>
<td>c.721A&gt;C</td>
<td>5</td>
<td>p.Thr241Pro</td>
<td>Neg</td>
<td>Neg</td>
<td>Pos</td>
<td>D-</td>
<td>RHCE*01</td>
</tr>
<tr>
<td><strong>RHD*884T&gt;C</strong></td>
<td>c.884T&gt;C</td>
<td>6</td>
<td>p.Met295Thr</td>
<td>Neg</td>
<td>Neg</td>
<td>Po</td>
<td>D-</td>
<td>RHCE*02</td>
</tr>
<tr>
<td><em><em>RHD</em>[178A&gt;G; 689G&gt;T]</em>*</td>
<td>c.[178A&gt;G; 689G&gt;T]</td>
<td>2, 5</td>
<td>p.[Ile60Leu; Ser230Ile]</td>
<td>Neg</td>
<td>Partial§</td>
<td>-</td>
<td>Partial weak D</td>
<td>RHCE*01</td>
</tr>
<tr>
<td><strong>RHD*492C&gt;A</strong></td>
<td>c.492C&gt;A</td>
<td>4</td>
<td>p.Asp164Glu</td>
<td>Weak</td>
<td>Partial</td>
<td>-</td>
<td>Partial weak D</td>
<td>RHCE*02</td>
</tr>
</tbody>
</table>

†Position as counted from ATG translation start site; homo = homozygous, hetero = heterozygous.
‡Position as counted from Met translation start site.
§The case positive for this variant allele carried a second variant allele RHD*10.01 that causes partial D expression, therefore we cannot exclude that this variant allele can also cause the D- phenotype.
yet a DEL phenotype was observed. Our analysis shows that in 0.22% (95% CI 0.17–0.26%) of cases (excluding the women carrying the \textit{RHD*06} variants) serological typing incorrectly indicated RhD negativity. Conversely, RH-MLPA incorrectly predicted the D+ phenotype in 0.05% (95% CI 0.03–0.08%) of serologically D- women.

Here we describe 15 novel alleles (11 D-null, two partial-weak and two DEL alleles). The D- phenotype was determined for a novel allele with a nonsense mutation (\textit{RHD*1084C>T}), two novel alleles with frame shift mutations (\textit{RHD*124_125del} or \textit{RHD*1174del}), four alleles with mutations that disrupt a splice site (\textit{RHD*335G>T}, \textit{RHD*634+1G>T}, \textit{1136C>T}), \textit{RHD*1073+1G>T} and \textit{RHD*1074-1G>A} and one allele with the deletion of exon 1 (\textit{RHD*(2-10)}). The presence of this last allele could not be unambiguously proven, because of the presence of another variant allele in this case.

The D- phenotype was also determined for a variant allele that contained mutations of both the \textit{RHD*03.03} and \textit{RHD*09.03} variant alleles (\textit{RHD*[361T>A; 380T>C; 383A>G; 455A>C; 602C>G; 667T>G; 819G>A]}). This was unexpected because the \textit{RHD*09.03} allele causes only a moderate weakening of the RhD expression and the \textit{RHD*03.03} allele has not been associated with an effect on RhD expression levels, although the mutations are all found in putative transmembrane and intra-cellular parts of the RhD protein, which might offer some explanation for our finding.

Interestingly, both an allele with a single missense mutation \textit{RHD*443C>G} (encoding p.Thr148Arg) and an allele with the deletion of a single amino acid \textit{RHD*424_426del} (p.Met142del) cause the D- phenotype, which was confirmed in a heterologous expression study in D- erythroblasts. Both alleles have mutations in the fifth putative transmembrane

Fig 1. RhD expression levels of the \textit{RHD*424_426del}, \textit{RHD*443C>G}, \textit{RHD*492C>A} and \textit{RHD*721A>C} variant alleles in a heterologous expression assay. Overlay plots of the fluorescence intensity, representative for the RhD expression levels of RhD-negative erythroblasts transduced with constructs containing the \textit{RHD*424_426del}, \textit{RHD*443C>G}, \textit{RHD*492C>A}, or \textit{RHD*721A>C} and the well-described \textit{RHD*01W.02} cDNA (black line). The wild-type \textit{RHD*01} cDNA (black dashed line) was transduced for quantitative analysis of the expression levels. The \textit{RHD*01W.02} sensitivity control showed weakened RhD expression levels compared to the \textit{RHD*01} wild-type allele. The \textit{RHD*424_426del}, \textit{RHD*443C>G} alleles had completely no RhD expression, the \textit{RHD*492C>A} allele had similar expression levels to the wild-type \textit{RHD*01} but showed weakened expression of epitope 3 (LHM76/55). The \textit{RHD*721A>C} showed very weak expression of epitope 3 (LHM76/55) and epitope 6/7 (LHM 169/80) weakened RhD expression, even weaker than the \textit{RHD*01W.02} allele. Histograms are representative figures (n = 3).
The DEL phenotype. Interestingly, the RHD*721A>C allele was detected in seven cases whereas all other novel alleles, except the RHD*1074-1G>A allele, were detected in single cases. This allele was not detected in previous studies performed in Germany, Austria, Poland and Belgium, respectively (Flegel et al, 2009; Polin et al, 2009; Orzinska et al, 2013; Van Sandt et al, 2015, respectively, indicating that this allele is specific for the Dutch population. All women positive for the RHD*721A>C allele had Dutch surnames but we have no indication that these women are related.

