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Sickle cell disease in the 21st century - from morbidity to molecular biology

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Sickle cell disease in the 21st century - from morbidity to molecular biology

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Editorial: Sickle cell disease in the 21st century—Morbidity to molecular biology

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sickle cell disease, molecular biology, newborn screening, iron overload, pain in sickle cell disease

Editorial on the Research Topic
Sickle cell disease in the 21st century—Morbidity to molecular biology

This Research Topic intends to review some recent developments in the management of sickle cell disease. It is now 112 years since the first description by James Herrick in 1910, while studying dentistry in Chicago, of a student from Grenada in the West Indies and knowledge of the disease has increased exponentially. The worldwide distribution of the genes contributing to sickle cell disease is now largely documented and the early assumption that the disease was confined to peoples of African descent has been rectified. Although the disease observed in the US, UK, Europe, Brazil, and the Caribbean is still predominantly among people of African descent, its presence and variability in other racial groups are increasingly recognized. These have provided models for understanding more about the disease as shown by the Asian haplotype which characterizes the disease in the Eastern Province of Saudi Arabia and throughout much of central, west and southern India. In these areas, the persistence of fetal hemoglobin (HbF) and often high frequencies of alpha thalassaemia have modified the disease which may present different features and require different models of care. The powerful contribution provided by DNA technology has also increased our understanding.

Against this background, contributions to the current topic have originated from the US, France, French Guiana, Oman, Brazil, and India. Some relate to transfusion therapy, which although sometimes beneficial in the short term, may present serious problems in chronic transfusion programmes including alloimmunization and iron overload. An IFN α/β gene signature in the majority of patients with SCD is described with the hypothesis that IFN α/β activity may determine the variable disease progression, a concept requiring further research. The use of determining EVS (Extra Cellular Vesicles) as a biomarker for sickle cell crisis is also proposed.

These topics and the mechanisms for red cell exchange are addressed and other papers look at features of chronic inflammation and chronic joint pain. Newborn screening for the disease, delayed by concerns that the high levels of HbF in the immediate post-natal period would obscure the genotype diagnosis, is now widely practiced and confers many benefits to patients if follow-up is ensured in

specialist clinics. High-pressure liquid chromatography (HPLC) is the mainstay in most programmes, aspects of which are addressed in two papers, one from the US and the other from western and central India. The first raises the issue of differential diagnosis of a HbSS phenotype in which studies (parental genetics or DNA) may be required to exclude the essentially benign HbS/HPFH syndrome, which is more common than previously thought. The second raises important questions on the degree to which models of care for sickle cell disease are universal or whether they should be tailored to meet the geographical differences which are emerging. Although the basic pathophysiology of sickle cell disease may be common in most geographic areas, it is increasingly clear that interaction with genetic and environmental factors changes the manifestations of the disease. In regions of Africa where falciparum malaria is common, it is still unclear the extent to which malaria and its therapy influence disease pattern. The epidemiology of parvovirus B19 infection and the aplastic crisis is poorly documented and commonly undiagnosed in situations where reticulocyte counts are unavailable. Profound differences may occur in the disease associated with the Asian haplotype which is almost always characterized by high levels of HbF and often frequent alpha thalassaemia, both of which may be expected to inhibit intravascular sickling. In Odisha, these factors are associated with the persistence of splenomegaly and a current contribution suggests that splenic function is also preserved. This may be crucially important in protection against pneumococcal sepsis where susceptibility falls rapidly after the age of 3 years. The result is that pneumococcal septicaemia has never been described in Indian HbSS patients and with the sophistication of many Indian laboratories, it is extremely unlikely that the diagnosis is being missed. In India, the cost of a regular prophylaxis programme including conjugate and regular vaccines and penicillin has been estimated as US$220 per child but, in addition to the cost, the logistical difficulties of providing and monitoring such programmes is considerable. As diligent Indian practitioners seek to implement pneumococcal prophylaxis for a disease that current data suggest does not occur, it seems appropriate to ask whether this is the best use of these resources. Indian colleagues must make this decision and whether this is the optimal use of limited resources. Other potential differences of the Asian haplotype include a relative infrequency of priapism and leg ulceration. The bone pain crisis, usually due to avascular necrosis of bone marrow is a major problem of HbSS in all geographic areas and, in developed societies, has been increasingly managed by daycare and effective analgesia. Although painful, the great majority of events are not pathologically serious, allowing daycare and avoiding hospital admission. In Jamaica, all patients are advised to keep paracetamol/codeine mixtures at home, the combination offering much more effective pain control along with other measures such as keeping warm and well hydrated. In Jamaica, combined tablets are available without prescription and play a major role in the control of symptoms. Yet in some areas, only paracetamol is allowed for domestic use and if inadequate for pain relief, forces the patient to travel to the nearest health center or hospital for pain relief, a journey only likely to make the pain worse. The moral, of course, is that models of care must be tailored to the manifestations of local disease; such documentation has been underway in populations of African descent, when living in temperate climates, but has yet to be established in other societies.

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Author contributions

The author confirms being the sole contributor of this work and has approved it for publication.

Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Increased Prevalence of Alloimmunization in Sickle Cell Disease? Should We Restore Blood Donation in French Guiana?

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Patients with sickle cell disease often undergo frequent blood transfusions. This increases their exposure to red blood cell alloantigens of donor units, thus making it more likely that they produce alloantibodies. This cross-sectional study aimed to describe the prevalence of allo-immunization in patients with sickle cell disease who were monitored at Cayenne Hospital in 2016. Of the 451 patients recruited during the study period, 238 (52.8%) were female. There were 262 (58.1%) homozygous sickle cell and 151 (33.5%) compound heterozygous sickle cell patients. The median age of the participants was 23.09 years (range, 0.5–68). We noted different red blood cell extended phenotypes: -in the Duffy system, the Fy^a- Fy^b–profile was found in 299 patients (66%);—for the Kidd system, the most represented profile was Jka+ Jkb-, with 213 patients (47%). The Jka antigen was present in 355 patients;—in the MNS system, the S-s+ profile was found in 297 patients (66%);—the Lea antigen of the Lewis system was absent in 319 patients. The most frequent Rh phenotype in our patients was D+ C- E- c+ e+ K-, representing 51% of the patients. A total of 6,834 transfused packed red blood cell units were recorded. Sixty-eight patients (23%; 95% confidence interval, 20–25%) had detectable RBC alloantibodies. In multivariate logistic regression, only the mean number of single transfusions was statistically higher for the alloimmunized patients ($p < 0.04$). Thirteen (19%) of the patients with alloimmunization developed a delayed hemolytic transfusion reaction, thus representing 4.4% of the total number of transfused patients. Whether differences between donors from France vs. recipients from French Guiana could explain this high prevalence of alloimmunization to be examined. In conclusion, careful transfusion strategies for patients with RBC alloantibodies should allow further reduction of the rate of alloimmunization.

Keywords: sickle cell disease, immunohematological issues, transfusion, alloimmunization, delayed hemolytic transfusion reaction

INTRODUCTION

Red blood cell (RBC) transfusions are often used to treat acute complications of sickle cell disease (SCD). The purpose of RBC transfusion is to increase oxygen distribution to the tissues and/or to replace the rigid sickle-shaped RBCs with healthy deformable RBCs (1). Transfusion can also be part of a regular long-term transfusion program aimed at the prevention of SCD complications. Seventeen percent of SC patients, 45% of S/β-thalassemia patients, and more than 60% of SS homozygous patients undergo at least one transfusion before the age of 18 years. It is estimated that 90% of adults with sickle cell disease will receive at least one transfusion in their lifetime (2). Many patients with SCD, however, undergo numerous transfusions over the course of their lives, thereby greatly increasing their exposure to RBC alloantigens of donor units. These patients are, therefore, more likely to produce alloantibodies. RBC alloimmunization can cause adverse effects, resulting in delayed hemolytic transfusion reactions (DHTRs) and limit the further safe transfusion. The incidence of alloimmunization in SCD has been reported to be 20–50% (3, 4). Recognition of RBC antigens, proteins and membrane glycoproteins by the recipient immune system leads to generation of antibodies and memory cells (5, 6). Alloimmunization is a frequent occurrence due to antigen disparities between patients and donors. Its severity varies according to the number of transfusions received and according to phenotypic differences between the recipients (who are often of African descent) and blood donors (who tend to be of European ancestry) (7, 8). Exact matching for ABO, Rhesus, Kell, Kidd, and Fy^a blood group antigens, and extending this match whenever possible, in patients who have not yet developed immunity is an effective strategy for reducing alloimmunization to RBC antigens in patients with sickle cell disease. French Guiana is an overseas French territory located in South America. It is bounded by Brazil to the south and east, Suriname to the west, and the Atlantic Ocean to the Northeast. The incidence of SCD in French Guiana is ∼0.41% (9). Following four deaths related to Chagas disease, a serological survey conducted in 2004 revealed a prevalence of Chagas disease of 0.5% among blood donors in French Guiana. In April 2005, blood drives in French Guiana were discontinued due to concerns of the safety of blood products (10). Other barriers to blood donation in French Guiana are a number of contagious infectious diseases with high risk for transfusion transmission, such as arboviruses (emergent and circulating), leptospirosis, Q fever, malaria, human immunodeficiency virus (HIV), and human T-cell leukemia virus (HTLV) infections. Two-thirds of French Guiana's supplies of packed RBCs comes from mainland France, and one-third is obtained from the Guadeloupian French Blood Establishment. The packed RBC units are transported by air to the Guianese French Blood Establishment. The latter facility only ensures their delivery to the hospitals where the transfusion is to be provided. This study aimed to describe the prevalence of allo-immunization in patients with SCD, since the transition away from blood donation in French Guiana.

MATERIALS AND METHODS

Study Design

This was a cross-sectional study designed to describe the following immunohematological characteristics of sickle cell patients followed in our center: the number of transfusion episodes, number of units of transfused blood, indication for transfusion, age at first transfusion, history of pregnancy if necessary, blood group ABO, D and the extended phenotypes, the possible type of alloantibodies, the rate of alloimmunization, and types of red blood cell alloantibodies in patients with SCD who were being monitored at the sickle cell center of Cayenne Hospital in 2016. In our center, all patients are systematically tested for autoantibodies during the annual check-up. The study population was part of a pre-existing cohort titled: Improving the quality of management of SCD in French Guiana: "Epidemiology of predictive factors of acute clinical events," that enrolled ∼1,000 patients.

Transfusion Procedures

In our center, as soon as SCD is diagnosed, ABO RH-K blood grouping and extended phenotyping are performed. The latter is sometimes made difficult when the patient has already been transfused, especially for transfusions performed abroad or in case of loss of records. The determination of the phenotype by molecular biology is then necessary. Before each transfusion, a test for irregular antibodies and a cross-matching test are systematically performed in the laboratory. Another cross-matching test is performed at the patient's bed. A control of irregular antibodies is planned 3–6 weeks after each transfusion.

