Review Article

Hybrids and microconversions in *RH* genes: investigation and implication in transfusion therapy

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Abstract: The discovery and characterization of the *RH* genes, i.e., *RHD* and *RHCE*, in the early 90s have been milestones in the field of blood group genetics. In addition to the single nucleotide variants (SNVs), which are commonly considered as the predominant molecular actors resulting in the variability of antigen expression, genetic exchange between both *RH* genes has been shown to be a frequent mutational mechanism. Indeed, dozens of hybrid alleles and microconversions associated with variant RH phenotype have been reported so far. Interestingly, these alleles display population-specific patterns of distribution. Also, they are directly involved in the qualitative and quantitative alteration of RH antigen expression, which has immediate consequences for diagnostics and transfusion management. Overall, hybrid alleles and microconversions contribute largely to the complexity of the RH blood group system. In the current state of knowledge, this narrative review: (I) defines and introduces the molecular determinants of the genetic exchange in the *RH* genes; (II) describes how these variant alleles may alter the expression of polymorphic and high-frequency antigens (HFAs) and/or result in the expression of low-frequency antigens (LFAs); (III) reports on their distribution at a low-resolution population scale; and (IV) discusses their relevance and identification in routine clinical practice for transfusion management at the laboratory level.

Keywords: Antigens; hybrid; microconversion; *RH* gene; variant

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Introduction

In the early nineties and more than 50 years after the identification of the D antigen, the molecular basis of the RH blood group system (ISBT #004) was deciphered. It did not take a long time thereafter before genetic variants associated with altered antigen expression were found in the genes. And still 30 years later, novel variations are reported on a regular basis in the scientific literature, notably by taking advantage of the contemporary technologies coming up in the market place, accounting for hundreds of simple and complex variant alleles. From a clinical point of view, expression of those variant alleles may result in variable expression of RH antigen(s) at both the quantitative and qualitative levels with potential clinical outcomes. In diagnostics, molecular immunohematology has become routine procedure in reference laboratories for predicting phenotype and guiding transfusion practice as a function of the risk of alloimmunization. The basic knowledge of *RH* molecular genetics is thus the key point of such a strategy.

In the *RH* genes, variant alleles mostly involve single nucleotide variant (SNVs) and/or structural variants (SVs), the latter basically altering large genomic sequences. A specific feature of *RH* molecular genetics is the transfer of genetic materials from one gene to another (= gene conversion), thereby creating 'hybrid genes' (that will be discussed in the next section). In this context, this review aims (I) to provide a global and nonexhaustive overview of this latter category of alleles from a genomic point of view, (II) to report their association with antigen expression, (III) to describe their frequency in various populations and, finally, (IV) to discuss their identification and relevance in daily practice in diagnostics and for the management of transfusion.

Molecular genetics of *RH* hybrid genes: a genomic perspective

The basics of RH molecular genetics

The paralogous *RHD* and *RHCE* genes involved in the RH blood group system were identified in the early nineties. Both genes are composed by ten exons and are separated by the *TMEM50A* gene within the *RH* locus on the short arm of human chromosome 1 (1-6). *RHD* is the duplicated product of the ancestral *RHCE* gene (7). *RHD* and *RHCE* encode the multipass transmembrane RhD and RhCE proteins, respectively. The former carries the D (or RH1) antigen, while the latter harbours the antithetical C/c (RH2/RH4) and E/e (RH3/RH5) antigens (8,9).

The generation of RH hybrid genes is directly linked to the structure of the RH locus

In 1994, soon after the description of the genes, Mouro and colleagues investigated the molecular basis of 'DVI category phenotype' in blood donors by Southern-blotting and reverse transcription-polymerase chain reaction (RT-PCR) analysis (10). For the first time, they described a gene conversion event encompassing exons 4, 5, and 6 within the *RHD* gene, i.e., *RHD-CE(4-6)-D*, which is currently referred to as the *DVI.2* (or *RHD*06.02*) allele. At the end of the year, the same team reported *RHCE-D-CE* hybrid alleles by probe hybridization and transcript analysis in samples presenting with the rare Dc– and DC^{W}– phenotypes (11). Both reports are milestones towards the identification and characterization of hybrid genes.

From a genomic point of view, homology, physical proximity and opposite orientation of the *RH* genes, as well as the homologous 5'- and 3'-*Rhesus boxes* at both sides of *RHD*, are actually critical and favourable factors supporting potential genetic rearrangements, such as unequal crossing-over—as illustrated by the deletion of the whole *RHD* locus generating the common D-negative *RHD*01N.01* allele –, and gene conversion (12). Typically, the latter mutational event involves the transfer of genetic materials from the 'donor' gene that replace the paralogous sequence within the 'recipient' gene (13). The novel alleles created by this mechanism are referred to as *RHD-CE-D* (from *RHCE* to *RHD*) and *RHCE-D-CE* (from *RHD* to *RHCE*) 'hybrid alleles'—or 'hybrid genes'—and exhibit both *RHD* and *RHCE* molecular specificity. The size of the genomic transfer is highly variable, ranging from the microconversion of a single nucleotide (e.g., *RHD*DFV*, **DCS2*, and **DUC2*) up to large genomic blocks, composed by exons and introns, spanning several dozen kilobases (e.g., *RHD*01N.03*, **01N.04*, and **01N.05*) (13-18).

The relationship between RH hybrid genes and antigen expression

Although the global genomic architecture of those variant alleles is not modified when compared to a 'normal' *RH* gene, i.e., 10 '*RH*' exons separated alternatively by nine '*RH*' introns, substitution of gene-specific coding DNA sequence by its counterpart changes the sequence of the translated 'hybrid' protein, notably by introducing missense variants from one gene to another, namely 'templated' variants. For example, variant alleles from the *DIVa* and *weak D type 4* clusters carrying the c.455A>C and c.667T>G microconversions in *RHD* (19,20), respectively, may be considered typically as 'hybrid alleles'. Change in the amino acid sequence has thereby the potential to alter quantitatively but also, and more importantly, qualitatively the expression of the polymorphic D antigen (if occurring at the *RHD* locus) or the C, c, E, and/or e antigens (if occurring at the *RHCE* locus). Indeed, those alterations directly disrupt the structure and/or the primary sequence of the protein product. The biosynthesized hybrid protein thus carries an incomplete number of epitopes, as revealed by the patterns of reaction obtained with various monoclonal antibodies (21-25), resulting in a so-called 'partial phenotype' (e.g., partial D, partial c, etc.). As an example, in the model of 30 epitopes defining the D antigen, the vast majority is lacking in DVI RBCs, while most of them are present in DVa RBCs (26,27). Consequently, from a clinical point of view, those individuals exhibiting a partial phenotype may produce an alloantibody if exposed to the corresponding normal antigen (= alloimmunization) [see (28) for a recent review on *RH* gene variants and antibody production]. The nature and specificity of alloantibodies have a direct impact on the severity of the hemolytic reaction that may occur potentially if exposed again to the same antigen(s) in a transfusion and/or obstetrical context(s).

