First investigation of *RH* gene polymorphism in patients with sickle cell disease and associated blood donors in Cameroon, Central Africa

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Background - Although genetic polymorphism of the RH blood group system is well known in sub-Saharan Africa, national/regional specificities still remain to be described precisely. For the first time in Cameroon, Central Africa, and in order to better characterize the molecular basis driving RH phenotype variability, as well as to identify the main antigens that may be potentially responsible for alloimmunization, we sought 1) to study the *RH* genes in a cohort of 109 patients with sickle cell disease; 2) to study the same genes in the corresponding donors whose red blood cells (RBCs) were transfused to the patients (108 donors in 98 patients); 3) to predict RH phenotype on the basis of the molecular data and compare the results with serologic testing; and 4) to identify retrospectively patients at risk for alloimmunization.

Materials and methods - In order to generate an exhaustive dataset, the *RH* genes of all patient and donor samples were systematically investigated 1) by quantitative multiplex PCR of short fluorescent fragments (QMPSF) for characterization of *RHD* gene zygosity and potential structural variants (SVs), and 2) by Sanger sequencing for identification of single nucleotide variants (SNVs). Subsequent to molecular analysis, the genotypes and RH phenotype were deduced and predicted, respectively, from reference databases.

Results - In a total of 217 Cameroonian individuals, as many as 24 and up to 22 variant alleles were identified in the *RHD* and *RHCE* genes, respectively, in addition to the reference alleles. Interestingly, 65 patients with SCD (66.3%) were assumed to be exposed to one or more undesirable RH antigen(s) with varying degrees of clinical relevance.

Discussion - Beyond the comprehensive report of the nature and distribution of *RH* variant alleles in a subset of Cameroonian patients treated by transfusion therapy, this work highlights the need for an extensive review of current practice, including routine serologic typing procedures, preferably in the near future.

Keywords: *epidemiology, genetics, RH antigen, sickle cell disease, variant.*

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INTRODUCTION

Sickle cell disease (SCD; MIM #603903) is a genetic disorder mostly encountered in populations of sub-Saharan African origin caused by variants within the *HBB* gene

resulting in abnormal hemoglobin function[1-3]. This hemoglobinopathy is typically characterized by red blood cells (RBCs) presenting with a so-called "sickle cell" shape[4]. Patients with SCD suffer from variable clinical symptoms, including anemia, vaso-occlusive pain and increased susceptibility to infections, which can be potentially life-threatening if severe complications occur. While there is still no cure available, the anemia and brain vessel occlusion may be prevented by long-term RBC transfusion, a major approach to patient management which has helped to dramatically improve quality of life[5,6]. However, an adverse effect of RBC transfusion therapy is the risk of alloimmunization, which has been demonstrated to be dependent on multiple factors, such as gender/age, inflammatory status, cellular antigen expression and total number of RBC transfusions in the patient; another factor is the extent of genetic diversity between donor and patient[6-11]. In fact, multi-transfused patients are potentially exposed to a broad range of RBC antigens expressed by various donors, and transfusion support may be seriously compromised in alloimmunized patients. To reduce the risk of alloimmunization, routine serologic matching for ABO, RH (D, C/c, E/e) and Kell (K) antigens has been recommended for RBC transfusion in clinical practice[12,13]. However, the rate of alloimmunization against RH antigens in African patients has remained significantly high due to the diversity of variant alleles frequently encountered[14-17], and *RH* genotyping in patients and donors has been proposed as a way of identifying those variant alleles and then further reducing alloimmunization by genotype-matching[14,17-25]. It is therefore crucial to acquire a basic knowledge of gene polymorphism in populations of interest.