All patients undergo the same anti-erythrocyte antibody screening protocol. This screening is performed using a three-cell panel and a gel test technique (indirect antiglobulin test). If antibodies are detected, a panel of 15 cells is used for identification, supplemented if necessary by a panel of 15 red blood cell cells treated with an enzyme (papain and/or trypsin). The distinction between alloantibodies and autoantibodies is based on the serological test, the phenotyping of red blood cells and, sometimes, the genotyping of red blood cells (in this case the sample is sent to a laboratory in France). Anti-M (anti-MNS1), anti-Lea (anti-LE1), anti-Leb (anti-LE2) and anti-Lex (anti-LE3) antibodies are considered as natural antibodies if they are present before any transfusion.

The transfusion protocol applied in our center, as in other French centers has been recently detailed in the paper by Clément et al. (11). Patients on a manual transfusion exchange program received 25 ml RBC/kg body weight every 4 weeks, preceded by phlebotomy if the hemoglobin level was >80 g/L. An HbS level of <30–40% was targeted depending on the indications. Since April 2012, manual exchange transfusion has been replaced by an automated exchange transfusion.

Study Site

This study was conducted at the sickle cell center of Cayenne Hospital. Cayenne Hospital is a 510-bed general hospital center that serves as a referral and teaching hospital, and it has the only

sickle cell center for children and adults (created in September 2014). The study took place between October 1 and December 31, 2016.

Study Subjects

Consecutive patients with SCD (homozygous SS, compound heterozygous SC and S-β-thalassemia) attending the sickle cell center at Cayenne Hospital who were 6 months of age or older, with or without a history of blood transfusion, and who freely provided their consent (or whose parents provided consent) to participate were included in the study. All individuals who did not meet the inclusion criteria were excluded from the study. Since 1990, all patients receive leukoreduced, non-irradiated RBC units that are phenotypically matched for D, C/c, E/e, and K. In case of alloimmunization, patients are expected to receive erythrocyte units matched for the relevant antigens and for Fya, Fyb, Jka, Jkb, S and s, if more than two alloantibodies are identified (11).

Data Collection

In the absence of a single computerized patient database, data from different sources (including the medical records from the French Guianese Blood Establishment, which monitored transfusions from 1995 to 2016) were collected in order to be as thorough as possible and to cross-check the information. The data were obtained directly from computerized hospital records or from paper records when computer data were not available. Records at the sickle cell center regarding the recruited SCD patients were reviewed in regard to their demographic characteristics and the transfusion history, the number of transfusions, the number of units of transfused blood, the date of the transfusions, the indication for transfusion, the age at the time of the first transfusion, and any history of pregnancy. The blood group ABO, D antigen, the extended phenotypes, and the possible type of alloantibodies were also recorded. All of these data were recorded in an anonymous Excel database. A delayed hemolytic transfusion reaction was suspected when a patient presented several days after RBC transfusion with the development or intensification of symptoms suggestive of a painful crisis, hemolysis, reticulocytopenia, and worsening anemia, reversing the effects of the previous RBC transfusion (12).

Statistical Analysis

We used as primary outcome the prevalence of alloimmunization. This prevalence was calculated by dividing the number of alloimmunized patients by the total number of transfused patients. The alloimmunization rate was calculated by dividing the number of alloimmunized patients by the total number of RBC units received (before first alloimmunization). Statistical software packages (LibreOffice Calc version 5.1.6.2 and *Stata 12.0 Statistical Software: Release 12*. College Station, TX: StataCorp LP) were used for data management and analysis, respectively. For univariate analysis of possible associations between alloimmunization and gender, age at the time of the first transfusion, pregnancy history, the number of transfusions, the clinical and laboratory findings, the Chi-squared test or Fisher's exact test was used for discrete variables. Unconditional logistic regression analysis was used for the continuous variables with a non-Gaussian distribution. $P < 0.05$ was chosen as the threshold for statistical significance.

Ethical Considerations

The patients, their parents, or authorized representatives provided written and informed consent to participate in this research. This study was approved by the hospital's ethics committee, and the database was declared to the Commission nationale de l'informatique et des libertés (Number 3Yj157849 3#).

RESULTS

Of the 451 patients recruited during the study period, 238 (52.8%) were female. There were 262 (58.1%) homozygous sickle cell and 151 (33.5%) compound heterozygous SC patients. The median age of the participants was 23.09 years (range, 0.5–68) (**Figure 1**). The most common blood group was O (51%), followed by group A (22%), and group B (17%). For 362 patients (80%), the RH/KEL group phenotype and an extended phenotype (Duffy, Kidd, MNS, and Lewis systems) were determined. Irrespective the extended phenotype, we noted different profiles, including:

(1) in the Duffy system, the Fy^a- Fy^b- profile was found in 299 patients (66%);
(2) for the Kidd system, the Jka+ Jkb- profile was the most common: 213 patients (47%). The Jka antigen was present in 355 patients;
(3) in the MNS system, the S-s+ profile was found in 297 patients (66%);
(4) the Lea antigen of the Lewis system was absent in 319 patients.

The most frequent Rh phenotype in our patients was D+ C- E- c+ e+ K-, representing 51% of all cases. Only 148 (33%) patients underwent molecular biology-based screening for a variant antigen or a rare phenotype. These are found in 40% of transfused sickle cell patients. Of the patients who were non-transfused or who had undergone few transfusions (<30 packed RBC transfusions), only 132 (33%) were assessed in this manner. Forty-two percent of the 103 patients who had undergone few blood transfusions were positive based on this molecular biology-based screening.

There were 298 (66%) sickle cell patients who had been transfused with at least one packed RBC transfusion, compared with 153 patients (34%) who had never been transfused.Twenty-one (4.7%) of the sickle cell patients had a history of at least one autoantibody, and 74 (16.4%) had a history of at least one alloantibody. The types of alloantibodies found in immunological reactions are detailed in **Table 1**. In total, 37 patients developed only one alloantibody, while 14 patients developed two different alloantibodies, including at least one alloantibody, while 23 other patients had at least three or more different alloantibodies, including at least one alloantibody. The number of alloimmunizations over time revealed an increase in these events, as shown in **Figure 2**. In parallel, the rate of RBC

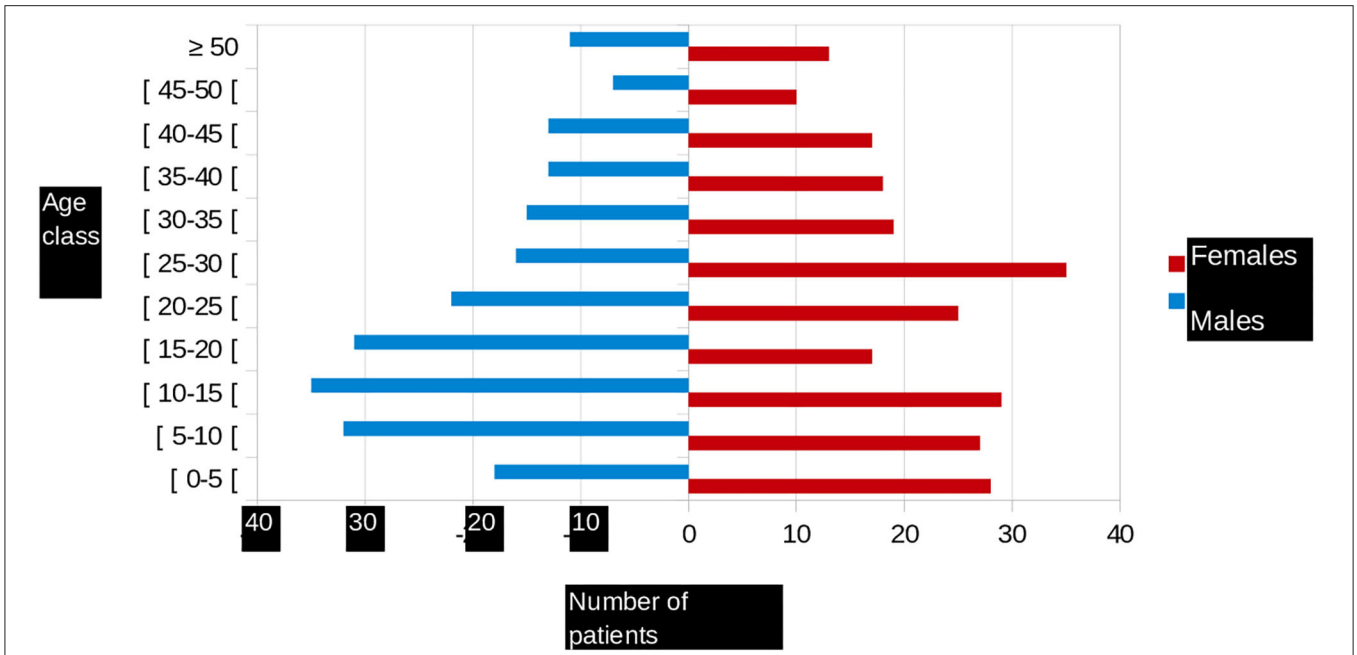

FIGURE 1 | Age pyramid of patients followed in French Guiana.

TABLE 1 | Specificities of the 134 RBC alloantibodies identified in 68 SCD patients in French Guiana.

Blood group system	RBC alloantobody specificity	Number of alloantibodies respectively	Total *n* (%)
Rhesus	C, Cw, D, E, e, RH20	6, 3, 7, 5, 2, 2	25 (19)
Kell	K1, K2, K6	3, 3, 2	8 (6)
Duffy	Fy1a, Fyb, Fy3	6, 1, 1	8 (6)
Kidd	Jya, Jyb	5, 7	12 (9)
MNS	M, S, MNS30	14, 13, 1	28 (21)
Lutheran	Lua	3-	3 (2)
Lewis	Lae, Leb, Le3ab	22, 13, 7	42 (31)
Non specific antibodies		8-	8 (6)

transfusions increased over time among the sickle cell patients, as shown in **Figure 3**.

Characteristics of the Transfused Patients

There were 298 sickle cell patients who had undergone at least one transfusion (51% aged under 18 years and 77% over 18 years of age). Twenty-six of them (8.7%) were transfused with only one packed RBC unit, 161 (54%) received fewer than 10 packed RBC units, and 137 (46%) 10 or more packed RBC units. The average number of packed RBC units received per transfused patient was 23, with a minimum of 1 and a maximum of 461 (standard deviation of 40.77). Fifty-nine (19.8%) of the transfused sickle cell patients had undergone exchange transfusions. Of these transfused patients, 68 (23%; 95% confidence interval, 20–25%) had detectable alloantibodies.

Characteristics of the Transfused Packed RBC

A total of 6,834 packed *RBC* unit transfusions were recorded. No ABO incompatibility was found. Seventy-six incompatibilities were found within the RH/KEL system. A total of 752 packed RBC units were transfused during a pregnancy or immediately after a delivery, representing 11% of the transfused packed RBC units. Specifically, 478 packed RBC units (7%) were transfused for pregnancy monitoring, and 274 packed RBC units (4%) were used to treat sickle cell complications during pregnancy. There were 544 packed RBC units that were transfused outside of French Guiana, and no information was found regarding the site of the transfusion for 361 participants.

Characteristics of the Alloimmunized Patients

Taking into consideration only the transfused patients ($n = 298$), 68 (23%) were alloimmunized. We did not take in account the alloimmunization rate, because the study design not allowed calculating incidences.

