Elucidation of the molecular bases of the Rh system and its contribution to transfusion and obstetric medicine—historical and current perspective: a review

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Abstract: The Rh system, the most polymorphic of the human blood groups, is of major clinical interest in transfusion and obstetric medicine due to the participation of Rh antibodies in phenomena of immune mediated red blood cell destruction. Rh antigens are highly immunogenic and are often involved in haemolytic transfusion reactions and in the Haemolytic Disease of the Newborn (HDN). Since the cloning and sequencing of the *RH* genes in the early 1990s and the development of numerous molecular techniques for the analysis of genetic polymorphisms, a plethora of allelic variants has been identified over time. Genotyping strategies are being increasingly incorporated in the field of transfusion medicine and have shown a great capacity to overcome the limitations of serology. In house and commercial DNA-based developments allow the characterization of clinically significant allelic variants and have proven to be remarkable for accurate donor-recipient matching to prevent alloimmunization, determining the risk for HDN or guiding immunoprophylaxis among other applications. In this work, the molecular bases of the Rh system and the new allele discoveries throughout this time will be reviewed. Also, the impact of the molecular identification of different *RH* allelic variants in the field of transfusion and obstetric medicine will be summarized.

Keywords: Rh system; transfusion medicine; *RH* alleles; *RH* genotyping

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Introduction

The Rh system is of major clinical interest in transfusion and obstetric medicine due to the involvement of Rh antibodies in phenomena of immune-mediated erythrocytes destruction. The five principal Rh antigens—D, C, c, E, and e—are highly immunogenic and play a central role in the pathogenesis of the Haemolytic Disease of the Newborn (HDN), haemolytic transfusion reactions and some cases of autoimmune haemolytic anaemia. Fifty-six different antigens have been serologically defined making the Rh system the most polymorphic of all the erythrocyte blood group systems (1,2). In this article, the molecular bases of the Rh system and the new allele discoveries throughout this time will be reviewed. Also, the impact of the molecular identification of different *RH* allelic variants in the field of transfusion and obstetric medicine will be discussed.

From antigens to genes

Levine and Stetson (3), in 1939, published the historic case

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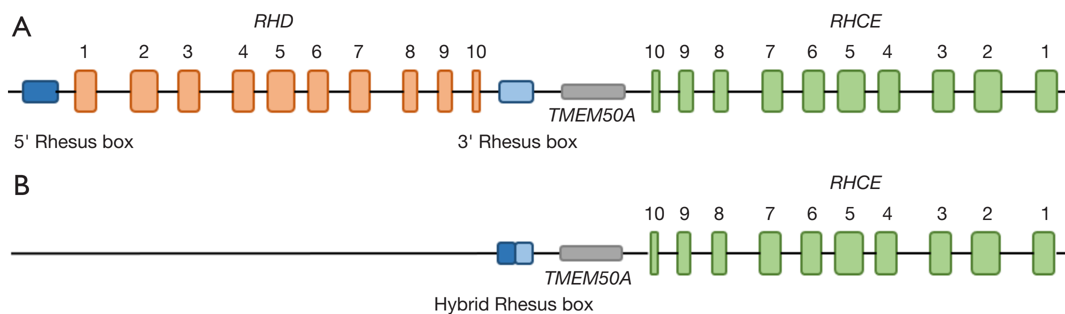

Figure 1 Schematic representation of the *RH* locus. The *RH* locus consists of two highly homologous genes denoted *RHD* and *RHCE*. Both genes have 10 exons encompassing approximately 58 kb of DNA each and reside in tandem in opposite orientation. They are separated by 31.8 kb, in which the *TMEM50A* is found. *RHD* is flanked by the 5' Rhesus box and 3' Rhesus box. Deletion of *RHD*, the usual cause of the D-negative phenotype in Caucasians, appears to have occurred through unequal crossing over involving the homologous 5' and 3' Rhesus boxes and the generation of a hybrid Rhesus box in the deleted region. (A) *RH* locus in a D-positive chromosome. (B) *RH* locus in a D-negative chromosome.

of a mother who required blood transfusion after giving birth a stillborn child and experienced an immediate and severe haemolytic reaction when she was transfused with blood from her husband. Subsequent studies detected an antibody in maternal serum that had an agglutination pattern similar to that obtained with the antibody generated by Landsteiner and Wiener (4) after immunization of rabbits with erythrocytes from Rhesus macaques. Further investigations demonstrated that the specificities of the human and the animal antibodies were different, denoting "anti-Rh" the human antibody and "anti-LW" the animal antibody (5).