RH is the most complex and polymorphic blood group system. It currently includes 56 antigens carried by the RhD and RhCE proteins at the surface of RBCs. Since the discovery of the molecular bases driving RH antigen expression in the early nineties[26-29], the population originating from sub-Saharan Africa has been known to be the most diverse in the homologous *RH* genes both in terms of nature and of frequency, as confirmed in several reports[30-35]. However, while these studies are definitely of great value, they have mostly focused on populations with heterogeneous African origins, and/or are limited in number. This observation suggests that knowledge about the molecular determinants resulting in RH variability at the national/regional level in sub-Saharan Africa is still poor, and needs to be investigated in an effort 1) to better characterize the fundamental *RH* gene polymorphism at a high resolution in Africa, 2) to define more precisely the epidemiology of RH antigen expression, and 3) ultimately to improve global transfusion settings in an African context.

In Cameroon, a Central African country of more than 28 million inhabitants, individuals presenting with sickle cell trait are estimated to account for ~20% of the whole population with regional/ethnic differences, and the prevalence of the disease is ~1% at birth[36]. So far, *RH* gene polymorphism has never been investigated in the Cameroonian population, including patients with SCD. In this context, and in parallel with routine serologic testing, we decided 1) to investigate for the first time the *RH* genes specifically in a subset of patients with SCD, as well as their corresponding donors; 2) to report the nature and frequency of the variant alleles; 3) to predict RH antigen expression, including partial, high-frequency (HFA) and low-frequency (LFA) antigens; and 4) to describe the potential incompatibilities related to RH antigen exposure.

MATERIALS AND METHODS

Ethics

This study was approved by the Institutional Ethics Committee "*Comité d'Ethique Institutionnel de la Recherche pour la Santé Humaine*" under the reference N°2019/0840/CEIRSH/ESS/DR and the National Research Ethics committee for Human Health (CNERSH) under the reference N°2019/12/1200/CE/CNERSH/SP. The research was conducted ethically, with all study procedures being performed in accordance with the requirements of the World Medical Association's Declaration of Helsinki. Written informed consent was obtained from each donor/patient for study participation and data publication.

Patients with sickle cell disease (SCD) and blood donors

The study was conducted in the cosmopolitan city of Yaoundé, which welcomes people from all ten regions of the country, and involved a total of 109 unrelated patients with SCD. In addition, *RH* genes were investigated in a total of 108 blood donors whose red blood cell units were transfused to 98 patients with SCD.