The Hemoglobin Phenotypes

No significant difference in the hemoglobin phenotypes was found between the alloimmunized and the non-immune patients. Molecular analysis of variant antigens and rare phenotypes was performed in 37 (54%) alloimmunized patients. For the alloimmunized patients who had undergone few transfusions (<30 packed RBC units), this molecular biology analysis

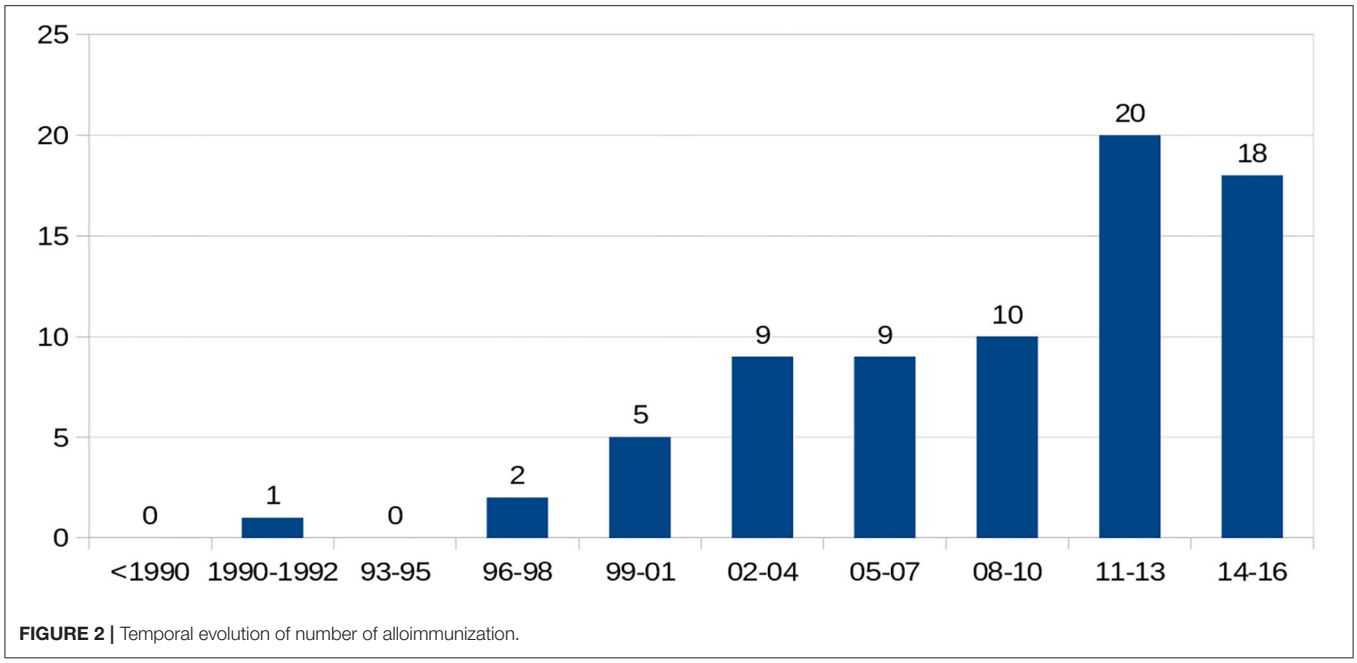

FIGURE 2 | Temporal evolution of number of alloimmunization.

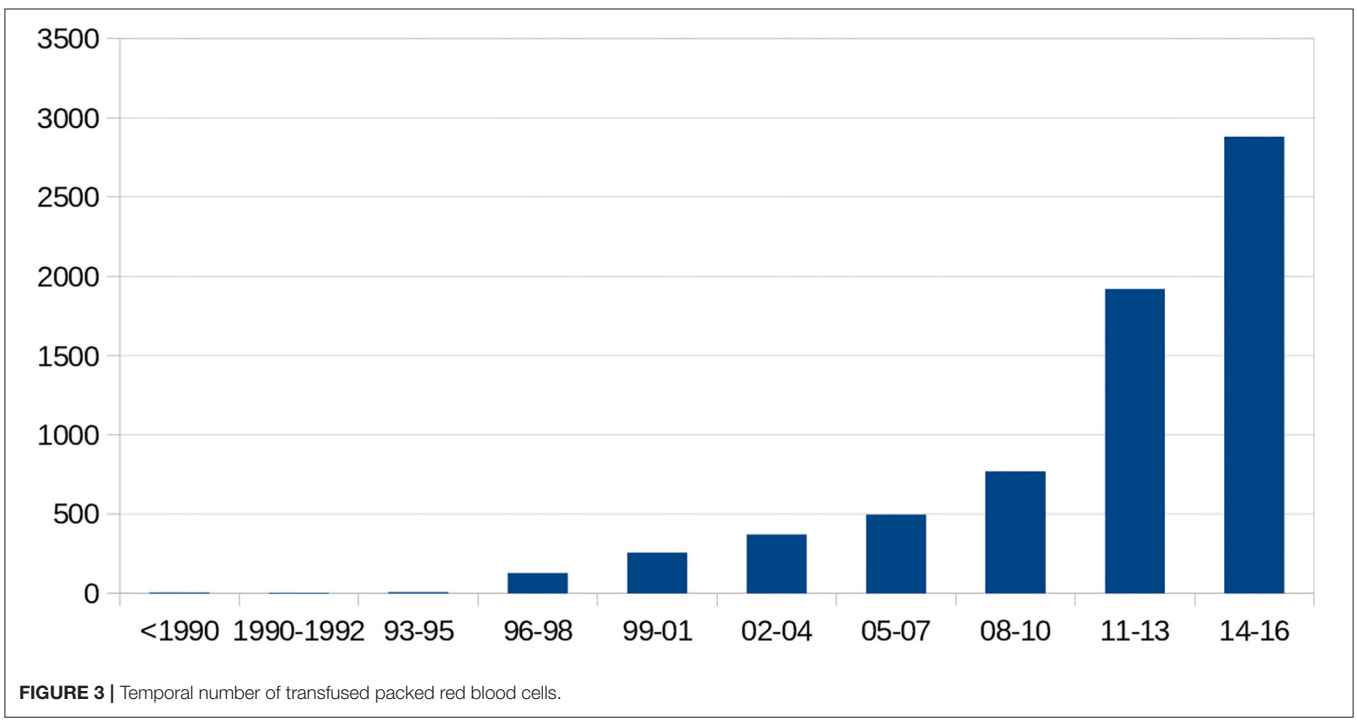

FIGURE 3 | Temporal number of transfused packed red blood cells.

was conducted in 28 patients (51%). Comparison of the alloimmunized vs. the non-alloimmunized patients revealed that the alloimmunized patients had undergone significantly more transfusions ($p = 0.02$), with more packed RBC units ($p = 0.01$) and in emergency settings ($p = 0.01$), as simple transfusions (as opposed to exchange transfusions). Twenty-five percent of the alloimmunized patients were in the exchange transfusion program vs. 18% of the non-alloimmunized patients, although this difference was not statistically significant. In multivariate logistic regression, only the mean number of single transfusions was statistically higher in the alloimmunized patients ($p < 0.04$).

RBC Antibodies

The 68 alloimmunized patients produced a total of 134 RBC alloantibody specificities. **Table 1** shows the specificities of the antibodies that were identified, with 42 (31%) belonging to the Lewis blood group system, of which 22 (52%) had anti-Le^a specificity. The MNS and Rh were the next most frequent

TABLE 2 | Specificities of RBC alloantibodies identified in the 13 patients with DHTR.

RBC Alloantibodies specificity	Number
Anti FY1 (Fya)	4
Anti FY2 (Fyb)	1
Anti JK1 (Jka)	2
Anti JK2 (Jkb)	3
Anti LE1 (Lea)	4
Anti LE2 (Leb)	2
Anti LE3 (Lea+b)	2
Anti MNS1 (M)	1
Anti MNS2 (N)	0
Anti MNS3 (S)	2
Anti MNS30	1
Anti RH1 (D)	1
Anti RH2 (C)	2
Anti RH3 (E)	2
Anti KEL1 (K)	1
Anti KEL3 (Kpa)	2
Anti LU1 (Lua)	1
Anti DI3	1
Anti Dumbrock 2	1
Non-specific	1

blood group systems involved, with 28 (21%) involving MNS and 25 (19%) involving the Rh system. The Kidd system accounted for 12 (9.0%) of the alloantibodies. The Kell and Duffy systems accounted for 8 (6.0%) alloantibodies each, and the Lutheran system for 3 (2%). Twenty-one transfused patients (7%) had at least one alloantibody that was known to be dangerous, namely anti-Jkb (JK2), anti-S (MNS3), and anti-Fya (FY1). Of the 68 alloimmunized patients, 10 (15%) had at least one associated autoantibody. One of the patients with multiple (three) antibodies had an antibody that we were unable to identify.

Characteristics of the Patients Who Developed a Delayed Hemolytic Transfusion Reaction

Thirteen (19%) of the patients with alloimmunization developed a DHTR, thus representing 4.4% of all of the transfused patients. The average age at the time of the DHTR was 29 (12–52) years. There were nine women. Ten of these patients were homozygous SS and three were SC. The SC patients were exclusively pregnant women. Among patients with DHTR, 11 were antibody positive and two antibody negative. Six of them had antibodies known to be dangerous. The specificities of these alloantibodies are detailed in **Table 2**.

DISCUSSION

Epidemiological Characteristics

Our study population was similar to other populations of sickle cell patients, with the usual predominance of young sickle cell patients and the homozygous SS phenotype as the most represented. There was a notably higher proportion of the SC phenotype compared to the proportion found in mainland France, the West Indies, and in South and Central America (13, 14). This proportion was, however, roughly the same as the proportion reported for Guadeloupe (15), and the higher frequency of SC SCD in the French West Indies and Guiana could be due to a different ethnicity of the populations (hemoglobin C is more common in West Africa, especially in Ghana) (16, 17). The characteristics of the sickle cell population in terms of the RH/KEL phenotype were comparable to those that have been described in other populations (18, 19). The distribution of ABO blood groups was the same as that observed in sickle cell patients monitored at the Henri-Mondor Hospital (Créteil, France); the most frequent Rh phenotype in sickle cell patients being D+ C- E- c+ e+ K- (18–20). There was limited expression of the Fya, Jkb, and S antigens in our population. These antigens have been reported to be expressed much more in the Caucasian population (18, 19). In our study, 60% of the sickle cell patients had been transfused. This proportion is identical to the proportion reported in Guadeloupe (15), although the proportion reported in the literature is often higher (21, 22). Chou et al. reported that nearly 90% of all adult sickle cell patients had been transfused at least once (21). The proportion of transfused adult sickle cell patients in the current study is reportedly 77%, which is lower than the rates in the study by Chou. The current study could have underestimated the number of patients receiving transfusions (e.g., lack of centralized reporting system as some hospitals have computer records and some have paper records). Or, perhaps the rates are lower due to the efficacy of therapeutic intensification with hydroxyurea, the high proportion of SC phenotypes in our study or a loss of information regarding transfusions for patients who were transfused outside of French Guiana. The number of transfused packed RBC units increased over time. There are several possible reasons for this increasing trend in blood transfusions among SCD patients. Firstly, with improvements in transfusion medicine, clinicians may be treating the acute complications of SCD more aggressively, with a more frequent use of exchange transfusions that require more blood (23). Secondly, the number of packed RBC units per transfusion is expected to increase with age and weight. There is no national database for transfused patients, and the regional nature of the current database implies a loss of data when patients move from one region to another. The same is true for transfusions performed outside of France. For some of the packed RBC transfusions, no information was available as to where these transfusions were performed. Group O packed RBCs accounted for a large proportion (65%) of the total units used, and this was 12% higher than the proportion of O blood group patients in our study population. This could be due to the particular transfusional logistics in French Guiana, which involves frequent use of group O packed RBCs. An evaluation carried out by the French Blood Establishment in 2016 also described overuse of Group O packed RBCs. Emergency distribution from the blood bank supplies of the Western French Guiana Hospital may underlie this phenomenon (unpublished data). Indeed, O-phenotype packed RBCs were used in nearly 20% of cases,