The DEL and RhCcCe phenotype was detected in three cases with a wild-type RHD*01 allele and without any mutation in the intron boundaries or in the promoter region of the RHD gene. The RhCc expression was normal in these two cases. Flegel et al (2009) also described a single case without mutations in the RHD exons and intron boundaries with the DEL and a normal RhCc phenotype. Possibly, in these cases a deep intronic mutation is present or a gene that is required for membrane expression of the RhD protein is mutated.

Furthermore, it is important to note that for the variants RHD*DEL5, RHD*DEL8, RHD*DEL9, RHD*01N.22, the D-phenotype determined in this study deviated from previously reported DEL phenotypes (Singleton et al, 2001; Flegel et al, 2009; Reid et al, 2012). A D- phenotype for RHD*DEL8 and RHD*DEL9 has been described (Wagner et al, 2001) and for RHD*DEL8, Kormocz et al (2005) described a partial D phenotype. Moreover, in agreement with our observed D-serology is the fact that alloimmunization has occurred in individuals carrying the RHD*DEL8 (Kormocz et al, 2005; Gardener et al, 2012) or the RHD*DEL5 (Daniels & Reid, 2010) allele.

Three large studies have been performed (46 133 D-donors in Germany (Flegel et al, 2009), 31 200 D- donors in Poland (Orzinska et al, 2013) and 23 330 D- donors Austria (Polin et al, 2009)) to ascertain the presence of RHD variant alleles in D- donors, in which considerably lower percentages of D- donors carried RHD variant alleles, 0-21%, 0-20% and 0-40%, respectively. The African RHD*Ψ allele was observed at low frequency (0-03%) in the German donor population (Flegel et al, 2009) and not among the Austrian and Polish donors (Polin et al, 2009; Orzinska et al, 2013). Furthermore, in our study the RHD*06 variant was tested as D- on purpose, while this was not the case in other studies. Even if the frequency of variant alleles in our study is recalculated excluding the RHD*Ψ and the RHD*06 alleles, 0-32% (95% CI 0-26–0-37%) of the D- women carry an RHD variant: this is still slightly higher than the frequency in the two largest blood donor studies, possibly reflecting the multiracial origin of the Dutch pregnant population.

In conclusion, 0-96% of the Dutch D- pregnant women carry a D variant allele harbouring RHD exon 5 and/or exon 7. The large majority of pregnant women with a variant allele carry an RH-null allele or partial RHD allele and need administration of anti-D prophylaxis to prevent anti-D immunization. Genotyping of this group of women has the
limited advantage in that the woman with weak D type 1, 2
and 3 are recognized and can be regarded in the current and
any subsequent pregnancy as D+. More importantly, our
study emphasizes the relevance of genotyping of blood
donors (Denomme, 2013; Sandler et al, 2015). Furthermore,
this cohort of extensively typed D- women can be used to
optimize RH-D genotyping assays, as it is essential that the
most frequently occurring D-null alleles are identified for
correct prediction of the D phenotype via a genotyping assay
(Gassner et al, 2005; Daniels, 2013b).

Author contributions

TS, BV, RB, PL and LHW performed the research. FT, BB,
GC, RJ, BV and PL collected and registered all the samples.
The data was analysed by TS, BV, LHW, CS and MH. The
study was designed by CS and MH. TS, LHW and CS wrote
the paper.

Conflicts of interest

The authors declare that they have no conflicts of interest
relevant to the manuscript submitted to British Journal of
Haematology.

Supporting Information

Additional Supporting Information may be found in the
online version of this article:

Fig S1. Schematic representation of MLPA results in a
single case with two RHD variant alleles.

Fig S2. Position of mutations of seven alleles in the RhD
protein.

Table S1. Primer and probe list.

Table SII. Calculation of number of cases with a RHD
variant allele in the total cohort of 37 782 D- pregnant
women.

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