Table I - *Rare RHD SNVs in 109 Cameroonian patients with SCD and frequency*

Exon	SNV	dbSNP ID	AA change	Occurence	Frequency (%)	MAF[1]
2	c.186G>T	rs199509194	p.Leu62Phe	15	6.88	0.06668
3	c.410C>T	rs113982491	p.Ala137Val	14	6.42	0.06600
	c.455A>C	rs17418085	p.Asn152Thr	14	6.42	0.06097
4	c.505A>C	rs17421137	p.Met169Leu	11	5.05	0.00007
	c.509T>G	rs17421144	p.Met170Arg	11	5.05	0.00006
	c.514A>T	rs17421151	p.Ile172Phe	11	5.05	0
	c.541C>T	rs139508538	p.Leu181=	1	0.16	0.00246
	c.544T>A	rs17421158	p.Ser182Thr	11	5.05	0.00004
	c.577G>A	rs1053352	p.Glu193Lys	11	5.05	0.00013
	c.579G>A	rs77813628	p.Glu193=	3	1.38	0.01392
	c.594A>T	rs569974439	p.Lys198Asn	11	5.05	0.00017
	c.602C>G	rs1053355	p.Thr201Arg	22	10.09	0.04878
	c.609G>A	rs114032679	p.Thr203=	5	2.29	0.04299
	c.611T>A	rs2124674196	p.Ile204Lys	1	0.46	N/R
5	c.654G>C	rs141540728	p.Met218Ile	5	2.29	0.04161
	c.667T>G	rs1053356	p.Phe223Val	27	12.39	0.10780
	c.674C>T	rs148014996	p.Ser225Phe	5	2.29	0.04266
	c.697G>C	rs1053359	p.Glu233Gln	11	5.05	0.01448
	c.712G>A	rs1053360	p.Val238Met	11	5.05	0.00034
	c.744C>T	rs1053362	p.Ser248=	12	5.50	0.01092
	c.787G>A	rs3118454	p.Gly263Arg	11	5.05	0
	c.800A>T	rs112907722	p.Lys267Met	11	5.05	0.00004
6	c.807T>G	rs141833592	p.Tyr269*	5	2.29	0.04126
	c.819G>A	rs150606530	p.Ala273=	7	3.21	0.03573
	c.835G>A	rs139704879	p.Val279Met	4	1.83	0.02509
	c.916G>A	rs590813	p.Val306Ile	12	5.50	0.00296
	c.932A>G	rs590787	p.Tyr311Cys	11	5.05	0.00004
7	c.941G>T	rs200762372	p.Gly314Val	13	5.96	0.00077
	c.957G>A	rs146292192	p.Val319=	1	0.46	0.00687
	c.968C>A	rs200415166	p.Pro323His	13	5.96	0.00004
	c.974G>T	rs377051051	p.Ser325Ile	13	5.96	N/R
	c.979A>G	rs780001468	p.Ile327Val	13	5.96	0.00004
	c.985_986delinsCA	N/R	p.Gly329His	13	5.96	N/R
	c.989A>C	rs751746562	p.Tyr330Ser	13	5.96	0.00013
	c.992A>T	rs755177266	p.Asn331Ile	13	5.96	0.00013
	c.998G>A	rs144996388	p.Ser333Asn	2	0.92	0.00068
	c.1006G>T	N/R	p.Gly336Cys	11	5.05	N/R
	c.1025T>C	rs138235491	p.Ile342Thr	14	6.42	0.00736
	c.1048G>C	rs41307826	p.Asp350His	14	6.42	0.01432
	c.1053C>T	rs41300142	p.Thr351=	13	5.96	0.00098
	c.1057_1059delinsTGG	rs200307239/rs1468536305	p.Gly353Trp	13	5.96	0/0.00079
	c.1060_1061delinsAA	rs753613761/rs756982993	p.Ala354Asn	13	5.96	0/0.00077
8	c.1136C>T	rs61740966	p.Thr379Met	52	23.85	0.69788

[1]Minor allele frequency from the Genome Aggregation Database (gnomAD v2.1.1; African/African American subpopulation)[48]; N/R: not reported in gnomAD v2.1.1.

Routine serological testing

The reactivity of D (RH1), C (RH2), E (RH3), c (RH4), and e (RH5) antigens was analyzed by agglutination in a gel matrix technique using two types of cards in conditions recommended by the manufacturer. D antigen expression was determined by the Diaclon ABO/D ID-Card (Bio-Rad Laboratories, Hercules, CA, USA) containing two monoclonal antibodies: anti-DVI– ([LHM59/20 (LDM3) + 175-2]) and anti-DVI+ ([ESD-1M + 175-2]). C/c and E/e expression was screened using the Diaclon Rh-Subgroups + K ID-Card ([Bio-Rad Laboratories] anti-C: MS-24; anti-c: MS-33; anti-E: MS-260; anti-e: MS-16, MS-21, MS-63).

Genomic DNA extraction and molecular analysis of the *RH* genes

Genomic DNA was extracted from 200 µL whole blood with the QIAamp DNA Blood Mini Kit (Qiagen, Les Ulis, France) in the conditions recommended by the manufacturer and eluted in 200 µL molecular-grade, distilled water.