Finally, hybrid proteins may not express high-frequency antigens (HFAs), which are thus absent from the RBC membrane, and/or create novel sequences and structural motifs carrying low-frequency (or rare) antigens (LFAs), as illustrated by the following examples throughout the paper. Overall, the study of the molecular structure of

hybrid genes and its related expression has gained much attention with an evident clinical interest, and has definitely contributed to the global understanding of the complex RH blood group system.

Expression of *RH* hybrid genes: an 'antigen' perspective

RH hybrid genes and variant phenotype

After the initial discoveries made by the French team, several other hybrid genes associated with the expression of the so-called DIIIc, DIVa, DIVb, DV and DFR partial phenotypes were characterized by the same group and others (29-31). These findings suggested that gene rearrangement is a redundant mutational mechanism resulting in RH variant phenotype and illustrated the growing complexity of *RH* molecular genetics at that time. Interestingly from a clinical point of view and for diagnostics, the resolution of hybrid variant alleles helped for defining the molecular basis of several LFAs, including Go^a (RH30), D^W (RH23), FPTT (RH50), RH32, and BARC (RH52) that are further discussed below (*Table 1*).

Table 1 Clinical relevance of antibodies directed against LFAs encoded by hybrid alleles and microconversions

Antigen	Clinical relevance (references)
VS (RH20)	Mild HDFN (32)
D^W (RH23)	Severe HDFN (33)
Go^a (RH30)	Severe HDFN (34-36), DHTR (37)
RH32	Severe HDFN (38-40)
Evans (RH37)	HDFN (41)
RH42	Mild HDFN (42)
STEM (RH49)	Mild HDFN (43)
DAK (RH54)	Severe HDFN (40)

No clinical significance has been yet recorded for antibodies directed against the RH33, RH43, FPTT (RH50), BARC (RH52) and CENR (RH56) LFAs. LFA, low-frequency antigen; HDFN, hemolytic disease of the fetus and newborn; DHTR, delayed hemolytic transfusion reaction.

DIV alleles and Go^a antigen expression

DIVa and DIVb RBCs can be distinguished by their differential expression of Go^a (44,45); the former being Go(a+), while the latter are Go(a–). Rouillac and colleagues proposed a tentative structure of the *RHD*DIVa* allele associated with Go^a antigen expression, consisting of two microconversions in exons 3 and 7, as well as an untemplated missense SNV in exon 2, resulting in three amino acid substitutions at the protein level: p.Asn152Thr, p.Asp350His and p.Leu62Phe, respectively (29). This allele has never been confirmed formally since, but a similar structure completed by the untemplated p.Ala137Val substitution, defining the likely *RHD*DIVa* (or **04.01*) allele presenting with the same phenotype, was later described (46) (*Figure 1*). In contrast, the *RHD*DIVb* (or **04.06*) allele expressed in the Go(a–) DIVb RBCs consists in a gene conversion from a partial exon 7 (from c.1048) up to exon 9, i.e., *RHD-CE(7:1048-9)-D*, accounting for a total of four templated missense changes in the hybrid protein (29).

Three additional *DIV* hybrid alleles have been characterized since (47-49), all of them being typed Go(a–) (*Figure 1*). On the basis of these observations, the templated p.Asp350His change in RhD appears to be required, but not sufficient, to the expression of Go^a (20).

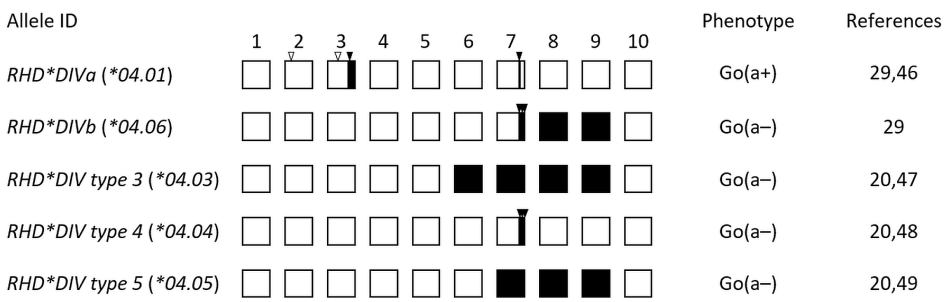

Figure 1 *DIV* hybrid alleles and Go^a (RH30) antigen expression. White box: *RHD*-specific exon/sequence; black box/bar: *RHCE*-specific exon/sequence; black arrowhead: templated missense SNV; white arrowhead: untemplated missense SNV; 1 to 10: *RH* exon numbering. Templated SNVs are indicated by a black arrowhead only when in hybrid exons. SNV, single nucleotide variant.

	RHD exon 5								Other variants	Phenotype	References
Nucleotide position	667	676	697	712	733	744	787	800			
Amino acid position	223	226	233	238	245	248	263	267			
Allele ID (ISBT)											
Reference (**01*)	Phe	**Ala**	Glu	Val	Val	Ser	Gly	Lys	-	D^W–	NP_057208.2
DV.1 (**05.01*)	Val	**Ala**	**Gln**	Val	Val	Ser	Gly	Lys	-	D^W+	(29,50,51)
DAU5 (**10.05*)	Val	**Ala**	**Gln**	Val	Val	Ser	Gly	Lys	Met379	D^W+	(16,18)
DV.8 (**05.08*)	Val	**Ala**	**Gln**	Met	Leu	Ser	Gly	Lys	-	D^W+	(50,51)
DV.2 (**05.02*)	Val	**Ala**	**Gln**	Met	Leu	Ser	Arg	Met	-	D^W+	(18,29)
DBS1 (**13.01*)	Val	Pro	**Gln**	Met	Leu	Ser	Arg	Met	-	D^W–	(53,54)
DV.10 (**05.10*)	Val	**Ala**	**Gln**	Met	Leu	Ser	Arg	Met	Ile306, Cys311	D^W+	(52)
DV.4 (**05.04*)	Phe	**Ala**	**Gln**	Val	Val	Ser	Gly	Lys	-	D^W+	(50,51)
DV.5 (**05.05*)	Phe	**Ala**	Lys	Val	Val	Ser	Gly	Lys	-	D^W–	(50,51)
DFV (**08.01*)	Val	**Ala**	Glu	Val	Val	Ser	Gly	Lys	-	D^W–	(15,18)