According to the theory put forward by Fisher and Race in the 40's, the Rh system was composed of three close genes, each with two alleles, *C* and *c*, *D* and *d*, and *E* and *e* (6). A person inherited a set of alleles of the three *RH* genes, known as haplotypes, from each parent. It was originally expected that each allele would be found to determine a corresponding antigen but only C, c, D, E and e had been recognized and it was presumed that d was amorphic. Later in 1951, Wiener proposed that the inheritance of Rh antigens was related to a single gene with multiple alleles (R_1, R_2, R_0, R_z, r, r', r'', r^y) (7). Each allele determined an agglutinogen which had multiple factors (Rh_0, rh', hr', rh'', hr''). For example, the agglutinogen produced by R_1 expresses three factors, Rh_0, rh', and hr'' (D, C, and e in Fisher-Race terminology). In 1986, Tippett (8) proposed another model, based on an abundance of serological data, in which Rh antigens are determined by only two *RH* genes, one encoding D with two different alleles (*D* and non-*D*, the latter not coding for a recognizable product), and the other encoding the CcEe antigens with four alleles (*ce*, *Ce*, *cE* and *CE*).

Colin *et al.* were the first to report, in 1991, the genetic organization of the *RH* locus in D-positive and D-negatives individuals (9). Southern blot analyses were key to demonstrating that there are two genes in the *RH* locus, per haploid genome of D-positive individuals. Later it was confirmed that one of these two genes was absent in the *RH* locus of D-negative individuals, suggesting that the missing gene encodes the D antigen (10-12). The two *RH* genes, namely *RHD* and *RHCE*, are highly homologous and similarly organized in all D-positive Caucasians. In summary:

- ❖ The *RH* locus is composed of two adjacent homologous structural genes denoted *RHD* and *RHCE* (*Figure 1*);
- ❖ Caucasian D-positive individuals have either one or two *RHD* genes per cell while the D-negative phenotype is mainly caused by the absence of the entire *RHD* gene;
- ❖ The *RHD* gene encodes the RhD protein that expresses the epitopes of the D antigen;
- ❖ The *RHCE* gene has 4 most common allelic forms: *RHCE*ce*, *RHCE*Ce*, *RHCE*cE* and *RHCE*CE* and each allele determines the expression of two antigens in ce, Ce, cE or CE combination carried by the RhCE protein (*RHCE* is the collective name of the 4 alleles).

Rh proteins

The *RH* genes (*RHD* and *RHCE*) encode two 417 amino acids Rh polypeptides (RhD and RhCE, respectively) but the initiating methionine residues are post translationally cleaved (13,14). The mature 416 Rh proteins are palmitoylated but not glycosylated and cross the erythrocyte membrane 12 times. The N-terminal and C-terminal amino acid residues reside on the cytoplasmic side and both

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RH allele	Nucleotide position						
	Exon 1	Exon 2					Exon 5
	48	150	178	201	203	307	676
*RHD*01*	G	T	A	G	G	T	G
*RHCE*ce*	**G**	**C**	**C**	**A**	**A**	**C**	**G**
*RHCE*Ce*	C	T	A	G	G	T	–
*RHCE*cE*	–	–	–	–	–	–	C
*RHCE*CE*	C	T	A	G	G	T	C

Rh protein	Amino acid position						
	16	50	60	67	68	103	226
RhD	Trp	Val	Ile	Ser	Ser	Ser	Ala
Rhce	Trp	Val	Leu	Ser	Asn	Pro	Ala
RhCe	Cys	–	Ile	–	Ser	Ser	–
RhcE	–	–	–	–	–	–	Pro
RhCE	Cys	–	Ile	–	Ser	Ser	Pro

Figure 2 Nucleotide and amino acid changes among the conventional *RHCE* alleles. *RHD*01* and RhD polymorphisms are shown for comparison. Reference sequence is highlighted in red. A dash (–) indicates identity to the reference sequence. The c.150C>T and c.201A>G are silent mutations. Trp, tryptophan; Val, valine; Ile, isoleucine; Ser, serine; Ala, alanine; Leu, leucine; Asn, asparagine; Pro, proline; Cys, cysteine.

proteins are predicted to have 6 extracellular loops. They are erythrocyte specific and only expressed on the red blood cell (RBC) membrane if the Rh associated glycoprotein (RhAG) is also present. Rh and RhAG together with the accessory proteins LW, glycophorin B and CD47 are assembled in the "Rh complex" (15). The core of the complex is predicted to be a heterotrimer involving RhD, RhCE and RhAG, stabilized by interactions between the N-terminal and C-terminal domains to which the accessory polypeptides are linked by non-covalent bonds (16). The Rh complex is associated with the Band3/glycophorin. A complex giving rise to a macromolecular structure that seems to participate in gas and cation transport across the membrane. Particularly, RhAG is involved in NH_4^+ and CO_2 exchange (16,17). This macrocomplex also interacts with the spectrin-based membrane skeleton through ankyrin and protein 4.2 and participates in the maintenance of the shape and mechanical properties of the RBC (18-21). As evidence, Rh_{null} individuals (lacking all Rh antigens) have a mild clinical condition, called Rh-deficiency syndrome, characterized by membrane abnormalities and some degree of haemolytic anemia. Typical hematological features include the presence of stomatocytes and some spherocytes in blood smears, reduced survival of autologous RBCs and increased erythrocyte osmotic fragility (22).

RhBG and RhCG are human RhAG homologues that are mainly expressed in the kidney and thus the "Rh family" currently consists of five proteins—RhD, RhCE, RhAG, RhBG and RhCG (23-25). Contrary to the three other members of the Rh family, RhD and RhCE wouldn't be a channel for ammonia and might be implicated only in CO_2 transport in RBCs but are functionally redundant (26,27).