Molecular investigation was carried out by two complementary approaches. A quantitative multiplex PCR of short fluorescent fragments (QMPSF)-based method with gene-specific sets of primers was performed to identify potential copy number variations, i.e. *RHD* zygosity, as well as structural variants (SVs) such as hybrid gene conversions, insertions/deletions, duplications, etc.[37]; while single nucleotide variants (SNVs) in the *RH* genes were analyzed by Sanger sequencing in exons and flanking intronic regions as described before[38-40]. The most probable genotypes/alleles were deduced using reference databases, including RHD and RHCE Blood Group Allele Tables from the Red Cell Immunogenetics and Blood Group Terminology ISBT Working Party[41,42], RHCE Table from the New York Blood Center (NYBC) Genomics Laboratory[43], the Human RhesusBase[44], Erythrogene[45], BloodAntigens.com[46], and RHeference[47].

RESULTS

RH gene polymorphism in patients with SCD

RHD QMPSF and Sanger sequencing of all *RHD* exons were carried out systematically in the 109 patients. Forty-three SNVs were found, of which c.1136C>T, c.667T>G, and c.602C>G were most common, presenting with a frequency above 10% in our subset (**Table I**): this is in line with the data reported in gnomAD v2.1.1 (African/African American subpopulation)[48]. Those 43 SNVs define a total of 18 variant *RHD* alleles (**Table II**). A conventional (reference) *RHD*01* allele was observed in

Table II - *RHD genotype and predicted D phenotype in 109 Cameroonian patients with SCD*

D phenotype	*RHD* genotype	Occurence	Frequency (%)
D-positive (D+)	**01/*01*	27	24.77
	**01/*10.00*	23	21.10
	**01/*01N.01*	11	10.09
	**01/*03N.01*	5	4.59
	**01/*08N.01*	3	2.75
	**01/*09.03.01*	3	2.75
	**01/*10.03*	3	2.75
	**01/*58*	2	1.83
	**01/*DIIIa(819G)*[2]	1	0.92
	**01/*01W.66*	1	0.92
	**01/*09.01.02*	1	0.92
	**01/*09.03*	1	0.92
	**01/*10.00.01*	1	0.92
	**01/*10.06*	1	0.92
	**01(541T)/*01N.01*	1	0.92
	Subtotal	84	77.06
Variant D[1]	**10.00/*10.00*	6	5.50
	**09.03.01/*01N.01*	2	1.83
	**10.00/*01N.01*	2	1.83
	**10.00/*03N.01*	2	1.83
	**01(186T)/*01N.06*	1	0.92
	**04.01*[3]*/*03N.01*	1	0.92
	**10.03/*03N.01*	1	0.92
	**10.06/*08N.01*	1	0.92
	**DIIIa(819G)*[2]*/*01N.01*	1	0.92
	**10.00/*01W.161*	1	0.92
	**10.00/*08N.01*	1	0.92
	**10.00/*09.03.01*	1	0.92
	**10.00/*10.00.01*	1	0.92
	**10.00.01/*03.01*	1	0.92
	Subtotal	22	20.18
D-negative (D–)	**01N.01/*01N.01*	2	1.83
	**01N.01/*03N.01*	1	0.92
	Subtotal	3	2.75
Total		109	100.00

[1]Variant (weak/partial) D phenotype in agreement with the recommendations of the Red Cell Immunogenetics and Blood Group Terminology ISBT Working Party[41]. [2]Allele associated with DAK (RH54) expression. [3]Allele associated with Go[a] (RH30) expression.

83 samples, and one sample was found to carry only the synonymous c.541C>T SNV. Overall, 84/109 samples (77.1%) supposedly expressed a "normal" D-positive phenotype, while three samples carried two *RHD*-negative alleles, in agreement with the D-negative phenotype observed by routine serologic testing (**Table II**). Finally, 22 patients harbored two variant alleles at either the homozygous or compound heterozygous state, and were thus considered to be expressing a variant (i.e. weak and/or partial) D phenotype (**Table II**).