Figure 2 Variant alleles, amino acid sequences and relationship with D^W (RH23) antigen expression. Amino acids in grey font indicate variations from the reference (wild-type) RhD sequence (NP_057208.2), except Ser248 (synonymous variant) (15,16,18,29,50-54). Amino acids p.Ala226 and p.Gln233, which are thought to be critical for D^W (RH23) antigen expression, are bold underlined. ISBT, International Society of Blood Transfusion

DV alleles and D^W antigen expression

First, *RHD*DV.1* and **DV.2* (29), and later *RHD*DV.4* (50,51), **DV.8* (50,51), **DAU5* (16,18), and **DV.10* (52), which all involve p.Glu233Gln in all or part of *RHCE* exon 5 (*Figure 2*), were shown to express D^W (55). Interestingly, among *RHD*DV.4*, **DV.5*, and **DFV* that differ from the reference sequence by a single missense variant (i.e., p.Glu233Gln, p.Glu233Lys, and p.Phe223Val, respectively), only the former expresses D^W (15,18,50,51) (*Figure 2*). Also, *RHD*DV.2* and **DBS1* alleles both consist in a *RHD-CE(5)-D* hybrid gene, but differ by the single polymorphism encoding E and e, respectively, i.e., p.Ala226 and p.Pro226 (29,53,54). RBCs from these allele carriers are respectively D^W+ and D^W– (*Figure 2*). These results indicate that both p.Gln233, induced by a microconversion, and p.Ala226, which is specific to both *RHD* and *RHCE* carrying a '*e*' allele, are critical to the expression of D^W.

Hybrid alleles and FPTT antigen expression

Expression of FPTT in DFR RBCs was shown in 1988 (56,57). The year after, Rouillac and colleagues reported the structure of the *RHD*DFR1* (or **17.01*) allele, which consists in the conversion of a discrete region of *RHD* by the paralogous sequence of *RHCE* in exon 4, i.e., *RHD-CE(4:505-514)-D*, thereby introducing the three p.Met169Leu, p.Met170Arg, and p.Ile172Phe missense variants in RhD (29). Other *DFR* hybrid alleles have been reported since, all of them sharing the first two amino acid substitutions (58-60) (*Figure 3*).

FPTT-positive expression has also been reported in RBCs expressing the *DHAR* (or *ceHAR*) haplotype in the rare R_0^{Har} RBCs, and the *RHCE*CeVa* (or **02.04*) allele, which are both composed by a *RHCE-D(5)-CE* hybrid gene, but on a different allelic background (61,62) (*Figure 3*). *DHAR* haplotype is defined by the single aforementioned hybrid allele and, although composed by a single '*RHD*-like exon', expresses a weak partial D antigen with a very limited number of D epitopes in addition to normal c, weak e, and LFA RH33 (67-69). The *CeVa* allele encodes weak e and RH33, but also partial C (62). As this allele was found in a D-positive individual, it is unknown whether it encodes or not a partial D antigen (*Figure 3*).

Finally, more than 50 years ago, a report described an Ivorian woman presenting with the so-called DIV(C)– (or DIVa(C)–) phenotype, who experienced a fatal haemolytic disease of the fetus and newborn in her third child (70). Her RBCs were shown to be positive for D (partial), C (very weak), G (RH12), as well as HFAs RH29, Nou (RH44), and Dav (RH47), and LFAs Go^a, RH33, Riv (RH45), FPTT; but negative for E and e (56,70-73). The *DIVa(C)*– haplotype was resolved in 2012 by genomic DNA study and transcript

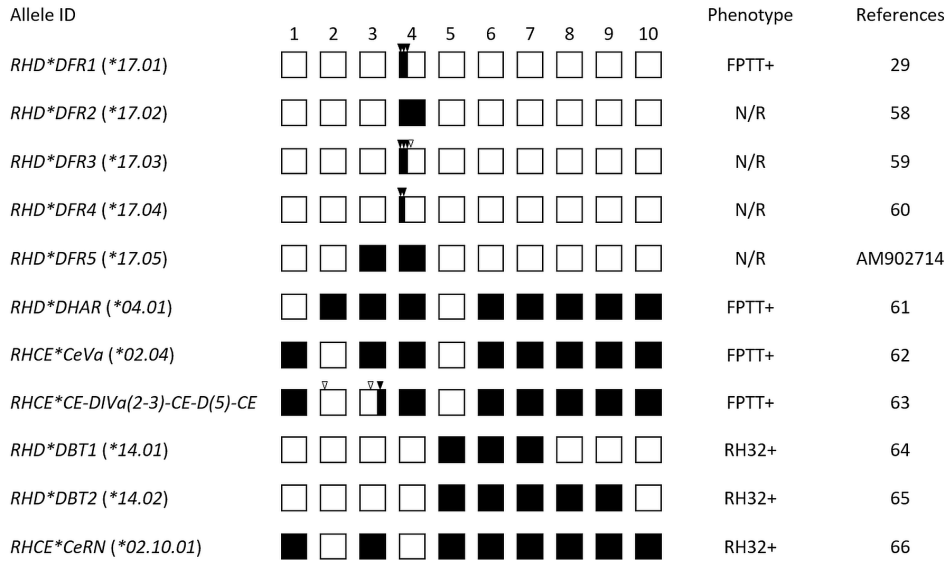

Figure 3 Hybrid alleles expressing FPTT (RH50) or RH32 antigen, and associated alleles (29,58-66). White box: *RHD*-specific exon/sequence; black box: *RHCE*-specific exon/sequence; black arrowhead: templated missense SNV; white arrowhead: untemplated missense SNV; 1 to 10: *RH* exon numbering. Templated SNVs are indicated by a black arrowhead only when in hybrid exons. In *RHCE* alleles, *RHCE*c* exon 1 and *RHCE*C* exon 2 are equivalent to *RHD* exons 1 and 2 (white box), respectively. N/R, not reported; SNV, single nucleotide variant.

analysis (63). It consists of the *RHD*DIVa* and complex *RHCE*CE-DIVa(2-3)-CE-D(5)-CE* hybrid alleles (*Figure 3*), the latter expressing RH33, Riv, and FPTT (63).

On the basis of the data in the literature, it is thought that amino acids p.Leu169, p.Arg170 and p.Phe172 encoded by exon 4 in *RHCE*, and p.Glu233 encoded by exon 5 in *RHD*, are required on a single 'hybrid protein' for FPTT antigen expression. It is important to mention that amino acids 169, 170, and 172 reside within the extracellular vestibule (74,75), in which substitutions have been extensively shown to be associated with partial D phenotype (18,19).