The RhD protein carries the D antigen. The presence or absence of the D antigen in the RBC membrane allows individuals to be classified as "D-positive" or "D-negative", respectively. The RhCE polypeptide bears the antithetical antigens C or c (involving the second extracellular loop) in addition to E or e (involving the fourth extracellular loop). RhD differs from RhCE by approximately 35 amino acids (depending on the RhCE isoform considered). In spite of this high homology between them, normal RhCE polypeptides do not express any D epitopes and the conventional RhD protein does not express CE antigens (28-30), clearly showing that Rh antigens are conformation-dependent structures. RhCE polypeptides expressing C and c antigens differ in 4 amino acid substitutions—p.Cys16Trp, p.Ile60Leu, p.Ser68Asn and p.Ser103Pro—of which, position 103 is critical for C/c specificity, whereas RhCE polypeptides expressing E and e antigens differ in 1 amino acid change—p.Pro226Ala (31-33) (*Figure 2*).

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RH locus

The *RH* locus (*Figure 1*) is located on the short arm of chromosome 1 (position 1p34.1–1p36) (34) and comprises *RHD* and *RHCE*. Both genes consist of 10 exons encompassing approximately 58 kb of DNA, reside in tandem in opposite orientation facing each other with their 3' ends, with *RHD* telomeric of *RHCE* (9-14,35-37). The region between both genes encompasses 31.8 kb and contains the *TMEM50A* gene (previously *SMP1*) (36). Each exon is shorter than 200 bp. *RHD* and *RHCE* share a high level of sequence homology (overall 93.8% gene sequence identity and 96.4% exon sequence identity), supporting the concept that these genes have evolved by duplication of a common ancestor (10-14,35,36). Despite their being very closely related, the immunologic heterogeneity within the resulting protein is remarkable. Two 9 kb regions of high homology (98.6%) flank the *RHD* gene, the so-called 5' Rhesus box and 3' Rhesus box. *RHD* deletion, the most frequent genetic background responsible for the D-negative phenotype in Caucasians, appears to have occurred through unequal crossing over involving the upstream and downstream Rhesus boxes and the generation of a hybrid Rhesus box in the deleted region. The analysis of the hybrid Rhesus box (a marker for *RHD* deletion) may be a predictor of the *RHD* zygosity (36,38).

The coding sequence of *RHD* (accession number NG_007494.1) contains 37 specific nucleotides that are not found in the 4 most common *RHCE* alleles (accession number NG_009208.3). *RHCE*c* has 5 specific nucleotides in exon 2 (c.150C, c.178C, c.201A, c.203A and c.307C), of which the c.307C encoding p.103Pro is best correlated with c expression (31,39). On the other hand, exon 2 of *RHCE*C* is equal to exon 2 of *RHD* and only c.48C in exon 1 encoding p.16Cys is *RHCE*C* specific. However, there is not a strict correlation between c.48C (p.16Cys) and C expression since, mostly in Africans, some *RHCE*c* alleles with normal expression of c also harbour c.48C in exon 1 (40). Hence *RHCE*C* genotyping assays must rely on the detection of a 109 bp insertion that is present in intron 2 of *RHCE*C* alleles only (41). *RHCE*E* differs from *RHCE*e* in one single nucleotide variation (SNV) at position 676 in exon 5 (c.676C>G) that leads to the p.Pro226Ala substitution in RhCE proteins expressing E or e respectively. The c.676G is also present in the conventional *RHD* (31) (*Figure 2*).

The 3' untranslated region of *RHD* covers more than 1,500 bp while a shorter stretch is known for *RHCE*. A deletion of approximately 600 bp in *RHD* intron 4 is another remarkable difference between *RHD* and *RHCE*. Other intronic insertions or deletions over 100 bp have been detected (12,35,42,43). Short tandem repeats are also present in some introns of the *RH* genes and can be used for polymorphism analysis (35,44,45). GATA-1, SP1 and Ets binding sites are found in the 5' flanking region of *RHCE* (32).

The discovery of *RH* alleles over time

Since the cloning and sequencing of the *RH* genes in the early 1990s and the development of many molecular techniques for the analysis of genetic polymorphisms, a plethora of allelic variants has been identified over time. *RH* alleles show substantial ethnic variability. Some variants are confined to specific ethnic groups whereas others are more dispersed.

An updated record of *RH* alleles is available in the International Society of Blood Transfusion (ISBT) website (46). At present (March 2023), 446 *RHD* alleles and 188 *RHCE* alleles have been recognized by the ISBT. *RHD* variants are also listed in the Rhesus Site (47) and many more alleles can be found in published articles and conference abstracts and in databases such as Genbank (48), Erythrogene (49) and RHeference (50).