which supports this hypothesis. It should be noted that the low frequency of the D+ C- E- phenotype in Caucasian donors leads to a frequent diversion of RBC phenotype D-, which are themselves not readily available because of their storage and use in emergency blood banks. For example, in Île-de-France, in nearly 20% of cases, sickle cell patients with the D+ C- E- phenotype receive D- C- E- packed RBCs (18, 24). According to the French Blood Establishment, there do not appear to be any problems with the supply of labile blood products in French Guiana, other than the additional time required for their delivery. On the other hand, the origin of the packed RBCs can vary according to the immunological needs, which allows optimized phenotypic adequacy. The need for packed RBCs is steadily increasing due to the increasing number of people being monitored, as well as the increase in individual needs per patient through the establishment of long-term transfusion programs. It is recommended that poorly preserved packed RBCs are transferred in these patients who are dependent on their transfusion support, within 15 days as a general rule. "Fresh" RBCs allow for better transfusion efficiency and thus an increase in the time between transfusions. In addition, a study in mice has shown an impact of RBC age on alloimmunization (23, 25).

High Prevalence of Alloimmunization

In our study, RBC alloimmunization occurred in 23% of the transfused SCD patients. This alloimmunization rate increased and the number of alloantibodies and transfused units increased with time. This prevalence would have been even higher if our study population did not exclusively received transfusions with blood systematically matched for C, c, E, e, and K antigens. This prevalence could have been lower if the search for antigenic variants by molecular biology had been carried out in all of the transfused patients. The rate of alloimmunization reported in the literature varies between studies from 4 to 47%, with a median of 25% (18, 26, 27). A meta-analysis has suggested a rate of alloimmunization ranging from 4.4 to 76% (28). Our prevalence was higher than in Guadeloupe (15). The prevalence of alloimmunization at the Henri-Mondor Hospital in Créteil (France) is higher, and this may well be due to the fact that this "reference center" monitors patients who require complex disease management (18). In Brazil, the rate of alloimmunization has been reported to be as low as 9.9% (29). The authors suggest that high rates of alloimmunization are due to the disparity of antigenic frequencies between blood donors of mostly Caucasian origin and the SCD recipients, who are more often Afro-Caribbean. This explains a lower rate of alloimmunization in countries where the donors and the recipients are of the same ethnic origin (19). In the United States of America, alloimmunization occurs in 30% of all transfused sickle cell patients, compared to only 2–5% for other non-sickle cell transfusion recipients. This may be explained by the immunocompetence of sickle cell patients compared with other transfused patients whose underlying pathologies often induce immunosuppression (30). According to Chou et al., alloimmunization affects 18–76% of sickle cell patients transfused with packed RBCs compatibilized based on the ABO and Rhesus systems, vs. 5–14.5% when the packed RBCs are compatibilized based on the ABO Rhesus D, C, E, and K system (23). Several other studies support this hypothesis, stating that extensive compatibility with the Jkb, Fya, and S antigens would further reduce the risk of alloimmunization (31, 32). RBC alloimmunization occurs due to genetic disparity between donor and recipient antigens. Indeed, the most frequent phenotype in sickle cell patients is D+ C- E-c+ e+ K-. Thus, the latter is present in 50 to 75% of individuals of Afro-Caribbean origin, while its frequency is <2% in individuals of European origin (18, 20). Sickle cell patients in Uganda and Jamaica, where donors and recipients tend to be from the same ethnic population, have lower alloimmunization rates of 6.1 and 2.6%, respectively. It should be noted, however, that the low use of transfusion in these countries in the management of SCD (due to reduced transfusion safety and product availability) also contributes to these lower rates of alloimmunization (19, 33), as highlighted in a study comparing Jamaica to the United Kingdom, where this rate was found to be 76% (34). We are inclined to presume that the rate of alloimmunization was lower when blood donations were available in French Guiana before 2006. However, we have no collected data to support this hypothesis, and this is one of the limitations of our study. A study published in 2015 showed a prevalence of 16% (35). Reliance on intra-ethnic transfusion might decrease the rate of alloimmunization. Some studies, however, suggest that intra-ethnic transfusions may not completely prevent the risk of alloimmunization, due to the existence of antigenic variants found in populations of African origin (19, 27). In our study, the most frequently encountered antibodies were anti-LE1, anti-MNS1, and anti-LE2, thus confirming the results published in 2015 (35). In a recent study, Allali et al. (36) had also reported a high level of these alloantibodies. Antibodies from the Rh system were found less often. The frequency of antibody types found in our study also appears to be comparable to that described in the United Kingdom (34). In the literature, the antibodies are mainly of the Rh system (especially anti-RH2 and anti-RH3 antibodies) and the Kell system (26, 28). This is the case for Guadeloupe, where the predominant antibodies are anti-RH2 and anti-RH3 (15). In addition to these two antibodies, anti-RH1 is also frequently found in the United States and Brazil (27, 29, 37). In our study, we noted the presence of natural antibodies. Indeed, these antibodies are said to be irregular when they are observed sporadically in the serum of individuals whose RBCs do not express the corresponding antigen. This is the case of antibodies directed against antigens of the Lewis system (26, 38). In addition, some authors claim that anti-Lewis alloimmunization can sometimes occur during pregnancy, due to a loss of expression of the Lewis system. The mechanism remains unknown at present. A bacterial origin or a placental interaction have been hypothesized (38). The main reported risk factors for RBC alloimmunization in SCD patients include age, age at the time of the first transfusion, the number of RBC units received, autoantibody formation, increased inflammation during transfusion, and the age of the RBC units (39, 40). In our study, no significant difference was found between alloimmunized and non-immune patients in terms of their average age, gender, or the hemoglobin phenotype. The data in

the literature on this subject are contradictory (26). A number of studies have, however, also not found differences between alloimmunized and non-immunized patients (26, 33). Other authors suggested that alloimmunization occurs more often in women, which may be partly explained by exposure during pregnancy for some, or independently of pregnancy for others. This alloimmunization increases with age and is independent of the number of transfusions (28, 29, 41). In a study by Murao et al., alloimmunization occurred more frequently in sickle cell patients with the SC phenotype (29). In our study, the average age of the patients with alloimmunization was 20 years, and they received an average of 7.15 packed RBC units, which is consistent with the data in literature (33, 37). Multiple studies have found that the risk of alloimmunization appeared early in the transfusion history and increased with the increase in the number of transfusions (2, 19, 28, 29). We also found this difference in the total number of transfusions in univariate analysis, which was not significant in multivariate analysis. In addition, there was a significant difference in the number of emergency transfusions as well as in episodic transfusions. Only the number of episodic transfusions was found to be significantly different in multivariate analysis. This corroborates the results of a meta-analysis that similarly found a risk of increased alloimmunization with episodic transfusions (28).

Delayed Hemolytic Transfusion Reaction

One complication of alloimmunization is delayed hemolytic transfusion reaction/hyperhemolysis (DHTR/H) syndrome. In our study, 4.4% of the patients developed hyperhemolysis. This result is in keeping with the data in the literature (7, 42). According to several authors, the occurrence of DHTR/H is underdiagnosed as this complication is still poorly understood and it can go unnoticed (19, 42). Indeed, there is no item dedicated to DHTR in the declaration of adverse reactive effects published by the French National Agency for the Safety of Medicinal Products. DHTR are, therefore, declared in other items, such as "Sickle cell hemolysis," "Hemolysis other," "Immunological incompatibility," "Transfusion inefficiency," "Unlisted/unspecified diagnoses." In addition, there is no coding of the underlying pathology.

Limitations

Other limitations of our study include information bias on the notion of episodically or chronically transfused patients, for patients transfused outside of French Guiana. Although it is highly likely that the phenotypes of the RBC of individuals from French Guiana (compared with blood from France) are more similar to those of transfused sickle cell patients, we could not collect data to demonstrate this. However, while the study shows that alloimmunization rates are increasing, and the blood is from other territories in France, the increased rates could be due to increased number of transfused units, not the increase in the number of transfused patients. Despite these limitations, this comprehensive cross-sectional study has demonstrated increased alloimmunization rates in a sickle cell population that is totally dependent on blood from donors in other territories of France.

CONCLUSIONS

This cross-sectional study was designed to determine the RBC alloimmunization rate in patients with SCD in French Guiana since the transition away from blood donation in French Guiana. The study reports a 23% alloimmunization rate, in which both the number of alloantibodies and transfused RBC units increases over time (years). In order to reduce this high rate of alloimmunization, since 2015, the French Blood Establishment has devised careful transfusion strategies for patients with RBC alloantibodies. However, the reestablishment of blood donation in French Guiana could help reduce this high prevalence of alloimmunization.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

SC and NE analyzed the data and drafted the manuscript. VV, MP, PS, and FM collected the data, provided the necessary logistical support, and read the manuscript. NE and FM provided critical comments on the manuscript. All authors contributed to the article and approved the submitted version.

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Potential Implications of a Type 1 Interferon Gene Signature on COVID-19 Severity and Chronic Inflammation in Sickle Cell Disease

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At the onset of the corona virus disease 19 (COVID-19) pandemic, there were concerns that patients with sickle cell disease (SCD) might be especially vulnerable to severe sequelae of SARS-CoV-2 infection. While two reports support this conclusion, multiple studies have reported unexpectedly favorable outcomes in patients with SCD. However, mechanisms explaining these disparate conclusions are lacking. Here, we review recent studies indicating that the majority of patients with SCD express elevated levels of anti-viral type 1 interferons (IFNα/β) and interferon stimulated genes, independent of COVID-19, during their baseline state of health. We also present our data from the pre-COVID-19 era, illustrating elevated expression of a well-characterized interferon stimulated gene in a cohort of patients with SCD, compared to race-matched controls. These type 1 interferons and interferon stimulated genes have the potential to contribute to the variable progression of COVID-19 and other viral infections in patients with SCD. While the majority of evidence supports a protective role, the role of IFNα/β in COVID-19 severity in the general population remains an area of current investigation. We conclude that type 1 interferon responses in patients with SCD may contribute to the variable COVID-19 responses reported in prior studies. Additional studies investigating the mechanisms underlying IFNα/β production and other clinical consequences of IFNα/β-mediated inflammation in SCD disease are warranted.