Hybrid alleles and RH32 antigen expression

Expression of LFA RH32 was reported in cells presenting with the variant R^N and partial DBT phenotypes (76-81). The molecular basis of R^N involves the *RHCE*Ce-D(4)-Ce* hybrid allele at the *RHCE* locus, namely *RHCE*02.10.01* (66) (*Figure 3*). Another R^N allele, i.e., *RHCE*02.10.02*, on the same background, but including the templated p.Thr152Asn missense variant encoded by *RHD* exon 3 (*RHCE*Ce-D(3:455-4)-Ce*), was described in the same study, but has never been reported in the literature since. In addition to RH32, R^N RBCs carry weak partial C and weak partial e antigens, and are positive for LFA DAK (RH54), but deficient for HFA Sec (RH46) (66,77,79). Two different *DBT* alleles altering the *RHD* locus have been described so far: *RHD*DBT1* (**14.01*) and **DBT2* (**14.02*), respectively structured as *RHD-CE(5-7)-D* and *RHD-CE(5-9)-D* (64,65).

Interestingly, the nature of amino acids critical for RH32 expression is assumed to be the mirror of FPTT. Indeed, p.Met169, p.Met170 and p.Ile172 encoded by exon 4 in *RHD*, and p.Gln233 encoded by exon 5 in *RHCE*, are required on a single 'hybrid protein' for RH32 antigen expression.

DVI alleles and BARC antigen expression

In 1989, Lomas and Mougey reported a differential expression of the BARC antigen in RBCs presenting with a DVI phenotype (82). Indeed, RH:2,-3,4,5 (or Ccee) and RH:-2,3,4,5 (or ccEe) DVI RBCs typed BARC-positive and -negative, respectively, suggesting heterogeneity in the molecular bases guiding the DVI phenotype (80,82). In 1997, the molecular structure of the gene in DVI RBCs was re-examined by Avent and colleagues (83), who characterized two different hybrid alleles: *RHD*DVI.1* (**06.01*), structured as *RHD-CE(4-5)-D*, with *RHCE* exon 5 specific of the '*E*' allele (i.e., encoding p.Pro226), was associated with BARC– DVI RBCs, as confirmed by

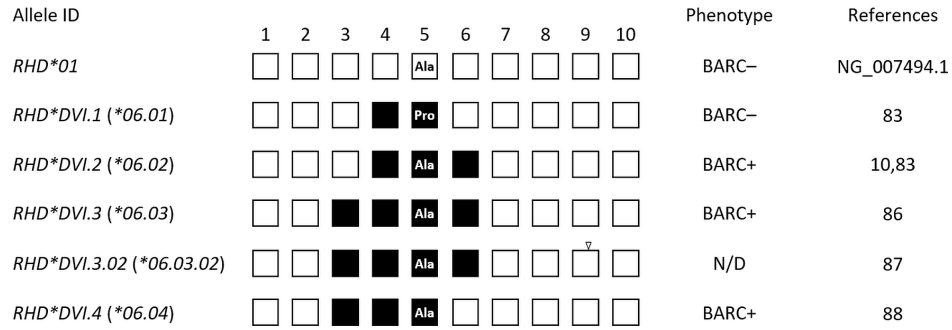

Figure 4 *DVI* hybrid alleles and BARC (RH52) antigen expression. White box: *RHD*-specific exon; black box: *RHCE*-specific exon; white arrowhead: untemplated missense SNV; 1 to 10: *RH* exon numbering. In exon 5, codon 226 is either Ala or Pro. SNV, single nucleotide variant.

Table 2 Examples of hybrid alleles and microconversions resulting in LFA expression and/or HFA deficiency

Phenotype[1]	Allele ID or designation	Molecular structure[2]	References
RH:42	*RHD*DIIIa-CEVS(4-7)-D* (**03N.01*)	*RHD(L62F,A137V,N152T)-CE(4-7)-D(G336C)*	(89
RH:54	*RHD*DIIIa* (**03.01*)	*RHD(L62F,A137V,N152T,T201R,F223V)*	(90-92)
RH:–18,–20	*RHCE*ceAR* (**01.04.01*)	*RHCE(W16C,M238V,L245V,R263G,M267K,I306V)*	(93,94)
RH:–18,49	*RHCE*ceBI* (**01.08*)	*RHCE(W16C,M238V,A273V,L378V)*	(15)
RH:43,–58	*RHCE*ceCF* (**01.20.06*)	*RHCE(W16C,Q233E,L245V)*	(95,96)
RH:–18,–34,–61	*RHCE*ceMO* (**01.07.01*)	*RHCE(W16C,V223F)*	(97,98)
RH:–18,49	*RHCE*ceSM* (**01.09*)	*RHCE(W16C,M238V,A273V)*	(99)
RH:20,–34	*RHCE*ceVS.01* (**01.20.01*)	*RHCE(L245V)*	(100,101)
RH:56	*RHCE*CeNR* (**02.08.02*)	*RHCE(Q41R)-D(6-10)*	(102)
RH:–46,54	*RHCE*CeRN* (**02.10.01*)	*RHCE-D(4)-CE*	(66,91)
RH:33	*RHCE*CeVa* (**02.04*)	*RHCE-D(5)-CE*	(62)
RH:37	N/A	*RHCE-D(2-6)-CE*	(14)

[1], phenotype regarding the expression of LFAs VS (RH20), RH33, Evans (RH37), RH42, Crawford (RH43), STEM (RH49), DAK (RH54), and CENR (RH56); and HFAs Hr (RH18), Hr^B (RH34), Sec (RH46), CELO (RH58), and RH61. [2], amino acid changes due to microconversions are underlined. LFA, low-frequency antigen; HFA, high-frequency antigen; N/A, not applicable.

independent studies (84,85); while *RHD*DVI.2* (**06.02*), structured as *RHD-CE(4-6)-D*, but with *RHCE* exon 5 specific of the '*e*' allele (i.e., encoding p.Ala226), as previously published (10), was found in BARC+ DVI RBCs. Three other *DVI* alleles, all encoding p.Ala226, have been formally described thereafter (*Figure 4*). On BARC typing, *RHD*DVI.3* (*RHD-CE(3-6)-D*) and **DVI.4* (*RHD-CE(3-5)-D*) were positive, while *RHD*DVI.3.02* (*RHD-CE(3-6)-D(A399T)*) was not investigated (86-88).

In addition to the templated missense SNVs brought in the hybrid alleles by the *RHCE*-specific sequences, including p.Leu169, p.Arg170 and p.Phe172, these observations reinforced the statement that p.Ala226 is critical to BARC antigen expression (83).