RH alleles have arisen through different mechanisms such as rearrangements between the *RHD* and *RHCE* genes by unidirectional segmental DNA exchanges. This gene conversion event, favoured for the high homology and opposite orientation of both genes, is responsible for the generation of hybrid *RH* alleles. Many kilobases, including multiple exons and introns can be converted, but often microconversion events, involving a few bases, produce templated mutations (51). In addition, SNVs are responsible for untemplated mutations, where the modified nucleotides do not come from the other gene. Insertions, deletions and duplications are also responsible for the genetic polymorphism of the Rh system. Nucleotide modifications may affect exonic, splice site, intronic and/or promoter regions and lead to amino acid(s) change(s) with subsequent loss or alteration of some epitopes and/or expression of a low incidence antigens or cause premature stop codons preventing protein synthesis. Base changes may also produce frameshifts and splicing alterations leading to shorter or longer polypeptides that will most likely not be integrated in the erythrocyte membrane (46,47,50).

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RHD alleles

The _RHD*01_ allele is the reference sequence for the _RHD_ gene and is responsible for the normal D-positive phenotype. _RHD*01.01_, characterized by a c.48G>C change, is also considered as to express normal D antigen while the other _RHD_ allelic variants are responsible for D-negative or D variant phenotypes. In this work, we will use the term "D variant" to refer to altered expression of D, such as weak D, partial D or DEL. D variants are serologically recognized because the strength of reactivity with anti-D reagents may be weaker than that of normal D-positive RBCs (≤2+ in tube immediate spin or detected in antiglobulin phase or through adsorption-elution tests) or similar to normal D-positive cells but with the concomitant presence of an anti-D alloantibody. Variants of D can also be recognized when discrepant results using different anti-D reagents are obtained (52).

RHD alleles have been classified according to the encoded phenotype in silent, partial D, weak D and DEL alleles (46,47).

Silent alleles

RHD silent alleles are responsible for a D-negative phenotype. Even though homozygosity for a whole deletion of the _RHD_ gene (_RHD*01N.01_) is the primary background for the D-negative phenotype in most populations, silent (non-functional) _RHD_ alleles exist that do not generate a complete RhD polypeptide, or the proteins encoded do not express any D epitopes (53-56).

Two _RHD_ silent alleles are frequent in Africans: _RHD*08N.01_ (_RHD*ψ_) and _RHD*03N.01_ (part of r'^{S} haplotype) and at least one copy is harboured by 67% and 15% of D-negative black Africans, respectively. _RHD*08N.01_ is characterized by a duplication of 37 bp located in the intron 3–exon 4 boundary and 5 nucleotide changes along exons 4 (c.609G>A), exon 5 (c.654G>C, c.667T>G, c.674C>T) and exon 6 (807T>G). This allele may be inactivated by the introduction of a reading frame shift and a translation stop codon at position 210 or by the presence of the nonsense c.807T>G substitution in exon 6 that lead to the p.Tyr269Ter change (57). _RHD*08N.01_ is generally linked to _RHCE*ce.16_ that harbours a c.48G>C change in exon 1 and a _RHCE-to-RHD_ gene conversion in exon 9. _RHCE*ce.16_ has been associated to altered e antigen expression (58). _RHD*03N.01_ is a hybrid allele in which exons 4 to 8 are derived from _RHCE_ and exon 3 is an _RHD-RHCE_ hybrid but may be completely _RHCE_ (_RHD*01N.06_). _RHD*03N.01_ is part of the r'^{S} haplotype together with _RHCE*ceS_ (59-61), characterized by the c.48G>C, c.733C>G, c.1006G>T changes (see _RHCE_ alleles below). In Caucasians, the hybrid _RHD-CE(2-9)-D_ (_RHD*01N.03_) is the most frequent silent allele encountered and is associated with a R_1 haplotype. It is estimated that one copy may be harboured by 0.05% to 0.15% of D-negative individuals (54,55). It is worth mentioning that the African _RHD*08N.01_ and _RHD*03N.01_ alleles are also found in whites (56).

There are many other silent _RHD_ alleles responsible for a D-negative phenotype generally harbouring inactivating mutations, such as nucleotide changes, insertions or deletion that give rise to premature stop codons or splice site changes (46,47). These variants are relatively unusual, and the most frequently D-negative genotypes found are homozygosity or compound heterozygosity for _RHD_ deletion, _RHD*08N.01_, _RHD*03N.01_ or _RHD*01N.03_. To note, _RHD*01N.75_, characterized by a G insertion between positions 581 and 582 (c.581_582insG) in exon 4, is the second null variant most frequently found in D-negative, C- and/or E-positive Argentines, after _RHD*03N.01_ (62).

RHD silent alleles confound D genotyping because they may lead to false positive results. Molecular strategies must be properly designed considering the distribution of the most frequent silent variants in any given population. Therefore, stringent protocols must be designed to obtain reliable results when D genotyping is performed.

Weak D alleles

Weak D alleles are responsible for a D variant phenotype characterized by a reduced expression of the D antigen. They are most often associated with single nucleotide changes in _RHD_ that lead to RhD proteins with amino acid substitutions predicted to be located in the transmembranous or intracellular segments. Such mutations may negatively affect insertion of proteins in the membrane resulting in reduced amount of D sites at the cell surface (63,64).