Keywords: sickle cell disease, type 1 interferons, COVID-19, myxovirus resistance interferon stimulated genes, SARS-CoV-2

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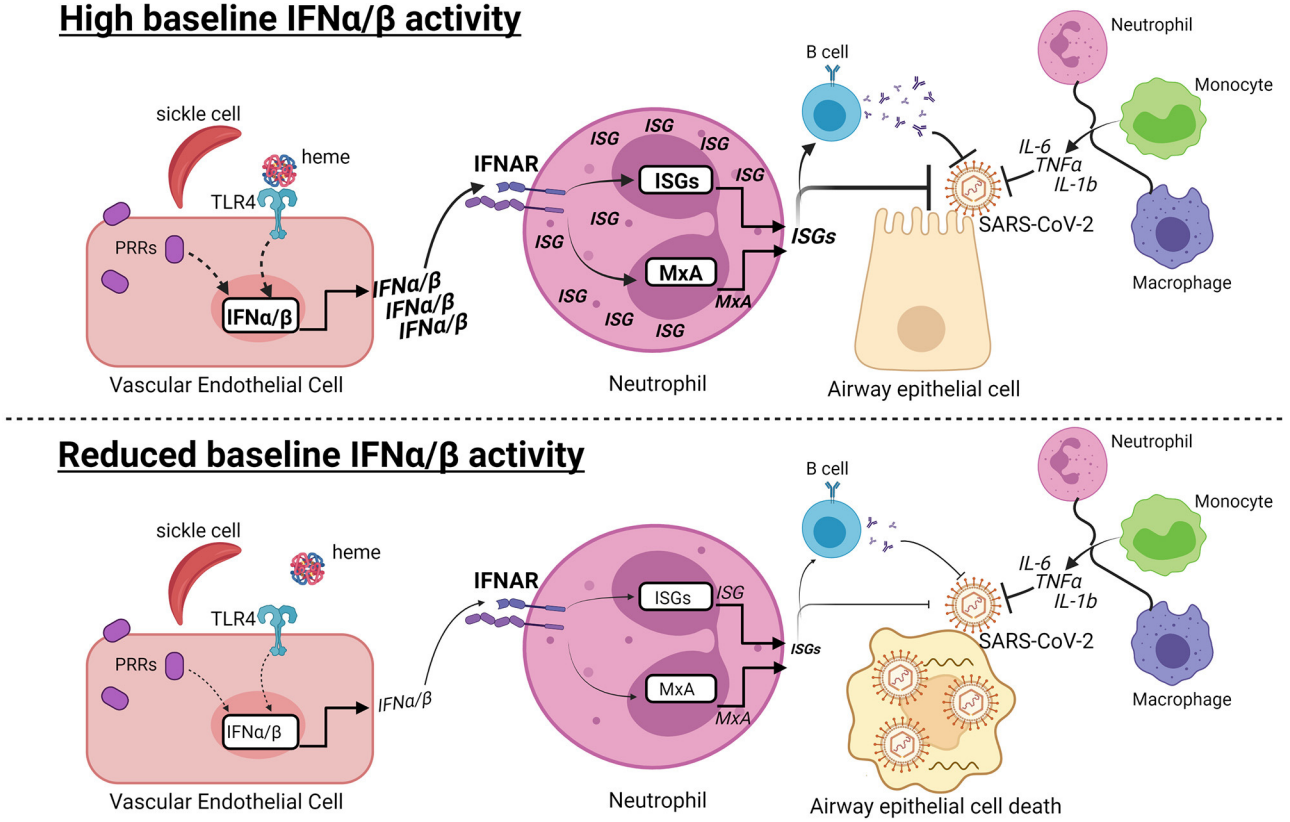
INTRODUCTION

Certain comorbidities are associated with an increased severity of corona virus disease 19 (COVID-19) resulting from SARS-CoV-2 infection (1). Given that patients with sickle cell disease (SCD) have underlying chronic inflammation, significant cardiopulmonary dysfunction, and immune deficiency due in part to hyposplenism, concern exists regarding the severity of COVID-19 in patients with SCD (2). In March of 2020, the National Haemoglobinopathy Panel in the United Kingdom deemed that patients with SCD and other rare hemoglobinopathies are exceptionally vulnerable populations requiring strict self-isolation (3). In November, the Center for Disease Control added SCD to the list



Graphical Abstract | Hypothesis: Baseline type I interferon activity may contribute to variable COVID-19 progression in SCD. (Top) At early stages of SARS-CoV-2 infection, high baseline IFNα/β activity may contribute to the anti-viral response in patients with SCD. Recognition of damage-associated molecular patterns by pattern recognition receptors (PRRs) induces IFNα/β production. Heme released from hemolyzed sickle cells binds Toll-like receptor 4 (TLR4), which may induce IFNα/β in vascular endothelial cells. IFNα/β bind to the IFNα/β receptor (IFNAR) in neutrophils and other cells types, leading to production of MxA and other interferon-stimulated genes (ISGs). ISGs can directly inhibit viral replication and promote B cell production of neutralizing antibodies. The IFNα/β response is one of multiple responses, including production of IL-6, TNFα, and IL-1b, by innate and adaptive immune cells that have the potential to limit COVID-19 progression. (Bottom) In contrast, reduced or absent IFNα/β activity may increase susceptibility to viral infection, leading to airway epithelial cell death and COVID-19. Dashed lines indicate potentially connected pathways, while solid lines are supported by prior studies.

of comorbidities that may increase the severity of COVID-19 (https://www.cdc.gov/coronavirus/2019-ncov/need-extra-precautions/people-with-medical-conditions.html).

Multiple case series and reports have described the disease course of patients with SCD and COVID-19. One common finding is that COVID-19, like H1N1 influenza and other respiratory infections, can trigger acute chest syndrome and vaso-occlusive crises (4, 5). However, it is unclear whether patients with SCD have a more or less severe COVID-19 disease course than those without SCD. Minniti et al. reported that pre-existing co-morbidities, including renal and cardiopulmonary disease, are associated with poor COVID-19 outcomes in patients with SCD (6). In addition, Singh et al. found an increase in hospitalizations and pneumonia, but no increase in mortality, when comparing patients with SCD to race-matched controls with comorbidities (7). In contrast, smaller studies have reported favorable outcomes in patients with SCD (4, 8–11). As reviewed by Sahu et al., most SARS-CoV-2 infected patients with SCD experience mild disease with few patients requiring respiratory support (12). While these outcomes may be due to early diagnosis and treatment in an at-risk population, underlying inflammatory mechanisms in SCD may also influence COVID-19 progression.

Following SARS-CoV-2 infection, multiple cell types produce cytokines, including IL-1b, TNFα, and IL-6, which contribute to the anti-viral response and inflammation (13). Patients with SCD have chronic inflammation at baseline, characterized by leukocytosis, endothelial damage, oxidative stress, and production of pro-inflammatory cytokines. Interleukin-6 is induced during COVID-19, and there are reports of improved COVID-19 outcomes following anti-IL-6 therapy in patients with SCD (14, 15). In addition, there is particular interest in the role of anti-viral type 1 interferons (IFNα/β) produced following SARS-CoV-2 infection. IFNα/β, including IFNβ, IFNω and 12 subtypes of IFNα, were discovered in 1957 for their

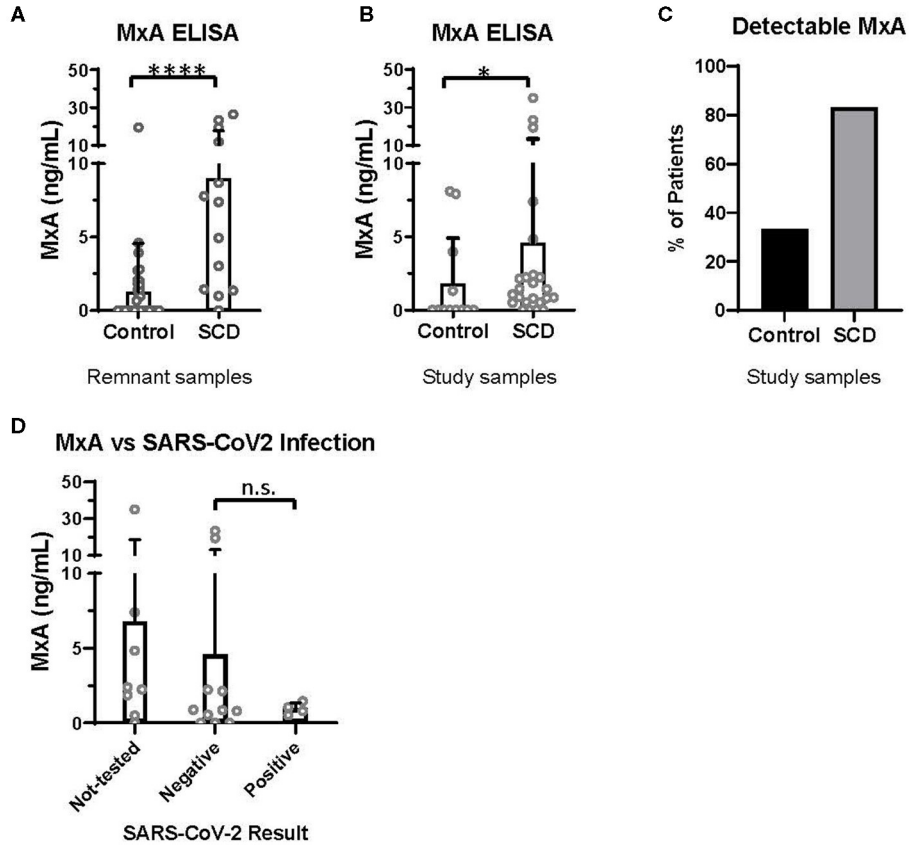


FIGURE 1 | MxA expression is increased in patients with SCD, compared to controls. MxA expression in whole blood measured by MxA Protein Human ELISA (BioVendor). **(A)** MxA expression in de-identified remnant samples from patients with ($n = 13$) or without SCD ($n = 37$). **(B)** MxA expression in patients with SCD ($n = 24$) and race-matched controls ($n = 12$) summarized in **Table 1**. **(C)** Percentage of subjects summarized in **Table 1** with detectable levels of MxA. **(D)** MxA expression of patients with SCD ($n = 24$) who were not tested or tested positive ($n = 4$) or negative ($n = 11$) for SARS-CoV-2 by PCR. n.s., not significant by Kruskal Wallis test with a Dunn's post-test. $^{*}p < 0.05$, $^{****}p < 0.0001$ by Mann-Whitney *U*-test. Bars represent the mean. Circles represent values from individual patient or control samples, and error bars represent the standard deviation.

critical role in anti-viral immunity (16). During infection, viral nucleic acids bind pattern recognition receptors and induce IFNα/β, which in turn activate signaling via the IFNα/β receptor to produce interferon-stimulated genes (ISGs) that interfere with viral replication and promote the production of neutralizing antibodies. Independent of COVID-19, Hounkpe et al. performed a meta-analysis of gene expression studies and identified a cluster of ISGs enriched in patients with SCD (17). In addition, Hermand et al. recently reported that serum IFNα and ISGs produced by neutrophils are elevated in children with SCD, compared to healthy blood donors (18). Here, we assessed the baseline expression of a well-characterized ISG, Myxovirus Resistance Protein 1 (MxA), in adults with SCD compared to race-matched controls, and describe SARS-CoV-2 test results and COVID-19 hospitalization outcomes in one patient cohort.