Other hybrid alleles, LFA expression and RH antigen deficiency

Many microconversions and/or conversions of large genomic regions in either *RHD* or *RHCE* variant alleles have been associated with the expression of several other LFAs, but also deficiency in HFA. A nonexhaustive list of such examples is provided in *Table 2*.

Also, it is important to remind that, when gene

Table 3 Hybrid alleles resulting in a D-negative phenotype

Allele ID or designation	Molecular structure	References
*RHD*03N.01*[1]	*RHD(L62F,A137V,N152T)-CE(4-7)-D(G336C)*	(101,103,104)
*RHD*01N.02*	*RHCE(1-9)-D*	(13)
*RHD*01N.03*	*RHD-CE(3-9)-D*	(13,105)
*RHD*01N.04*	*RHD(S68T)-CE(3-9)-D*	(17)
*RHD*01N.05*	*RHD-CE(3-7)-D*	(13,17)
*RHD*01N.06*[2]	*RHD-CE(4-7)-D(G336C)*	(89)
*RHD*01N.07*	*RHD-CE(4-7)-D*	(13,17,106)
RHD-CE(3-10)-D	*RHD-CE(3-10)*	(17,107)
*RHD*01EL.44*[3]	*RHD-CE(4-9)-D*	(108,109)

[1], *RHD*03N.01 = (C)ce*^{s} *type 1*. [2], *RHD*01N.06 = (C)ce*^{s} *type 2*. [3], *RHD*01EL.44*: initially reported as a *DEL* allele (108), but found in serologically D-negative, DEL-negative samples in India (109).

conversion involves large genomic regions, including those encoding all epitopes, hybrid genes may result in the complete abolishment of D or CcEe antigen expression if occurring at the *RHD* or *RHCE* locus, respectively. As soon as in 1994, Blunt and colleagues reported the identification of *RHD*-specific sequences in D– donors of African origin (103). The authors hypothesized the presence of a recombinant *RHD/CE* gene in the so-called '*dCe*^{s}*/dce*' genomes, which was later fully characterized and referred to as the *(C)ce*^{s} *type 1* (**03N.01*) allele (101,104). Several other D– hybrid alleles have been described since (*Table 3*).

Finally, hybrid genes in *RHCE* causing a complete deficiency in CcEe antigen expression have been less documented. However, beyond the alleles involved in the rare Dc– and DC^{W}– phenotypes discussed above (11), investigation of rare D-- individuals has contributed to the identification of several variant alleles, including *RHCE-D(3-7)-CE*, *RHCE-D(3-9)-CE*, *RHCE-D(4-9)-CE*, *RHD(1-9)-CE*, and *RHCE-D(2-6)-CE* [reviewed in (110)], the latter expressing the Evans (RH37) antigen (14). Identification and registration of the donors and patients deficient in CcEe antigens are definitely critical for the management of these precious resources for future transfusions.

Molecular epidemiology of RH hybrid genes: a population-specific perspective with variable outcomes

Although *RH* hybrid genes have been found and characterized in all populations, allele structure and frequency are typically population-dependent with high specificity. Numerous molecular epidemiology studies have contributed to document and enrich the catalogue of *RH* molecular genetic polymorphism.

The African situation

In 1997, Daniels *et al.* reported that, while the *RHD* gene was systematically absent in serologically D– White Europeans, *RHD*-specific sequences were found in as many as 22/25 D– Black South Africans blood donors (111). For the first time, it was shown that the molecular basis of the D– phenotype is highly dependent on the ethnicity of the population of interest.

As indicated above, variant alleles from the *DIVa* and *weak D type 4* clusters found in populations of African ancestry are basically hybrid alleles, including the D-negative *(C)ce*^{s} *type 1* allele in the former cluster. The nonfunctional *RHD* pseudogene in the latter cluster, known as *RHDψ* (**08N.01*) (112), also completes the definition. However, the D– phenotype induced by this allele is not directly due to the c.667T>G microconversion, but to a 37 bp-microduplication at intron 3/exon 4 junction and a putative premature stop codon, thus exhibiting a different mechanism resulting in a similar D– phenotype. Therefore, considering *RHDψ* as a 'D– hybrid allele' may be abusive in the strict sense. Importantly, it was initially described at various frequencies in serologically D– individuals of African ancestry: 54/82 (65.9%) in Black Africans from South Africa, Zimbabwe, and Ghana; 13/54 (24.1%) in African Americans; and 7/41 (17.1%) in mixed-race South Africans (112). In a preliminary study of 58 randomly

Table 4 *RHD* allele distribution in serologically D-negative donors of African ancestry

Country	Total number	*RHD* alleles (occurrence, %)				References
		*RHD*01N.01*[1]	*RHD*08N.01*[2]	Hybrids[3]	Others[4]	
South Africa/Ethiopia/Curaçao	112	78 (69.6)	15 (13.4)	17 (15.2)	2 (1.8)	(114)
Congo	110	59 (53.6)	35 (31.8)	16 (14.6)	0	(46)
France (African origin[5])	36	24 (66.7)	7 (19.4)	4 (11.1)	1 (2.8)	(115)

See the respective references for information about sample selection. [1], **01N.01/*01N.01* (**01N.01* homozygous). [2], **08N.01/*08N.01* or **08N.01/*01N.01* (**08N.01 = RHDψ*). [3], Including alleles from the *DIVa* (e.g., *(C)ce*^{s} *type 1*) and/or *weak D type 4* clusters in *trans* with **08N.01*. [4], Including SNVs. [5], Selected by their Fy(a–b–) phenotype.

selected blood donors from Mali, Wagner and colleagues identified *(C)ce*^{s} *type 1* and *RHDψ* in five and seven samples, respectively, by exon-specific PCR using sequence-specific primers (PCR-SSP) and PCR-restriction fragment length polymorphism (PCR-RFLP), confirming that those alleles are commonly found in the general population in Africa (113). By using different techniques, other reports have shown that hybrid alleles are found in ~10-15% of the serologically D– donors of African origin (*Table 4*), although the data currently available have mostly involved a limited number of samples.