Weak D alleles are classified into different "types" according to the mutation responsible for the decreased expression of the D antigen. Some weak D alleles are further divided into several subtypes, for example, _weak D type 1.1_, _weak D type 2.1_, _weak D type 4.0_, _weak D type 4.1_, etc. The number of weak D alleles is currently greater than 160. However, _weak D type 1_, _weak D type 2_, _weak D type 3_ and _weak D type 4_ are the most frequently alleles found in D variant individuals, reaching up to 95% in certain

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populations. DNA-based genotyping protocols have been described to identify the most common weak D alleles in D variant individuals (65-67).

Partial D alleles

Partial D alleles encode RhD proteins with amino acid substitutions predicted to be located in exofacial loops that cause the loss of one or more D specific epitopes (63,64). Partial D individuals can produce anti-D alloantibodies against the absent epitopes following immunization with D-positive RBCs after transfusion or pregnancy. Partial D phenotypes can react weakly with commercial anti-D reagents (for example, the DFR phenotype encoded by *RHD*17* alleles), they can also show a strength of hemagglutination equivalent to that observed in D-positive RBCs (for example, the DBT phenotype encoded by *RHD*14* alleles) or, even, show an overexpression of the D antigen (for example, the DIVa phenotype encoded by *RHD*04* alleles) (1). *RHD**06 alleles responsible for the DVI phenotype have been involved in many anti-D alloimmunization events with severe consequences (47,68-70). This has given rise to the development of IgM monoclonal anti-D typing reagents that do not recognize the partial DVI phenotype, the most frequently partial D found in Caucasians. Using this strategy, hemizygous (or the rare homozygous) *RHD*06* carriers are typed D-negative and managed accordingly (71,72).

Partial D alleles are mainly generated by DNA segment exchanges between *RHD* and *RHCE*. These gene conversion events give rise to hybrid *RHD-CE-D* or *RHCE-D-CE* alleles that encode chimeric proteins in which not only some D specific epitopes are missed but also low incidence antigens can be expressed, or high incidence antigens may be lost. Other molecular events such as missense SNVs in one or multiple positions can also lead to partial D alleles (1,46,47).

As mentioned before, monoclonal anti-D reagents cannot reliably distinguish some partial D and weak D phenotypes as they show similar reactivity. DNA-based testing can overcome these limitations and clearly discriminate weak D from partial D phenotypes. The molecular characterization of D variants is useful to implement an appropriate use of D-negative units and a rational administration of anti-D immunoprophylaxis.

DEL alleles

DEL alleles encode mutated RhD proteins that express very low levels of D antigen. The so called DEL phenotype is not detected by standard serology even when D typing is performed with a sensitive indirect antiglobulin test. Therefore, DEL RBCs are frequently mistyped as D-negative unless adsorption-elution tests are performed. The DEL phenotype is almost exclusively found in "seemingly D-negative" individuals expressing the C and/or E antigens (1).

DEL alleles are mainly generated by SNVs in exons or splice sites leading to amino acid changes or affecting RNA splicing. Deletions, insertions, intron polymorphism and hybrid structures have also been described to be responsible for DEL alleles (46,47).

In eastern Asia, between 10% and 33% of individuals who type as D-negative in routine testing are DEL and the most common *RHD* allele responsible is *RHD*DEL1*, the so called Asian-type DEL allele (1). It is characterized by a synonymous mutation in the last nucleotide of exon 9 (c.1227G>A, p.Lys409=), that interferes with efficient splicing and results in skipping of exon 9 in the mRNA (73,74).

The incidence of DEL varies from 0.1% to 0.5% among phenotypically D-negative Caucasians and *RHD*11* is the most common *DEL* allele. It carries a c.885G>T substitution in exon 6 leading to p.Met295Ile change in the RhD protein. *RHD*11* may occur in two haplotypes, in R_0 encodes a weak D phenotype easily demonstrable in the indirect agglutination test but in R_1 the antigen density is much lower resulting in a DEL phenotype. The second most common *DEL* allele in Caucasians is *RHD*DEL8* [RHD(IVS3+1G>A)] characterized by the splice site mutation c.486+1G>A and is associated with a R_1 haplotype (54-56). To note, *RHD*DEL43*, characterized by the c.46T>C change in exon 1, is the *DEL* variant most frequently found in Argentina (56).

DEL alleles may be responsible for genotype-phenotype discrepancies and their putative presence must be taken into account when performing *RHD* genotyping.

RHCE alleles

The *RHCE* gene also bears its own heterogeneity, which is responsible for C, c, E and e polymorphisms. The conventional *RHCE* alleles are designated as *RHCE*ce* (*RHCE*01*), *RHCE*Ce* (*RHCE*02*), *RHCE*cE* (*RHCE*03*), and *RHCE*CE* (*RHCE*04*) which encode RhCE proteins coexpressing C or c together with E or e per polypeptide. *RHCE*ce* is the reference sequence for the *RHCE* gene. *RHCE*c* and *RHCE*C* differ in 6 nucleotide substitutions

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while the difference between *RHCE*e* and *RHCE*E* is a single nucleotide (31) (*Figure 2*).