METHODS AND RESULTS

Discovered in 1962 for its role in resistance to orthomyxoviruses, including influenza, MxA is a GTPase that targets nucleocapsid proteins and inhibits viral transcription and replication (19, 20). MxA has since been reported to be a clinically applicable biomarker of IFNα/β activation (21). In the pre-COVID era, we utilized an ELISA-based assay (BioVendor, Czech Republic) to quantify MxA levels in the whole blood of patients with and without SCD, according to manufacturer instructions. Initially, 13 SCD and 37 control de-identified remnant type and screen samples were randomly selected from the blood bank. All samples were analyzed within 72 h of the blood draw. In this initial analysis, patients with SCD expressed a significantly elevated level of MxA, compared to controls (**Figure 1A**).

Given that these initial samples were de-identified and the health status of patients and controls was unknown, a second study comparing MxA levels in patients with SCD seen in out-patients clinics for routine care and race-matched controls was initiated. Blood was drawn from 24 patients and 12 controls in their baseline state of health. 22 patients had Hgb SS, 1 had Hgb SC, and 1 had $S\beta^0$ disease (**Table 1**). Exclusion criteria included acute illness or crisis, pregnancy, and lack of competency to provide informed consent. The study was approved by the Yale Institutional Review Board.

TABLE 1 | Demographics of subjects enrolled in second study.

		SCD	Control
Gender	Male (%)	7 (29%)	6 (50%)
	Female (%)	17 (71%)	6 (50%)
Mean age in years (Std Dev)		29.3 (9.8)	41.2 (13.7)
Hgb S disease	SS (%)	22 (92%)	
	SC (%)	1 (4%)	
	$S\beta^0$ (%)	1 (4%)	

Gender, age, and hemoglobinopathy of subjects. SCD, sickle cell disease; Std Dev, standard deviation.

In this second study, MxA was also significantly elevated in patients with SCD compared to control subjects (**Figure 1B**). 83% of patients with SCD had detectable MxA, compared to 33% of controls (**Figure 1C**). It is noteworthy that expression of MxA, as well as IFNα and other ISGs (18), is highly variable amongst patients with SCD, which may contribute to differences in anti-viral immunity. In addition, MxA was detected in four of 12 controls, which may reflect the role of IFNα/β and MxA in other diseases, including autoimmunity and viral infections (19, 21).

Finally, we retrospectively examined SARS-CoV-2 testing results and COVID-19 severity of patients with SCD enrolled in the second study. Fifteen of the 24 patients had test results of SARS-CoV-2 PCR documented in the electronic medical record. Eleven patients had only negative test results and four patients tested positive. Three of the four with positive PCR results were admitted to the hospital: two were diagnosed with acute chest syndrome and received supplemental oxygen but neither required intubation; all patients survived. MxA levels of these patients, according to SARS-CoV-2 test results, are shown in **Figure 1D**. There were no significant differences in MxA levels between untested patients, patients with negative results, and patients with positive results.

DISCUSSION

Data presented here, in combination with prior studies (17, 18), indicate that patients with SCD express an IFNα/β gene signature. As IFNα/β and ISGs are upregulated as a result of SARS-CoV-2 infection and vaccination (22), it is worth noting that these laboratory studies were completed prior to the COVID-19 pandemic. This allowed for baseline measurement of MxA without the potential variable of SARS-CoV-2 infection or vaccination. While SARS-CoV-2 test results and COVID-19 hospitalization outcomes were examined, based on the sample size, no definitive conclusions can be drawn about baseline MxA levels and the risk of SARS-CoV-2 infection or disease severity. While it is plausible that elevated baseline anti-viral IFNα/β and ISGs, including MxA, contribute to the variable progression of COVID-19 and other viral infections in patients with SCD, it is certain that other inflammatory responses, including production of IL-1b, TNFα, and IL-6, also significantly contribute to the anti-viral response (23).

Multiple groups have investigated the role of IFNα/β in COVID-19 disease progression, independent of SCD. Utilizing mass cytometry and Nanostring technology, Hadjadj et al. performed immune profiling of 50 COVID-19 patients and observed an impaired IFNα/β response in severe and critically ill patients, compared to those with mild to moderate disease (24). Also in support of a protective role of IFNα/β, another group found inborn errors of IFNα/β immunity and an increased prevalence of autoantibodies against IFNα/β, including IFNα and IFNω, in critically ill COVID-19 patients, compared to those with mild disease or asymptomatic infection (25, 26). These results are consistent with recent reports of reduced COVID-19 associated mortality in patients treated with IFNβ (27, 28), and reduced duration of detectable virus and inflammation in a cohort treated with IFN-α2b (29).

Conversely, another group concluded that the IFNα/β response can also exacerbate deleterious COVID-19 induced inflammation. Utilizing single cell RNA sequencing, Lee et al. observed that an IFNα/β response was absent in mild disease but co-existed with Tumor Necrosis Factor α (TNFα) and IL-1b production in severe COVID-19 disease (13). However, it is unclear whether the correlation between IFNα/β and severe disease in this study is causal or responsive. In addition, Ziegler et al. demonstrated that angiotensin converting enzyme 2 (ACE2), a receptor for the SARS-CoV-2 spike protein, is an ISG expressed by multiple pulmonary cell types, suggesting that the virus may exploit the IFNα/β response to gain viral entry (30).

Although evidence supporting a protective role of IFNα/β outweighs that supporting a deleterious role in COVID-19 disease progression, it remains an area of current investigation. Ongoing clinical trials have shown conflicting results. A phase 2 trial of inhaled IFNβ showed clinical improvement (31), while data from the World Health Organization Solidarity trial indicate that IFNβ is not effective in improving mortality or other endpoints (32). Others have postulated that the timing of IFNα/β responses may impact the outcomes of the aforementioned studies (33). Early robust responses and early treatment with inhaled IFNβ may lead to reduced viral load; whereas delayed and dysregulated IFNα/β responses or IFNα/β-based treatments may exacerbate deleterious hyperinflammation at later stages of disease (34, 35).

It is noteworthy that IFNα/β has also been implicated in the pathogenesis of autoimmune diseases, including rheumatoid arthritis, myositis, Sjögren's syndrome (SS), systemic sclerosis and systemic lupus erythematosus (SLE) (36–40). Approximately two-thirds of adult patients and nearly all children with SLE express an IFNα/β gene signature (41, 42), and more than 50% of SLE-associated genetic variants have been linked to the IFNα/β pathway (43). In addition, approximately 25% of patients with SLE produce anti-IFNα/β autoantibodies, which are associated with decreased disease activity (44, 45). Due to the IFNα/β gene signature and the use of hydroxychloroquine in patients with SLE, many groups have investigated COVID-19 disease progression in patients with SLE and have reported variable results (46, 47). Unfortunately, it has been difficult to separate the effects of baseline IFNα/β activity and the use

of immunosuppressive therapies, which result in an increased incidence of multiple viral infections in patients with SLE (48).

Specific mechanisms leading to IFNα/β activation during viral infection or SLE are fairly well-characterized. However, IFNα/β-inducing stimuli and pathways in SCD have not been identified. Heme-induced inflammation is one of many candidates yet to be investigated. Heme, released from hemolyzed RBCs, binds to Toll-like receptor 4 and induces NFκb-mediated production of pro-inflammatory cytokines, including IL-6, IL-12, and TNFα (49). However, its role in IFNα/β activation has not been reported, and many other ligands and pathways are worthy of investigation. Moreover, while anti-IFNα/β antibodies that prevent IFNα/β-induced inflammation have been described in patients with autoimmune polyendocrinopathy syndrome, SLE and COVID-19 (25, 50, 51), it is unclear whether they are produced by patients with SCD and whether they contribute to the variable disease progression during viral infection.

Finally, unlike in viral infection and autoimmunity, the contribution of IFNα/β activation to chronic inflammation and the numerous sequelae of SCD are unknown and warrant investigation. For example, endothelial damage is a hallmark of SCD associated with vaso-occlusive crises, including acute chest syndrome and cerebrovascular accidents, and mortality (52, 53). IFNα/β has been shown to induce endothelial damage in patients with anti-phospholipid syndrome, thrombotic microangiopathy, and SLE, and recipients of IFNα/β therapy (54–57). However, the role of IFNα/β in endothelial damage, and its associated adverse events, in SCD has not been investigated. In addition, prior studies reported that IFNα/β is a critical regulator of RBC alloimmunization in transfusion mouse models (58–61). Alloantibody production against minor RBC antigens can lead to significant hemolytic events and severely limit availability of blood products for alloimmunized patients. As patients with SCD have the highest incidence of RBC alloimmunization (62) compared with any other disease population, the role of IFNα/β in RBC alloimmunization in patients with SCD warrants further study.

In conclusion, data presented here, in combination with other studies, indicate that the majority of patients with SCD express an IFNα/β gene signature. While the impact of IFNα/β and ISGs on SARS-CoV-2 infection risk or COVID-19 disease severity remains to be fully determined, baseline IFNα/β activity may contribute to the variable disease progression reported in prior studies. Additional studies investigating mechanisms underlying IFNα/β production and clinical consequences of IFNα/β-mediated inflammation in SCD are needed. Future studies elucidating the role of IFNα/β in chronic inflammation, RBC alloimmunization, and vaso-occlusive events could lead to targeted therapies to mitigate severe sequelae in patients with SCD.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Yale University Institutional Review Board. Written informed consent to participate in this study was provided by the participants or the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

Project design and data analysis were completed by EM, DO-D, JEH, and DRG. Experiments were completed by DRG, EM, and RB-M. DRG wrote the initial draft of the manuscript. All authors contributed to edits of the manuscript.

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REVIEW
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Extracellular Vesicles in Sickle Cell Disease: Plasma Concentration, Blood Cell Types Origin Distribution and Biological Properties

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Prototype of monogenic disorder, sickle cell disease (SCD) is caused by a unique single mutation in the β-globin gene, leading to the production of the abnormal hemoglobin S (HbS). HbS polymerization in deoxygenated condition induces the sickling of red blood cells (RBCs), which become less deformable and more fragile, and thus prone to lysis. In addition to anemia, SCD patients may exhibit a plethora of clinical manifestations ranging from acute complications such as the frequent and debilitating painful vaso-occlusive crisis to chronic end organ damages. Several interrelated pathophysiological processes have been described, including impaired blood rheology, increased blood cell adhesion, coagulation, inflammation and enhanced oxidative stress among others. During the last two decades, it has been shown that extracellular vesicles (EVs), defined as cell-derived anucleated particles delimited by a lipid bilayer, and comprising small EVs (sEVs) and medium/large EVs (m/lEVs); are not only biomarkers but also subcellular actors in SCD pathophysiology. Plasma concentration of m/lEVs, originated mainly from RBCs and platelets (PLTs) but also from the other blood cell types, is higher in SCD patients than in healthy controls. The concentration and the density of externalized phosphatidylserine of those released from RBCs may vary according to clinical status (crisis vs. steady state) and treatment (hydroxyurea). Besides their procoagulant properties initially described, RBC-m/lEVs may promote inflammation through their effects on monocytes/macrophages and endothelial cells. Although less intensely studied, sEVs plasma concentration is increased in SCD and these EVs may cause endothelial damages. In addition, sEVs released from activated PLTs trigger PLT-neutrophil aggregation involved in lung vaso-occlusion in sickle mice. Altogether, these data clearly indicate that EVs are both biomarkers and bio-effectors in SCD, which deserve further studies.