Twenty-one SNVs were found in *RHCE* beside the common variants defining the four reference alleles in exons 1, 2, and 5 (**Table III**). By far the most common was c.733C>G, found in as many as 70/218 (32.1%) alleles, while 7 and 13 other SNVs had a frequency of >1% and <1%, respectively (**Table III**). The most prevalent allelic background was *RHCE*01* (**ce*; No.=189, 87.7%), followed by **03* (**cE*; 18, 8.3%), **02* (**Ce*; 10, 4.6%), and the rare **04* (**CE*; 1, 0.4%), and a total of 17 potential variant alleles was assumed (*Online Supplementary* **Table SI**).

Table III - *Common and rare RHCE SNVs in 109 Cameroonian patients with SCD and frequency*

Exon	SNV	dbSNP ID	AA change	Occurence	Frequency (%)	MAF[1]
Common SNVs						
1	c.48G>C	rs586178	p.Trp16Cys	105	48.17	0.54705
2	c.150C>T	rs200955066	p.Val50=	11	5.05	0.00016
	c.178C>A	rs181860403	p.Leu60Ile	11	5.05	0.00006
	c.201A>G	rs1053343	p.Ser67=	11	5.05	0.00016
	c.203A>G	rs1053344	p.Asn68Ser	11	5.05	0.00017
	c.307C>T	rs676785	p.Pro103Ser	11	5.05	0.00008
5	c.676G>C	rs609320	p.Ala226Pro	19	8.72	0.10501
Rare SNVs						
1	c.105C>T	rs142971926	p.Asp35=	11	5.05	0.03229
2	c.254C>G	rs57992529	p.Ala85Gly	4	1.83	0.05445
4	c.505C>A	rs1020280601	p.Leu169Met	1	0.46	0.00020
	c.509G>T	rs987753117	p.Arg170Met	1	0.46	0.00020
	c.514T>A	rs1053349	p.Phe172Ile	1	0.46	0.00012
	c.544A>T	rs1053350	p.Thr182Ser	1	0.46	0
	c.577A>G	rs1384157219	p.Lys193Glu	1	0.46	0.00008
	c.594T>A	rs1053354	p.Asn198Lys	1	0.46	0.00012
	c.602G>C	rs141398055	p.Arg201Thr	1	0.46	0.00048
5	c.667G>T	rs147357308	p.Val223Phe	7	3.21	0.01282
	c.712A>G	rs144163296	p.Met238Val	2	0.92	0.01092
	c.733C>G	rs1053361	p.Leu245Val	70	32.11	0.23115
	c.744T>C	rs149352457	p.Ser248=	1	0.46	0.00068
	c.787A>G	rs1132763	p.Arg263Gly	2	0.92	0.00511
	c.800T>A	rs1132764	p.Met267Lys	2	0.92	0.00427
6	c.916A>G	rs1132765	p.Ile306Val	1	0.46	0.00529
7	c.941T>C	rs79321360	p.Val314Ala	6	2.75	0.02016
	c.1006G>T	rs116261244	p.Gly336Cys	13	5.96	0.03980
	c.1025C>T	rs1053374	p.Thr342Ile	2	0.92	0.01981
9	c.1170C>T	rs630931	p.Leu390=	5	2.29	N/R
	c.1193T>A	rs630612	p.Val398Glu	5	2.29	0

[1]Minor allele frequency from the Genome Aggregation Database (gnomAD v2.1.1; African/African American subpopulation);[48] N/R: not reported in gnomAD v2.1.1.

Molecular-based *vs* serologic phenotype and RH antigen expression

On the basis of the genotyping data, RH phenotype was deduced by following the official recommendations of the RCIBGT ISBT Working Party[41,42]. Concordance between prediction and serologic testing was 97.6% for the five major RH antigens: 100.0% for RH1 (D), RH3 (E), and RH4 (c), while RH2 (C) and RH5 (e) yielded two false-positive and as many as eleven false-negative results, respectively (*Online Supplementary* **Table SII**).