Like the *RHD* gene, numerous allelic variants of *RHCE* exist and were generated by the same molecular mechanisms that are responsible for *RHD* alleles (46,47).

RHCE variants are associated with quantitative and qualitative RhCE changes leading to partial, weak, or no expression of the principal antigens, with altered C and e most frequently encountered. As two copies of *RHCE* are present per genome, partial CcEe antigens are not regularly detected by serologic tests because they are masked by normal antigens expressed on the RhCE proteins encoded in the other chromosome (1). For instance, RBCs expressing an e variant may wrongly be assumed E homozygous if such variant is not detected by routine typing reagents, or a weakly reactive c variant may be missed if RBCs also carry a normal c antigen.

*RHCE*CeCW* (*RHCE*Ce.08.01*) encode the low frequency antigen C^w (Rh8) that is found in approximately 2% of Caucasians and 1% of Blacks. This allele is characterized by a c.122A>G variation in exon 1 leading to a p.Gln41Arg in the RhCe polypeptide. Patients carrying this allele type C-positive; however, they can develop anti-C (or anti-Ce) antibodies following transfusion (75). Another important allele responsible for a variant C antigen is *RHD*03N.01* (see *RHD* silent alleles above). It is mainly found in African Blacks and hence in patients with sickle cell disease (SCD) but also occurs in admixed populations. Even though the polypeptide encoded by this allele does not express any D epitopes it carries a partial C antigen that react weakly with polyclonal anti-C but strongly with commercial monoclonal anti-C reagents. *RHD*03N.01* is linked to *RHCE*ceS* (*RHCE*ce.20.03*) encoding partial c and e (designated e^S) antigens. *RHD*03N*.01 carriers can produce anti-C, anti-e, and/or anti-Ce alloantibodies following transfusion or pregnancy. These complex and multiple specificities are dubbed anti-hr^B (59-61,76).

One further aspect to be considered for *RHCE* variants is the coding of RhCE polypeptides expressing D-like epitopes. Two significant examples are *RHCE*ceHAR* (*RHCE*ce.22.01*), found in individuals of German ancestry, and *RHCE*ceCF* (*RHCE*ce.20.06*), found in individuals of African ancestry. In *RHCE*ceHAR*, exon 5 was replaced by the homologous *RHD* sequence rendering the hybrid *RHCE-D(5)-CE* allele. *RHCE*ceCF* is characterized by the templated nucleotide variations c.48G>C in exon 1, c.697C>G and c.733C>G in exon 5, leading to the amino acid changes p.Trp16Cys, p.Gln233Glu and p.Leu245Val in the encoded Rhce protein. These two *RHCE* alleles are remarkable because, in the absence of RhD protein, individuals carrying *RHCE*ceHAR* or *RHCE*ceCF* type D-positive (3+/4+) with some monoclonal anti-D reagents but D-negative with others. Consequently, if wrongly typed D-positive they can become alloimmunized after transfusion of D-positive units (77,78).

*RHCE*ce* variants are frequent in individuals with African ancestry, and, as an additional complication, are often linked to partial *RHD* variants. Patients harbouring haplotypes composed of partial *RHD* and altered *RHCE*ce* are at risk of developing clinically significant alloantibodies with complex specificities that are difficult to identify serologically. Genotyping and molecular matching will be beneficial for transfusion support in these patients.

Some *RHCE* variants do not encode E or e and may even not encode C or c. Individuals homozygous or compound heterozygous for these alleles carry the rare Dc-, DC^W- and D-- phenotypes and may develop anti-Rh17 (clinically significant antibody against the high prevalence Rh17 antigen) if exposed to conventional RBCs during transfusion or pregnancy. Homozygosity or compound heterozygosity for totally inactivated *RHCE* alleles in a D-negative person (lack of *RHD*) gives rise to the Rh_{null} phenotype (amorph type). It is worth mentioning that inactive *RHAG* alleles are also responsible for the Rh_{null} phenotype (regulator type) in homozygotes or compound heterozygotes, even if they carry unaltered *RHD* and *RHCE*. Rh_{null} patients generally develop anti-Rh29 (clinically significant antibody against the high prevalence Rh29 antigen) after transfusion or pregnancy (1,46,47).

Contribution of the molecular study of the Rh system to transfusion and obstetric medicine fields

Hemagglutination is the "gold standard" method for testing Rh antigens. It is a simple and inexpensive technique that has the appropriate specificity and sensitivity for the correct typing of most patients and donors. However, there are some clinical situations in which serological techniques cannot determine the Rh phenotype accurately. When serology turns inapplicable or inconclusive, an alternative approach is to infer the RBC phenotype identifying the *RH* alleles at the genomic level. *Table 1* summarizes the clinical applications of *RH* genotyping.