Keywords: extracellular vesicles, sickle cell disease, inflammation, coagulation, oxidative stress, endothelial dysfunction

INTRODUCTION

Sickle cell disease (SCD) is one of the most frequent autosomal recessive genetic disorder that affects about 3.2 million people worldwide (1, 2). SCD is an umbrella term encompassing several sickle cell syndromes having in common the production of an abnormal hemoglobin, named hemoglobin S (HbS) instead of the normal hemoglobin A. HbS is produced as a result of a single base mutation (rs334) in exon 1 of the β-globin gene, leading to the replacement of a hydrophobic glutamic acid residue by a hydrophilic valine residue at the sixth position of the mature β-globin chain (3). Sickle cell anemia (SCA) results from the homozygous inheritance of the β^S mutation, whereas co-inheritance of β^S with other mutations such as β^C, $\beta^{DPunjab}$, β^{OArab} or β-thalassemia alleles lead to the other most frequently encountered sickle cell syndromes, namely HbSC, HbSDPunjab, HbSOrab and HbS-β-thalassemia, respectively, the latter one being subdivided in HbSβ^0-thal and HbSβ^+-thal (4).

Chronic anemia is a common clinical feature associated with the disease, as well as the occurrence of frequent and recurrent vaso-occlusive crises. In addition, SCD patients may exhibit various acute and chronic complications affecting a large number of organs such as the lungs, heart, kidneys, brain, skin and bones (5). Among the four drugs approved for prophylaxis and treatment of complications related to SCD, namely, hydroxyurea (HU), L-glutamine, voxelotor, and crizanlizumab; HU is the most commonly prescribed treatment (6). It is worthwhile to notice that SCD is characterized by a huge inter-individual variability in its clinical presentation, including for patients sharing the same sickle cell syndromes (7). This clinical variability could be related to the complex pathophysiology of this hemoglobinopathy for which new features and/or actors have recently been identified.

After a presentation of the main interrelated pathophysiological processes of SCD, we will present, in this review, compelling evidence showing that extracellular vesicles (EVs) are not only biomarkers of cellular activation and/or alterations occurring in SCD, but also bio-effectors able to modulate the different pathophysiological mechanisms.

SCD PATHOPHYSIOLOGY: A COMPLEX SCHEMA AND INTERRELATED PATHWAYS

HbS polymerization is the primary molecular event of SCD pathophysiology. In deoxygenated conditions, HbS proteins aggregate, form fibrous precipitates, and ultimately lead to red blood cell (RBC) sickling. These sickled RBCs are more rigid, fragile and therefore prone to disruption. HbS polymerization induces oxidative damage of the cytoplasmic membrane responsible for the stiffness of these cells and their shortened half-life (8–10). Increased RBC fragility and decreased deformability have been associated to chronic anemia and recurrent painful vaso-occlusive event, respectively (11). However, it has been recognized more than four decades ago that the transit time of RBCs in deoxygenated vascular areas, the territories affected by vaso-occlusive processes, would theoretically be too short to allow the sickling of RBCs (12). Activation and increased adhesiveness of various blood cell types such as neutrophils, monocytes and platelets to the endothelium (13–16), may trigger vaso-occlusion by decreasing blood flow and thereby increasing the RBC transit time in vascular bed with low oxygen content, leading to the sickling of RBCs before they can escape from the microcirculation (16). Sickled RBCs and stress reticulocytes, detected at abnormal level in the blood of SCD patients in response to anemia, also interact with endothelial cells (16). Aggregates of activated platelets and RBCs, monocytes or neutrophils, observed at abnormal levels in SCD patients (17–19), may also contribute to decreasing blood flow. The percentage of aggregates has been correlated with disease severity (20, 21). Finally, patients with the highest blood viscosity would also be prone to frequent vaso-occlusive crises because of the rise in vascular resistance and the slowing of blood flow (22–24).

Pro-inflammatory State and Oxidative Stress

SCD has long been recognized as a chronic inflammatory disease associated with enhanced oxidative stress. A key role of intravascular hemolysis in these two conditions has been identified as shown and summarized in **Table 1**. In SCD patients and more particularly in those with SCA or Sβ^0-thalassemia, hemolysis exceeds the capacity of plasma heme-binding proteins such as haptoglobin and hemopexin, leading to their depletion and thus the cell-free circulation of two toxic and oxidative molecules: hemoglobin and heme (29–31). Enhanced auto-oxidation of HbS induces the production of reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide and hydroxyl radical as well as the release of heme from sickle RBCs (25, 26, 42). Another significant source of ROS is related to the repeated episodes of ischemia-reperfusion occurring during repeated vaso-occlusive events and inducing high plasma levels of xanthine oxidase and NADPH oxidase (27, 28). Although conflicting results on antioxidant levels in SCD patients have been reported (43–45), the antioxidant capacity is insufficient to neutralize the excess of ROS, resulting in chronic oxidative stress (32). Enhanced oxidative stress may lead to endothelial damages through peroxidation of the lipid membrane and/or DNA fragmentation and ultimately cellular apoptosis (33) and has been linked to vascular alterations in SCD patients (34). In addition to these deleterious effects, ROS may promote vascular inflammation and NF-κB endothelial activation through the activation of redox-sensitive transcription factors such as (35). More recently, it has been shown that free heme may activate monocytes/macrophages (36, 37), neutrophils (38), platelets (39) and endothelial cells (40) inducing the secretion of pro-inflammatory cytokines and the activation of cell adhesion pathways, key events in heterocellular interactions leading to vaso-occlusion. Several studies have demonstrated that these heme-dependent cellular activations involved the Toll like receptor 4 (TLR4) and the NLRP3 inflammasome signaling pathways in endothelial cells and monocytes/macrophages (40, 46, 47). In addition, activation of neutrophils, one of the blood cell type playing a key role in vaso-occlusive process (16), by heme also induces the formation of neutrophil extracellular

TABLE 1 | Involvement of intra-vascular hemolysis in oxidative stress and chronic pro-inflammatory state in SCD.

References	Main findings
(23, 25, 26)	Enhanced auto-oxidation of HbS leading to the production of ROS and hemolysis
(27, 28)	Repeated episodes of ischemia-reperfusion inducing high plasma levels of xanthine—oxidase, NADPH and ROS
(29–32)	Exceeding antioxidant capacity of the patient, including low levels of plasma heme and hemoglobin binding proteins such as hemopexin and haptoglobin leading to enhanced cell-free circulation of heme and hemoglobin
(33–35)	Vascular inflammation and endothelial activation mediated by ROS through the NF-κB pathway
(36–40)	Activation of monocytes/macrophages, neutrophils, platelets and endothelial cells by cell-free heme
(38, 41)	Production of neutrophil extracellular trap by activated neutrophils leading to higher endothelial expression of VCAM-1 and ICAM-1

traps (NETs) for which high plasma concentration has been detected in SCD patients at steady-state with a further rise during crisis (38). NETs could participate to the chain of deleterious events occurring in SCD by promoting VCAM-1 and ICAM-1 endothelial expression (41), two proteins involved in the abnormal interactions between RBCs and endothelial cells (16), and by providing a scaffold for platelets, RBCs and pro-coagulant molecules (48).

Decreased Bioactivity/Bioavailability of Nitric Oxide

Another deleterious effect of intravascular hemolysis is its impact on the bioactivity/bioavailability of nitric oxide (NO). NO, produced by endothelial NO-synthase, play a key role in the vascular physiology. This free radical induces vasodilation by relaxing perivascular smooth muscles, down-regulates the expression of endothelial adhesion molecules such as ICAM-1, VCAM-1, E- and P selectins and inhibits platelets activation (49, 50). Cell-free hemoglobin inactivates NO in a dioxygenation reaction leading to the production of methemoglobin and the release of heme into the plasma (51). This inactivation of NO is very efficient and 1,000 times faster than the one mediated by hemoglobin encapsulated into RBCs (52). Another consequence of hemolysis is the release of arginase by RBCs into the plasma, an enzyme that consumes plasma L-arginine, the precursor of NO, producing ornithine and urea, and thereby exacerbating the decrease of NO bioavailability (53). The decrease of NO bioactivity/bioavailability and thus the resulting endothelial/vascular dysfunction has been linked to a greater risk of developing several SCD complications such as pulmonary hypertension (54), legs ulcers (55), priapism (56), stroke (57) and proteinuria (58).

Pro-coagulation State

Chronic activation of coagulation is another feature of SCD pathophysiology (59, 60). High plasma levels of markers of thrombin production such as prothrombin fragment 1.2 (F1.2), thrombin—antithrombin (TAT) complexes, D-dimers and plasmin—antiplasmin (PAP) complexes have been constantly detected in the plasma of SCD patients (61). Additionally, SCD patients exhibit low levels of protein C and protein S, two endogenous anticoagulants, presumably because of their chronic consumption related to ongoing coagulation activation (61). Tissue factor (TF), the primary initiator of extrinsic coagulation pathway, is one of the identified triggers responsible for the coagulation activation. In SCD patients, increased TF expression in monocytes (62), neutrophils (63) and circulating endothelial cells (64) have been detected. In agreement with the reported association between hemolysis marker levels and those of the coagulation activation in SCD patients (62), it has been shown that heme is able to promote TF expression in endothelial cells and blood mononuclear cells (40, 65). Moreover, the low levels of contact system proteins also suggest a contribution of the intrinsic pathway to thrombin generation (66). Externalized RBC phosphatidylserine (PS), detected in SCD patients in high amounts, may provide a negative charge surface allowing the docking of tenase and prothrombinase complexes, which in turn may promote the activation of the intrinsic pathway. Significant correlations between PS-positive sickle RBCs and plasma F 1.2, D-dimer and PAP complexes have been reported (67, 68). Additionally, high levels of cell-free DNA and nucleosomes released from neutrophils, two other triggers of the contact system activation, have been reported in the plasma of SCD patients (69–71). However, no correlation studies have been performed to our knowledge. If SCD chronic hypercoagulable state has been associated with an increased risk of limited complications such as venous thrombosis (72, 73), pulmonary hypertension (74) and *in situ* thrombosis of small vessels, it is worthwhile to notice that increased thrombin generation may also contribute to vascular inflammation (75).

This brief overview of SCD pathophysiology illustrates the fact that numerous abnormal pathways have been identified so far with multiple inter-relationships between these pathways. During the last decades, the involvement of the so-called extracellular vesicles in this complex pathophysiology has been documented.