In terms of RH antigen expression deduced from genotyping, RH5, RH4, and RH1 were supposed to be the most common (100.0%, 99.1%, and 97.2%, respectively) in the subset of patients, while RH2 and RH3 were less common (18.3% and 17.4%, respectively). In these, partial RH1, RH5, RH4, and RH2 antigens may be expected in 21, 20, 13, and 10 patients, respectively, while no partial RH3 was suspected (*Online Supplementary* **Table SII**).

Potential expression of both LFAs and HFAs could be also predicted. In the 109 patients, 47 (43.1%) and 58 (53.2%) patients are supposed to express the RH10 (V) and RH20 (VS) LFAs, respectively, which are common in sub-Saharan Africans[49,50]; and RH30 (Go^a), RH32, and RH54 (DAK) LFAs are predicted in one (P058), one (P060), and three (P017, P060, and P114) patients, respectively (*Online Supplementary* **Table SII**). Finally, the RH59 (CEAG) and RH31 (hr^B) HFAs, which are not considered clinically relevant, are thought to be defective in one patient (P075), and in the range of 4 to 16 patients, respectively (*Online Supplementary* **Table SII**).

RH genotype in donors and molecular investigation of donor-patient genotype matching

Of great interest to us was the genomic DNA of our 108 blood donors, whose red blood cells were transfused to a subset of 98 patients with SCD (one-to-four donors per patient), and which was available for molecular analysis. We decided to investigate the *RH* genes in these donors using a similar genotyping strategy (*Online Supplementary* **Table SIII**), and did a retrospective study of the risk of RH antigen exposure in our patients subsequent to phenotype prediction in the donors.

Serologic testing yielded two false-negative samples for RH2 testing, and a very significant number of 40 false-negative samples (37.0%) for RH5, which raises great concern and definitely questions the sensitivity of the current method. Overall, concordance between prediction and serologic testing was 92.2% for the five major RH antigens (*Online Supplementary* **Table SIII**). Comparison of predicted phenotype in donors and patients suggested that 33 patients (30.3%) were not at risk of being exposed to undesirable antigen(s), i.e. patients with a partial antigen or antigen-negative patients transfused by antigen-positive donor RBCs, with a potential subsequent risk of alloimmunization. Therefore, 65 patients with SCD were potentially exposed to a total of ten undesirable antigens with varying degrees of clinical significance (*Online Supplementary* **Table SIV**, **Figure 1**). Unfortunately, no data could be obtained about subsequent alloimmunization in these patients.

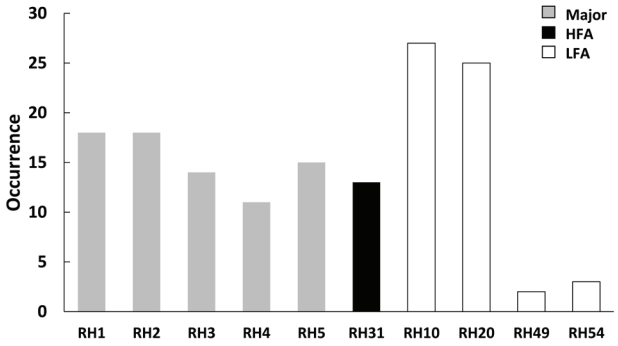

Figure 1 - Occurrence of theoretical undesirable exposure to RH antigens in 98 Cameroonian patients with SCD
HFA: high-frequency antigen; LFA: low-frequency antigen.

DISCUSSION

For the first time in Cameroon and more broadly speaking, in sub-Saharan Africa, we provide a comprehensive description of the genetic polymorphism of the RH blood group system in a cohort of patients with SCD. This work contains important epidemiological data for understanding the molecular basis of the RH variant phenotype in the Cameroonian population by documenting the nature and frequency of variant alleles. But it also paves the way for future guidelines in terms of blood typing and supply, selection of reagents for routine diagnostics, and identification of the clinically relevant antigens which might result in alloimmunization, all of which are crucial factors in improving the management and safety of patients, including those suffering from hemoglobinopathies.