Prenatal RH gene genotyping

One of the first clinical applications that arose after the

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Table 1 Clinical applications of *RH* genotyping

Clinical applications
Type patients who have been recently transfused
Type RBCs coated with immunoglobulin
Type fetus to determine risk for HDN or to guide immunoprophylaxis
Type pregnant women to identify weak D and partial D phenotypes to determine candidates for Rh immunoglobulin
Type blood recipients to identify weak D and partial D phenotypes to avoid use of limited D-negative blood for transfusion
Detect *RHD* alleles that encode a DEL phenotype
Type *RH* alleles for accurate molecular matching in SCD
Determine paternal zygosity for *RHD*
Resolve Rh typing discrepancies

RBCs, red blood cells; HDN, Haemolytic Disease of the Newborn; SCD, sickle cell disease.

elucidation of the molecular bases associated with the D-positive and D-negative polymorphism was the prenatal prediction of the fetal D phenotype. In the early 1990s it was already possible to determine the fetal D status by analyzing DNA obtained from amniotic fluid or chorionic villi (79). This fetal *RHD* genotyping strategy has been used for almost 10 years to identify the fetus who is not at risk of HDN (i.e., predicted to be antigen-negative) in D-negative mothers sensitized with anti-D. If the fetus is D-negative, the pregnancy does not require further monitoring other than normal. On the other hand, if the fetus is D-positive, the pregnancy needs more exhaustive monitoring and, sometimes, medical treatment. Strategies have also been developed to predict expression of antigens carried by RhCE or other blood group proteins in fetus at risk of HDN.

Nowadays, non-invasive testing of cell-free fetal DNA (cffDNA) obtained from maternal plasma is the method of choice for determining fetal antigens since it allows avoiding the adverse events associated with amniocentesis or chorionic villus sampling (80,81). Particularly, non-invasive fetal *RHD* genotyping, based on the analysis of cffDNA in maternal plasma, not only allows an early risk assessment of HDN in pregnancies with anti-D alloantibodies but also has the potential to avoid antenatal anti-D prophylaxis in the 38% to 40% of D-negative pregnant women carrying an *RHD*-negative fetus (82). In this scenario, it is particularly relevant to take into consideration that the presence of *RH* allelic variants (mainly *RHD* silent alleles and *DEL* alleles) in D-negative mothers (*RHD*+, D−) may impede the analysis of cffDNA in maternal plasma or inaccurately predict the D status when such alleles are carried by fetuses. The frequency of *RHD*+, D− individuals has been estimated to vary between 0.2% and 2.1% in different ethnic groups (54-56). Several protocols considering the genetic background of the population under study have been described to reach high sensitivity and specificity in non-invasive fetal *RHD* detection (greater than 99.5%) (82).

Molecular characterization of D variants

Commercial anti-D serology reagents cannot reliably distinguish partial D and weak D phenotypes and identify which patients are at risk of anti-D development. Anti-D immunization in weak D patients is unusual, but some cases have been well documented in *weak D type 11*, *weak D type 15*, *weak D type 21*, *weak D type 41*, *weak D type 42*, *weak D type 45* and *weak D type 4.2* (also known as *DAR*) carriers (47,83-90). It has been reported that D variant patients carrying *weak D type 1*, *weak D type 2* or *weak D type 3* alleles do not produce anti-D (84). However, a few cases of anti-D in patients with weak D types 1, 2 and 3 phenotypes were described (91-99), being most of them not clinically significant autoantibodies. The consensus among immunohematologists is to consider that *weak D type 1*, *weak D type 2* or *weak D type 3* alleles produce a normal D antigen. In 2015, a work group in USA (100) recommended that molecular *RHD* characterization be implemented in the management of obstetric patients and potential recipients of a blood transfusion with a serologic weak D phenotype, as it was estimated that approximately 80% were *weak D type 1*, *weak D type 2* or *weak D type 3* carriers not at risk for alloimmunization and not RhIg candidates. Based on research published since 2015, they updated in 2020 (101) their previous recommendation to include *weak D type 4.0* and weak D type 4.1 carriers.

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In conclusion, considering the available high-quality evidence from observational studies, it can be assumed that patients with weak D antigen expression caused by *weak D type 1*, *weak D type 2*, *weak D type 3*, *weak D type 4.0* or *weak D type 4.1* alleles have a very low risk of developing clinically significant anti-D.

Partial D carriers may produce alloantibodies directed to D epitopes missing on their RBCs. Particularly, females of child-bearing potential should receive D-negative RBCs for transfusion and pregnant women must be given RhIg prophylaxis. However, antibody-based typing does not have the capacity to accurately identify partial D phenotypes. Transfusion services often adopt a behaviour aimed at protecting patients from alloimmunization to the D antigen and manage as D-negative all women of child-bearing age with variable or weak D typing. The molecular identification of *weak D type 1*, *weak D type 2*, *weak D type 3*, *weak D type 4.0* or *weak D type 4.1* carriers in D variant patients may allow managing them safely as D-positive and thus, rationalize the use of D-negative stock units, always in short supply, which could be reserved for patients who are really at risk for anti-D alloimmunization. Moreover, weak D genotyping in D variant pregnant women is a useful tool for guiding the anti-D immunoprophylaxis avoiding unnecessary administration. Molecular characterization of the variant D phenotype also benefits patients who are candidates for chronic transfusions since it would help to decide on adequate transfusion compatibility.