But more importantly and as illustrated in the previous section, hybrid alleles are not confined to D-negativity, but have also been associated to a broad range of partial phenotypes. Molecular studies in D+ individuals from sub-Saharan areas have described the remarkable complexity and heterogeneity of both *RH* genes in Africans, including the prevalence of hybrid alleles. By allele-specific primer-PCR (ASP-PCR) and sequencing approaches, Touinssi and colleagues identified several partial *RHD* alleles involving gene conversions from the *DIVa*, *DAU* and *weak D type 4* clusters in 8/40 D+ samples from the Teke ethnic group in Central Congo (46). Granier and collaborators reported comprehensively the variability of both the *RHD* and *RHCE* genes in a total of 347 pygmoid and nonpygmoid individuals from sub-Saharan populations, including Western, Central, and Eastern Africa (116). At the *RHD* locus, *RHD*weak D type 4.2.0* (**DAR1.00*) and **DIIIa* (**03.01*) were found to be the most common partial *D-CE-D* hybrid alleles in 41 (11.8%) and 23 (6.6%) samples, respectively. In *RHCE*, the total number of alleles that may be considered as 'hybrid' was very high, most of them involving microconversions. Beyond the 'typical' African *RHCE*ce(48C)* (**01.01*), *RHCE*ce(733G)* (**01.20.01*), and *RHCE*ce(48C,733G)* (**01.20.02*) alleles found in a total of 230 samples (66.3%), the most prevalent hybrid *CE* allele was *RHCE*ceAR* (**01.04.01*) observed in as many as 57 individuals (16.4%), followed by *RHCE*ceTI* (**01.02.01*; 5.2%), *RHCE*ceMO* (**01.07.01*; 4.0%), and *RHCE*ce*^{s} (**01.20.03*; 3.7%) (*Figure 5*). Logically, those four later alleles, which encode partial antigens, have been frequently linked to *RHD*DAR*, **DIVa*, **DAU0*, as well as **DIIIa* and *(C)ce*^{s}, respectively (93,94,98,104,116,117). Another study in 46 Fulani individuals from Mali carried out by the same group pointed out the high prevalence of the partial R^{N} allele (allele frequency: 0.195) (118), which is known to be clinically relevant. Finally, it is worth mentioning that a hybrid *RHCE*ce-D(9)-ce* allele has been found to be *cis*-associated with the D-negative *RHDψ* allele systematically (119,120). It is still unknown whether or not this allele is clinically relevant.

These studies in various regions of sub-Saharan Africa have been of the greatest interest, but have been definitely too rare. It will be critical to investigate systematically more countries and populations in a near future that will undoubtedly result in very valuable findings. So far, the heterogeneous *RH* gene variability in the African population has been mostly addressed in large-scale studies performed 'outside' Africa. For example, in 806 individuals presenting with an altered RhCE antigen expression or producing anti-RhCE in France, ~80% being of African ancestry, the most common variant genotypes were R^{N}/R^{N} in 71 samples (8.8%), followed by *ceMO/ceMO* (n=15), *(C)ce*^{s} *type 1/ceAR* (14), R^{N}*/ceMO* (13), *(C)ce*^{s} *type 1/(C)ce*^{s} *type 1* (10), and *ceAR/ceAR* (7), as well as by many other alleles and combinations with a lower frequency (121). Several other studies have documented the complexity in *RHD* and/or *RHCE*, as well as the prevalence of hybrid genes, in populations of African ancestry in the USA, France, or Brazil, including in patients with sickle cell disease (SCD) (115,122-131), a clinically-relevant category of patients who are particularly prone to

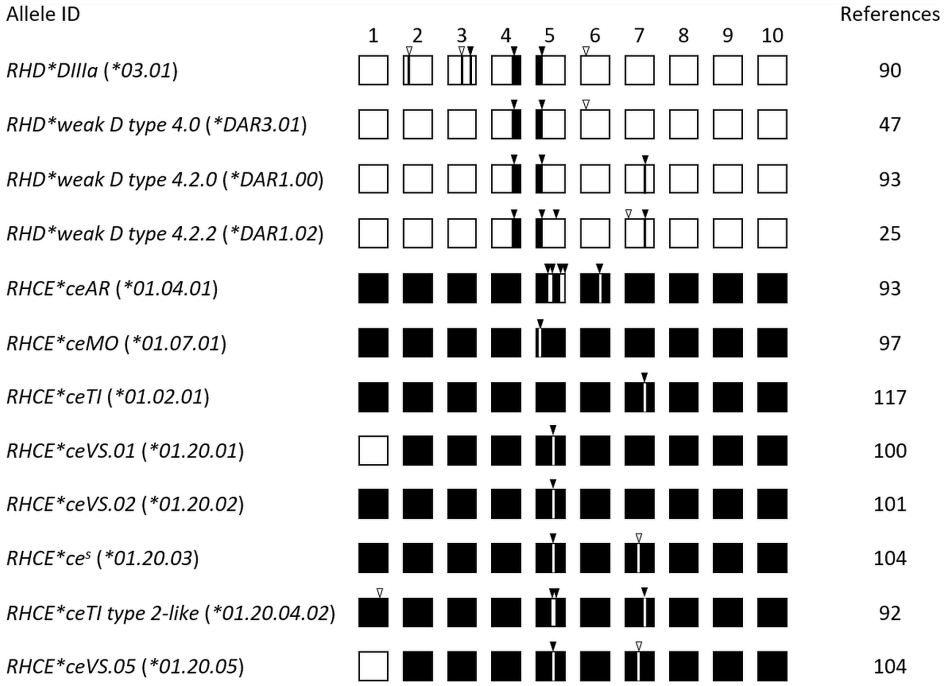

Figure 5 Examples of *RH* hybrid alleles in sub-Saharan and Northern African populations. White box: *RHD*-specific exon/sequence; black box/bar: *RHCE*-specific exon/sequence; black arrowhead: templated SNV; white arrowhead: untemplated SNV; black/white line: missense SNV; 1 to 10: *RH* exon numbering. In *RHCE* alleles, *RHCE*c* exon 1 and *RHCE*C* exon 2 are equivalent to *RHD* exons 1 and 2 (white box), respectively. N/R, not reported; SNV, single nucleotide variant.

RH alloimmunization.

Beside the population studies, another line of evidence in favour of the higher frequency of templated SNVs, which are markers for hybrid alleles, in the African population, comes from both reference human genomic and blood group gene databases, such as gnomAD (132), Erythrogene (133), Blood Antigens and Blood Group Database (134). Although the data are not finely organized at a high resolution as a function of ethnicity/geography, they strongly support the preferential association of some SNVs with African ancestry. For example, allele frequency of c.667T>G (rs1053356) in *RHD* and c.733C>G (rs1053361) in *RHCE*, which are both associated with several hybrid alleles, is 0.1062 and 0.2303 in the latter population, respectively, while only 0.0010 and 0.0024 in non-Finnish Europeans (gnomAD v3.1.1), thus highlighting the variable distribution of those changes as a function of populations.