Although it was obvious from serologic testing that routine typing was deficient to some extent, as shown in particular by the unsurprisingly high number of individuals presenting with the rare RH:–3,–5 phenotype (*Online Supplementary* **Tables SII** and **SIII**), genotyping helped to identify those issues. Two patients typed as RH2-positive actually carried *RH* alleles that do not encode this antigen (*Online Supplementary* **Tables SII**). The reason for these false-positive results is thus still unknown, but cross-contamination cannot be ruled out. On the other hand, it is well known that variant *RHCE* alleles may severely weaken expression of RH antigens at the surface of RBCs, which can give rise to a false-negative reaction in routine typing[51]. From genotyping-based phenotype prediction, it would appear that two donors and a remarkable total number of 51 patients and donors are supposed to express RH2 and RH5, respectively, while being typed negative for these antigens. While it cannot be excluded that antigen expression may be severely reduced in some of these individuals due to the variant alleles, it is also worth mentioning that some of the donors yielding a false-negative result carried at least one conventional *RHCE* allele unambiguously (*Online Supplementary* **Table SIII**: *RHCE*01* and/or **02* in donors typed "RH5-negative" and "RH2-negative", respectively), suggesting that routine typing very likely failed initially in some individuals. Taken together, these observations clearly point to the need for an extensive review of the reagents, techniques, in-house procedures and overall methodologies adopted, in order to identify the stage (or stages) responsible for mistyping, and resolve the problem, especially as regards RH5, which has been singled out as the main challenge in current settings.

As expected, the variants found in the subset of patients with SCD are typically "sub-Saharan African-type" SNVs. It is interesting to note that the frequency reported in reference databases, such as gnomAD, may diverge very much from the experimental data, more specifically in *RHD* (data not shown). For example, the frequency of the c.1136C>T change in *RHD*, which is the specific marker of the *DAU* allele cluster[52], is 69.8% in the African/African-American subpopulation in gnomAD v2.1.1, while in our cohort, the variant was carried by only 23.9% (**Table I**; Fisher's exact test: $p<10^{-42}$). Various factors may explain this kind of discrepancy, including the relative homogeneity of the patient cohort originating from a localized region compared to the geographic and ethnic heterogeneity of the gnomAD subpopulation in terms of their African ancestry[48]. Also, variant frequency in gnomAD was compiled on the basis of short-read exome/genome sequencing, which is known to be inappropriate for identifying and assigning SVs correctly[53-55], especially when homologous genes with rearrangements are involved as in *RH* genetics[56]. The genotyping strategy adopted in the present study allowed the robust identification of SVs in hybrid alleles and precise reporting of all variations with a high resolution at the nucleotide level. Generally speaking, reference databases are excellent sources of information, but the data they contain need to be handled with care, taking into account the context of the study, the gene(s) of interest, the prevalence of complex mutational mechanisms, and other variables.

The nature and frequency of *RH* variant alleles identified in our study are mostly in line with previous findings in other sub-Saharan populations. As observed in this work, the *RHD*10.00*, **09.03.01*, and **10.03* alleles, as well as *RHCE*01.01*, **01.20.01*, and **01.20.03*, are globally the most common in Congo, Mali, and heterogeneous subsets originating from different sub-Saharan African countries[32,34,35]. However, certain specificities can be noted. The *RHD*09.01.00* (or **weak D type 4.2.0* or **DAR1*) allele[57], reported as having a frequency in the range of 3.4-11.8% in heterogeneous non-pygmoid and pygmoid populations[34], was not found in the 109 patients with SCD (Fisher's exact test: $p=3.72\times10^{-3}$); and the commonly *cis*-associated *RHCE*01.04.01* allele was observed only once in our subset, while in the other study, its frequency ranged from 6.4% to 11.4% ($p<10^{-3}$). Also, the clinically relevant *RHCE*02.10.01* (or **CeRN*) allele[58] was almost absent here (*Online Supplementary* **Table SI**: 1/218, 0.46%), while it accounted for 19.6% of the total alleles in 46 Fulani individuals in Mali ($p<10^{-8}$)[35]. Although further large-scale studies are required to confirm our data, these observations seem to indicate a trend towards regional/local patterns of *RH* polymorphism which are highly dependent on the ethnicity of the population of interest.