DEL in patients and donors

DEL individuals express a minimum amount of D antigen sites in the RBC membrane (<100 versus 10,000 to 33,000 sites on regular D-positive erythrocytes) (1) and are better diagnosed by *RHD* genotyping.

Asian-type DEL patients (the DEL phenotype produced by *RHD*DEL1*) are not prone to produce anti-D. It was reported that no Chinese Asian-type DEL women with a history of pregnancy had developed anti-D, however, this alloantibody was found in truly D-negative pregnant women (102). A recent clinical trial has shown that individuals with Asian-type DEL do not develop anti-D alloantibodies when exposed to D-positive RBCs after transfusion or pregnancy (103). On the contrary, DEL Caucasian individuals with anti-D have been documented, with one of the antibodies causing mild HDN (104,105). It has been suggested that the D antigen that is present in DEL phenotype may have a partial or complete expression. Studies based on adsorption-elution tests with monoclonal anti-Ds reagents have revealed that *RHD*DEL1* (the Asian-type DEL allele) encode a RhD protein that express all D epitopes (104). Similar findings have been found with *in vitro* expression studies (103). In contrast, *RHD*DEL8* and *RHD*11* (the most frequent Caucasian DEL alleles) lead to partial D antigens, lacking some D epitopes (104).

DEL management should be adapted to needs of the population (106,107). Due to the low frequency of the DEL phenotype among serologically D-negative Caucasian donors, alloimmunization caused by DEL red cell transfusions to D-negative recipients pose a very low risk. However, the *RHD* genotyping of D-negative donors at first donation is being performed in some blood services to screen for DEL (54,55,108,109).

RHD zygosity

Hemagglutination has limitations to predict *RHD* zygosity (*RHD* copy number) in D-positive individuals as it is deduced from haplotype frequency estimates in any given population (1). An accurate determination of *RHD* homozygous or *RHD* hemizygous status in fathers is of significance to provide genetic counselling to women alloimmunized with anti-D. *RHD* zygosity determination is also important in the study of Rh variants. The analysis of the *RHD* gene copy number can be carried out based on the molecular identification of a hybrid Rhesus box that is present in the *RHD* deleted chromosome or by quantitative polymerase chain reaction (PCR) strategies to determine *RHD* gene dosage (36,38,110-112). Both approaches are complementary and require proper controls. It should be considered that zygosity determination can be confounded by altered hybrid Rhesus boxes or by the presence of certain *RHD* allelic variants. The most accurate approaches for assessing *RHD* zygosity are likely to be a multiplex ligation-dependent probe amplification (MLPA) assay (113) and a quantitative multiplex polymerase chain reaction of short fluorescent fragments (QMPSF) method (114) that investigate the copy number of all exons of both the *RHD* and the *RHCE* genes.

Molecular matching

Patients with SCD are likely to develop clinically significant anti-C, anti-c or anti-e when compatibility tests are based on serology. Moreover, SCD patients sometimes make complex Rh antibodies that are difficult to identify.

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This problem arises mainly because African black ethnic groups have an increased incidence of allelic variants that encode partial Rh antigens which serologic reactivity is difficult to analyse. Additionally, altered *RHCE* are commonly linked to altered *RHD* encoding partial D, so serologically D-positive patients can also make anti-D. *RH* genotyping is very useful to aid antibody identification and transfusion decision-making in these patients. It has been demonstrated that molecular *RH* matching is one potential strategy to reduce alloimmunization in SCD patients needing long-term chronic transfusion support and improve red cell use (115-117).

Conclusions

The progress made in the technology of gene targeting has certainly provided invaluable information to expand our knowledge of the Rh system. A plethora of allelic variants has been thoroughly characterized and the association with Rh antigens, phenotypes and haplotypes has been established. Despite this progress, the biological functions of the Rh system are still under study.

A large amount of clinical data has been accumulated and was of value in defining the clinical importance of different *RH* alleles in the field of transfusion and obstetric medicine.

Classical haemagglutination is still a robust and straightforward technique with a specificity and sensitivity suitable for typing most patients and donors. Considering that DVI is the most common partial D found in Caucasians, anti-D reagents non nonreactive with partial DVI RBCs in direct tests are selected for typing transfusion receptors and pregnant women. Performing only a direct test avoids the risk of sensitization by classifying DVI as D-negative for transfusion and RhIg prophylaxis. On the other hand, detecting DVI and weak D expression is necessary in blood donors and infants of D-negative mothers at risk for D immunization. Anti-D reagents formulated for the antiglobulin phase (generally IgM/IgG blends) are selected to type this cohort.

Nevertheless, there are some clinical situations in which serologic techniques are not appropriate for determining the Rh phenotype accurately. Molecular methods can be applied to achieve a deeper analysis of the Rh system and overcome the limitations of serology. Both methodologies, in conjunction, are undoubtedly useful to prevent incompatibilities, avoid alloimmunization and haemolytic transfusion reactions, diagnose fetal Rh status, guide anti-D immunoprophylaxis, and optimize RBC survival in patients requiring chronic transfusions.

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