As expected, the distribution of hybrid alleles in North Africa differs significantly from sub-Saharan Africa. In Tunisia, *D-CE-D* hybrid alleles, including the clinically-relevant *(C)ce*^{s} haplotype(s), have been reported in 0.8-2.5% of serologically D– donors (135-139), and in as much as 23.0% of the subpopulation expressing C and/or E (140). Molecular typing by various methods has identified *RHD*weak D type 4.0* as the most common variant *RHD* allele by far. Individuals carrying this allele were found in 34/2000 (1.7%) Tunisian and 11/4458 (0.25%) Moroccan donors with variant D phenotype (137,141,142). *RHD*DBT1* and **DOL1* were reported once each (134,140). Investigation of *RHCE* has been rare in this subset of population. However, the partial *RHCE*ceTI type 2-like*, **ceVS.01*, **ceVS.05*, **ceMO* and **R*^{N} hybrid alleles were identified in Moroccans (142).

The Caucasian situation

As well known in the molecular immunohematology laboratories and early illustrated by the investigation of serologically D– donors (111), but also in (variant) D+ donors, variability in *RH* genes is much lower in Caucasians than in Africans.

The most common partial D phenotype in Caucasians is DVI. Its prevalence in European populations, including in the Netherlands, UK, USA, Australia, Germany and

Austria (143-149), which was determined by serological studies using monoclonal antibodies before the molecular bases of DVI phenotype were defined, has been known to be 0.02–0.05%. The five hybrid alleles resulting in DVI were initially identified in Caucasians, as indicated above. Molecular studies have shown a various distribution of allele frequency in different European regions. For example, *RHD*DVI.1* and **DVI.2* are more prevalent in Germany, Austria, and Belgium, although with interregional variations (86,87,149), while **DVI.4* is the most common in Spain (88). Definitely many other clinically-relevant hybrid alleles at both the *RHD* and *RHCE* loci have been identified, characterized and reported in Caucasians, but to a much lower extent than *DVI*.

In 1997, Avent and collaborators investigated the molecular basis of serologically D–individuals from UK (150), which typically account for ~15% of the whole population in Europeans (151). By using a simple multiplex PCR approach targeting the *RHD* gene in this subgroup of individuals, they showed that 7 and 11 out of the 85 samples typed C+ and/or E+ (C/E+) were positive for intron 4 and exon 10 amplifications, respectively, whereas all 55 C–E– samples were negative. While the genotypes were not fully characterized at that time, the results clearly suggested that (I) D– phenotype is not strictly due to the homozygous deletion of the *RHD* gene in Caucasians and, interestingly, (II) nonfunctional hybrid alleles at the *RHD* locus are preferentially carried by individuals expressing C and/or E antigen(s). The later statement was subsequently confirmed in large-scale studies carried out in Central Europe. Indeed, *D-CE-D* hybrid allele frequency in serologically D– C/E+ Caucasians, including various large genomic conversions (*Table 3*), was estimated to be 0.006-0.018 (13,17,152).

The Asian situation

In East Asia, D+ phenotype is dramatically prevalent, typically >99% (151). In the remaining serologically D– individuals, Okuda and colleagues demonstrated that specific regions of the *RHD* gene were amplified by PCR in ~30% of Japanese donors, suggesting the presence of *RHD* variant alleles (153). Further investigations of such subsets in South Korea, China, Taiwan, and Japan, have identified hybrid alleles with gross rearrangements in ~8% (range, 2.8–16.6%), with a strong positive bias towards C/E+ individuals (153-165). *RHD*01N.03* is usually the most common hybrid allele resulting in the so-called 'true' D– phenotype except in Japan, where *RHD*01N.04* is more prevalent (*Table 3*). In the course of comprehensive large-scale and case studies, many hybrid alleles exhibiting a partial D phenotype have been reported, including *RHD*DIV*, **DV*, **DVI*, **DFR*, **DBS*, **DCS*, **DBT*, and **DLX* (50,51,65,160,161,164,166-174). Among these, alleles from the *DV* and *DVI* (mainly *RHD*06.03*) categories appear to be the most common, although at a very low frequency, typically $<10^{-5}$ (160,161,166,172,174).

Beside the numerous studies carried out in East Asia, comprehensive investigations in other Asian countries have been limited so far. In Thailand (Southeast Asia), where individuals presenting with a 'non-D+' phenotype are also <1% (D–: 0.30%; weak D: 0.01%) (175), a distribution comparable to that observed in East Asia has been found. In brief, ~11% of the serologically D– samples carry a hybrid gene, *RHD*01N.03* being the most common; while *RHD*06.03* is harboured by 24% of the weak/partial D donors in the Bangkok area, and even up to 37% at the nation-wide level (176,177). Finally, in India, where 3–7% of the whole population types D–, *RHD*01N.03* is more frequent in C/E+ donors (29/171, 17.0%), followed by **01N.05* (8.8%) and **01EL.44* (2.9%) (109). Hybrid alleles appear to be rare and private in weak/partial D samples (178).

Analysis of the *RHCE* locus has not been carried out at a large-scale level so far, notably as variability in antigens carried by the RhCE protein and the related risk of alloimmunization is globally limited in Asia. Nonetheless some hybrid alleles have been reported, such as *RHCE*D(1-3)-cE* (**03.02*) and *RHCE*cE-D(5*:*697-712)-cE* (**03.03*), which both encode a partial E antigen (179,180), as well as other negative *RHCE* alleles identified in rare D-- individuals (110).

RH hybrid genes in daily practice: a clinical perspective

The key factors for an efficient molecular typing strategy

Transfusion medicine has been known as the pioneering discipline applying a personalized approach, which is logically based on both donor and patient 'features', herein blood group antigens carried by RBCs, and how they 'match' together. Therefore, efficient typing strategies are critical to this achievement. In immunohematology, the gold-standard for typing has remained serological testing. However, low- to medium-, and even high-throughput molecular approaches have been extensively implemented in laboratories as valuable complementary tools to gain

into accurate prediction of the phenotype and optimization of patient safety, which is the ultimate goal (181-184). As illustrated above, hybrids and microconversions in *RH* genes are multiple, polymorphic, population-specific and confer a broad range of RH phenotypes with variable clinical interests. Therefore, the extent to which molecular testing is required as a complementary approach, as well as the strategy to be used thus basically depends on the status of the individual (donor or patient), his/her phenotype, ethnicity (African *vs.* Caucasian *vs.* Asian *vs.* admixed), as well as pathological status potentially.

Research and diagnostics in transfusion medicine

Since the identification of the *RH* genes, various approaches taking advantage of the successive technological advances in the fields of genetics and genomics have been designed and experienced. The simple PCR-SSP strategy, declined in both simplex and multiplex formats, followed by an agarose gel electrophoresis analysis, has been recognized for years as a simple and convenient approach to screen for the presence of specific *RH* segments in both transfusion and obstetrics, but also to genotype several hybrid alleles (86,185-195). As an example, identification of those clinically-relevant hybrid alleles in DVI individuals, who were proposed to be considered as 'D-positive donor, D-negative recipient' in as early as 1995 (26), was thus rapidly implemented in several specialized laboratories. Since the nineties, many low- to medium-throughput analytical methods, as well as commercial approaches, including genotyping platforms using PCR-based assays coupled with various detection systems for data acquisition, have been developed and shown to be robust to this purpose (183,196).