Beyond the basic reporting of variants and alleles in the cohort, in transfusion therapy, the crucial point to

consider is without doubt the expression of RH antigens. In our subset, 40/109 patients carried conventional *RHD* alleles only (**Table II**: **01* and **01N.01*). Also, on the basis of experimental and epidemiological data, it has been proposed that patients with **10.00* (and very likely **10.00.01*) and **09.03.01* should be considered and treated as "wild-type" D+ individuals[49,59,60]. Overall, then, in addition to the three D– individuals, only five patients (4.6%) should be transfused with D– RBCs, in order to prevent potential anti-D alloimmunization (**Table II**: patients carrying the **01(186T)*, **04.01*, **10.03*, **10.06*, and **DIIIa(819G)* alleles). The situation regarding *RHCE*-encoded antigens is slightly more complex, because 26 patients (23.9%) expressed partial RH2 (9.2%) and/or RH4 (11.9%) and/or RH5 (18.3%), including three (*Online Supplementary* **Table SII**: P058, P082, and P089) who expressed the three partial antigens, and even one (P058) carrying also the partial *RHD*04.01* allele. Considering the reported frequency for RH4 and RH5 in Black Africans (i.e. ~98%)[61,62], it seems reasonable to state that finding antigen-negative RBC units for patients alloimmunized to these antigens in the Cameroonian population would represent a real challenge. Finally, the frequency of the other RH LFAs and HFAs in our cohort was in line with that of previous reports[61,62]. In the subset of donors, who were selected on the basis of routine serologic testing, beside the discrepancies discussed above and in addition to RH10 and RH20, only RH49 (STEM) and RH54 LFAs were suspected and transfused to a limited number of antigen-negative patients (**Figure 1**: two and three patients, respectively). Most importantly, our data showed that a significant number of patients carried partial antigens, which suggests that attention should be paid above all to these antigens. Ultimately, there is a pressing need to investigate the alloimmunization rate in future, larger-scale retrospective studies, aimed in particular at investigating how conventional D+ patients may form anti-D when transfused with RBCs from donors carrying *RHD* variant alleles[17].

CONCLUSIONS

To sum up, current practice for preventing alloimmunization after blood transfusion in patients with SCD is limited to ABO/D matching in Cameroon, and clear guidelines to avoid this risk for these patients have not yet been adopted. Despite the difficulty in collecting RBC units and the fact that much of the blood transfusion in this population is just beginning, this study suggests that it may be worth extending routine typing to RH C/c and E/e antigens in a standard setting. Moreover, it would be highly desirable to carry out systematic immunological surveillance by looking for irregular antibodies, and investigate the immunogenicity of specific variants in these patients in future studies in the Cameroonian context[63]. Lastly, implementation of genotyping approaches dedicated to screening the most prevalent variants found in Africans would be invaluable for preventing alloimmunization, not only in sub-Saharan Africa, but also in countries with large communities originating from Africa.

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AUTHORSHIP CONTRIBUTIONS

JMMM, FNS, ANN and YF designed the study. JMMM, EW, HNP, SNA and JT collected the samples and performed serological testing. CB carried out the molecular analysis. JMMM, CB, FNS and YF analyzed the results and interpreted the data. JMMM and YF wrote the paper. CB, FNS, EW, HNP, SNA, JT and ANN reviewed and approved the paper.

The Authors declare no conflicts of interest.

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