A significant advance in hybrid identification for the past ten years has been the implementation of strategies dedicated to the quantification of exon copy number variations (CNVs), namely the (commercial) multiplex ligation-dependent probe amplification (MLPA) (119) and the (in-house) quantitative multiplex PCR of short fluorescent fragments (QMPSF) (120), currently used in several laboratories (personal communications). Indeed, both cost-effective approaches have proven valuable and potent for the characterization of complex genotypes and alleles, notably in compound heterozygous conditions that had remained unresolved by conventional methods, such as PCR-SSP, Sanger sequencing and commercial microarrays (109,142,164,176-178).

Finally, for the very past years, the strategies that have shown great promise for blood group genotyping have been high-density DNA arrays and next-generation sequencing (NGS). Although the former has not formally proven yet its efficiency for resolving complex cases in RH to my knowledge, the latter has been used to investigate *RHD* zygosity initially, but also and more importantly for the identification of those hybrids in the *RH* genes. Indeed, since the preliminary NGS studies in the blood group field, which aimed to identify SNVs primarily (197-198), many investigators have addressed successfully the CNVs by using different platforms, as well as various strategies to generate [e.g., whole-genome sequencing (WGS), whole-exome sequencing (WES), and targeted-exome sequencing (TES)] and process the data (read depth analysis, automated software…) (128,131,134,199-205).

The challenge of patients with sickle cell disease (SCD)

Sickle cell disease (SCD, OMIM #603903) is a genetic blood disorder caused by variations in *HBB*, the gene encoding the β-globin polypeptide, a subunit of the oligomeric haemoglobin protein (206). RBC transfusion has been a common therapeutic strategy for the management of acute and chronic symptoms of the disease (207). Preventing alloimmunization in these multitransfused patients is thus a major critical challenge to guarantee their safety in future transfusions.

Patients with SCD are of African descent while, in Western countries, blood donors are mostly of European ancestry. This discrepancy in the respective origin of donors and patients illustrated by the differential qualitative and quantitative distributions of antigens between the individuals is critical (208). It has been well documented that patients with SCD, who are thus prone to be exposed regularly to foreign (donor) antigens, are far more at risk to alloimmunization following transfusion, but also to delayed hemolytic transfusion reaction (DHTR), than any other patients (209). In the RH system, antibodies involved in DHTR are frequently directed against C and E (~40%), which are less common in Africans (210).

Although there is no international consensus among the transfusion community, the strategy for transfusing patients with SCD that initially relied on the delivery of ABO/D-matched RBC units determined by serological testing progressively evolved towards extended matching for C, E, and K antigens (the latter from the Kell blood group system), which is standard practice currently, and for other antigens from the MNS (S), Duffy (Fy^a, Fy^b), and

Kidd (Jk^a, Jk^b) blood group systems when possible. This trend towards extended RBC antigen-matching has helped to decrease dramatically the rate of alloimmunization in patients with SCD: from 18–75% with ABO/D only to 5–24% when extended to C/E/K, and up to 0–7% for optimal matching with additional antigens (211-213). Introduction of DNA-based approach for blood group genotyping has further contributed to facilitate transfusion support with extended antigen-matched blood, notably by predicting those RBC antigens that cannot be investigated by serological testing (214-219).

Even though RH antigen-matching has been carried out for the most common antigens, Chou and collaborators showed that production of RH alloantibodies occurred in 80/182 patients with SCD transfused primarily with RBC units from African American donors (123). Among the 146 antibodies found in the cohort, unexplained RH specificity was demonstrated for 91, including against D, C, E, and e, but also the Go^a, V/VS, RH32, and C^W LFAs, which are known to be due to *RH* variant alleles (see above). Upon molecular investigation of 226 patients with SCD in the *RH* genes, the authors showed a great allele diversity in both genes: 217 (48.0%) and 259 (57.3%) were found to be *RHD* and *RHCE* variant alleles, respectively, of which the majority involves hybrids alleles or microconversions, as previously reported in individuals of African descent (15,121,122,220,221). Importantly from a clinical point of view, those variant alleles are mostly associated with a partial antigen and/or LFA expression and/or HFA deficiency. Overall, this study elegantly confirmed the challenge of supplying RH antigen-matched RBC units to patients with SCD, even with donors originating from the same community. It also suggested to carry out *RH* genotyping in donors, additionally to patients, to improve identification of antigen-negative RBC units, thus increasing compatibility by molecular matching and reducing the risk of alloimmunization ultimately. The conclusions of this study were later supported by others (126,128,130). The latter strategy was further investigated in different laboratories, notably in the USA and Brazil, where the supply of compatible RBCs to patients with SCD is particularly challenging. All authors concluded invariably on the relevance and superior benefit of the prophylactic *RH* molecular matching over serological matching to optimize resources and delivery of compatible RBC units to patients with SCD, and recommended to promote blood donation in individuals of African descents (125,127,129,222-227). Implementation of this strategy routinely at a large-scale level, which requires high-throughput genotyping tools accompanied by bioinformatics resources and medical expertise, still remains a major challenge to achieve.

Conclusions

Since its discovery in the 1940s, the RH blood group system has been the matter of thousands of studies guided by the curiosity of clinicians, immunohematologists, biochemists, and latter geneticists and molecular biologists, as a fascinating example of transdisciplinary efforts towards its knowledge. *RH* molecular genetics is a complex field. Hybrid alleles and microconversions, not only from a genetic point of view, but also as the sources of the RH phenotypic variability through the expression of variable antigens and their clinical consequences, perfectly illustrate that complexity. Since the discovery of the *RH* genes 30 years ago, the successive advances in genetics and genomics, which have had a major impact in research and/or diagnostics, have contributed considerably to document the molecular repertoire at the origin of the variant phenotypes (184,228). Although most of the *RH* variant alleles can be resolved by the current tools nowadays, there is no doubt that other methods, including the third-generation (or long-read) sequencing technology that is becoming more and more available and attractive, will be key actors of future discoveries. At the population level, while numerous studies have been carried out, there is likely still much to learn in terms of *RH* molecular epidemiology in Africa, Asia, and many more isolated populations, but also many specific variant alleles to discover. Overall, while the current number of hybrid genes and microconversions has considerably increased since the characterization of the molecular basis of DVI category phenotype in 1994 (10), the catalogue is not completed yet.

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Footnote

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