EXTRACELLULAR VESICLES IN SCD

Classification of Extracellular Vesicles

Extracellular vesicles (EV) are a generic term for various particles delimited by a lipid bilayer, released from cells and detectable in numerous biological fluids (76). According to their genesis pathways, three main subtypes have been identified and named exosomes, microparticles (MPs) also called microvesicles, and apoptotic bodies. Exosomes, deriving from the endolysosomal pathways or from the outwards budding of the cytoplasmic membrane, are formed within the multivesicular bodies (MVBs) and released upon fusion of MVBs with plasma membrane (77).

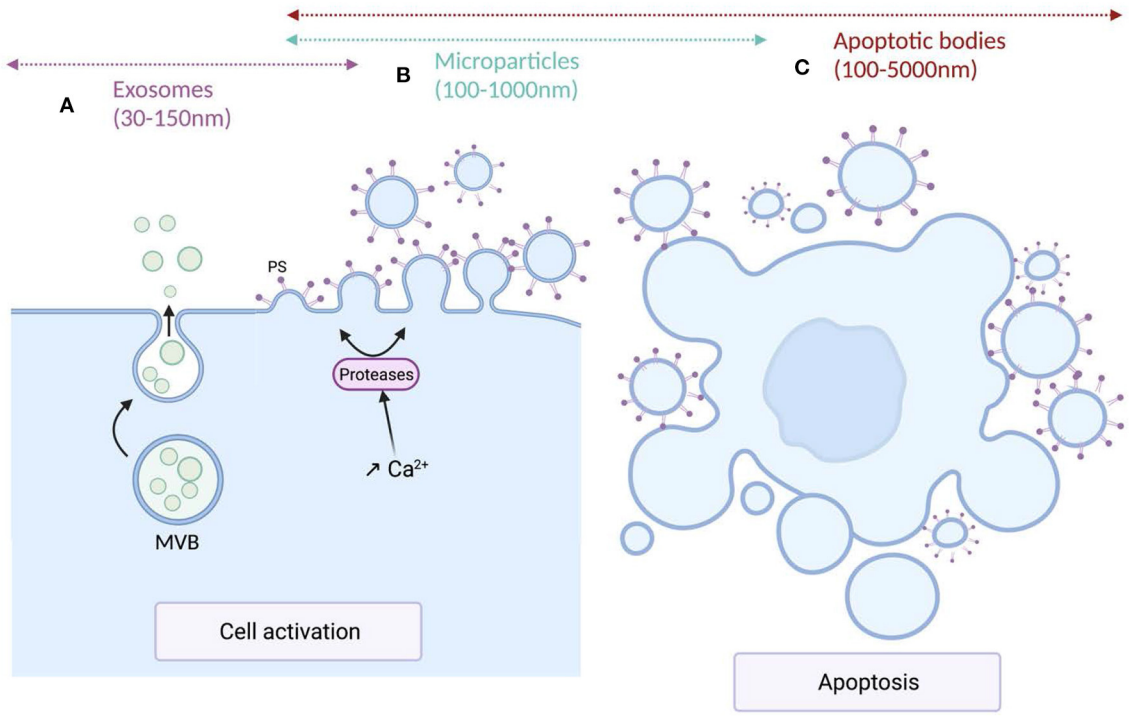

FIGURE 1 | Mechanisms of production and size of the different extracellular vesicles types. **(A)** Exosomes are formed within multivesicular bodies (MVBs) and released upon fusion of MVBs with plasma membrane. Exosomes (sEVs) exhibit a narrow diameter ranging between 30 and 150 nm. **(B)** Microparticles (m/lEVs) diameter ranges from 100 to 1,000 nm. MPs derive from the cytoplasmic membrane of activated or apoptotic cells. Cell activation induces an increase of intracellular Ca^{2+} concentration leading to the translocation of phosphatidylserine (PS) to the outer leaflet of the cytoplasmic membrane and the activation of proteases that cleave the cytoskeleton, weaken its interaction with the cytoplasmic membrane, ultimately leading to the release of m/lEVs. **(C)** Apoptotic bodies are the largest EV subtypes exhibiting the wider size distribution (100–5,000 nm). They result from cell fragmentation and decomposition of the cell membrane of apoptotic cells.

Compared to the other EV subtypes, exosomes exhibit a narrow size ranging from 30 to 150 nm in diameter. Microparticles, ranging from 100 to 1,000 nm in diameter, derive from the cytoplasmic membrane of activated, stressed or apoptotic cells. These conditions induce the increase of intracellular Ca^{2+} leading to the translocation of phosphatidylserine (PS) to the outer leaflet of the cytoplasmic membrane, a structural characteristic of these EV subtype, and to the activation of proteases that cleave cytoskeleton, weaken its interaction with the cytoplasmic membrane and ultimately allowing the release of MPs (78). Apoptotic bodies, the larger EV subtypes exhibiting the wider size distribution (100–5,000 nm) result from cell fragmentation and decomposition of the cell membrane of apoptotic cells (79, 80). The size distribution and the biogenesis pathways of the different EV subtypes are illustrated in **Figure 1**.

Over time, several techniques have been implemented for quantitative and/or qualitative analysis of EVs such as flow cytometry, dynamic light scattering, nanoparticles tracking analysis, scanning and transmission electron microscopy, cryo-electron microscopy and atomic force microscopy (81). Up to now, flow cytometry is clearly the most commonly used technique for EV analysis. Using fluorescent probes such as labeled Annexin V, a protein with high affinity for PS, and labeled antibodies directed against membrane proteins specific of each blood cell types, plasma concentration and cellular origin of EVs could be theoretically established. However, flow cytometry encounters several shortcomings including limited sensibility and resolution, leaving uncharacterized a significant proportion of the smallest EVs even with the most sensitive flow cytometers (82). Besides, it has been shown that numerous parameters such as technical characteristics of flow cytometers, pre-analytical and analytical conditions among others, significantly impact on both quantitative and qualitative EVs analysis. Since these pitfalls and limits have been extensively reviewed (83–85), they will not be discussed in the present review. To overcome these limitations, specific recommendations and guidelines have been produced (86, 87). However, most of the studies that aimed to characterize EVs in SCD, have been performed previously to their publication or did not apply these recommendations. Conflicting results found in the literature and described later are undoubtedly related to non-standardized procedures.

Originally described as dust cells in the late sixties (88), it has been demonstrated since then that EVs can mediate intercellular communication in both physiological and pathophysiological conditions (89–91) through the transfer to the recipient cells of their biological content, i.e., proteins, lipids, mRNA and miRNA (90, 92). In addition, high plasma levels of EVs have been detected in various diseases such as cardiovascular diseases,

TABLE 2 | Comparison of blood cell type-derived EVs determined by flow cytometry between SCD patients and healthy controls.

References	Type of EVs	Patients included	EV cellular origin assessed[a]	Compared to healthy controls
Feng et al. (95)	m/lEVs	27 SCD patients	– RBCs (CD235a), – Platelets (CD41a), – Monocyte (CD14), – Endothelial cells (CD114)	– ↗in SCD – ↗in SCD
Witwer et al. (96)	m/lEVs	50 SCD patients:	– RBCs (CD235a), – Platelets (CD41a),	– ↗in SCD – ↗in SCD
Shet et al. (97)	m/lEVs	92 SCD patients:	– RBCs (CD235a), – Platelets (CD41a),	– ↗in SCD – ↗in SCD
Tantawy et al. (98)	m/lEVs	29 SCA patients	– RBCs (CD235a), – Platelets (CD41a),	– ↗in SCA – ↗in SCA
Gerotziafas et al. (99)	m/lEVs	45 SCD patients	– RBCs (CD235a), – Platelets (CD61), – Monocyte (CD14), – Endothelial cells (CD106)	– ↗in SCD – ↗in SCD – ↗in SCD – ↗in SCD
Kasar et al. (100)	m/lEVs	138 SCD patients	– RBCs (CD235a)	– ↗in SCD
van Tits et al. (101)	m/lEVs	232 SCA patients	– RBCs (CD235a), – Reticulocytes (CD71) – Platelets (CD61), – Leucocytes (CD45), – Endothelial cells (CD106)	– ↗in SCA – ↗in SCA – ↗in SCA – ↗in SCA
Dembélé et al. (102)	sEVs	22 SCA patients	– RBC (CD235a), – Platelets (CD31/CD42b), – Monocytes (CD45/CD14), – Endothelial cells (CD309/CD133) – Progenitor cells (CD309/CD34)	– ↗in SCA – ↗in SCA – ↗in SCA – ↗in SCA – ↗in SCA
Garnier et al. (103)	sEVs	33 SCD patients	– RBCs (CD235a), – Platelets (CD31/CD42b), – Monocytes (CD45/CD14), – Endothelial cells (CD309/CD133) – Lymphocytes (CD45) – Progenitor cells (CD309/CD34)	– ↗in SCA – ↗in SCA – ↗in SCA – ↗in SCA – ↗in SCA

[a] *In bracket is indicated the blood cell specific CD used to determine the cellular origin of EVs.*

artherosclerosis, cancer and diabetes (93). All these disorders share several key pathophysiological components with SCD such as, increased risk of thrombosis, endothelial dysfunction, enhanced oxidative stress and high level of inflammation which may lead to increased release of EVs. For example, pro-inflammatory state and ischemic-reperfusion induce cellular activation and/or apoptosis and thus the production of EVs from various blood cell types such as endothelial cells, leukocytes, platelets and red blood cells as observed in ischemic coronary disease (94). In diabetic patients, endothelial dysfunction, evaluated by endothelial-dependent flow-mediated dilation, has been positively correlated with EV concentration released mostly by apoptotic endothelial cells (95). Altogether, these data strongly suggest that pro-inflammatory-state, pro-thrombotic-state and endothelial dysfunction are among the pathophysiological pathways associated with increased release of EVs.

It is important to notice that most of the reported studies in the field of EVs in SCD, do not provide any information on the biogenesis of these vesicles and used either their size and/or their density to classify them. Therefore, in the present review, we will use the terms of small EVs (sEVs) and medium/large EVs (m/lEVs), for exosomes and microparticles/microvesicles, respectively, according to the classification proposed by the International Society of Extracellular Vesicles (96). Knowing that there are significant overlaps of both size and density parameters between each EV subtype, this classification could be partly artificial and some of the reported associations and/or biological functions could be related to a mixture of different EV subtypes instead of one specific subtype.

Plasma Levels of Extracellular Vesicles in SCD

In Steady State Condition

As indicated in **Table 2**, several reports have documented higher levels of EVs in SCD patients at steady state, i.e., at distance of acute complication and blood transfusion, compared to healthy individuals (97–102, 104, 105). Since most of these studies were performed using flow cytometers unable to detect sEVs, the previous observations are undoubtedly related to m/lEVS. Increased plasma concentrations of m/lEVs were detected not only in SCA patients but also in SC patients, although the levels reached in the latter population were not as high as in the former one (103). So far, no study specifically dedicated to patients with Sβ-thalassemia has been conducted. These m/lEVs derived mainly from platelets and RBCs while those originated from the other blood cell types such as endothelial cells, monocytes or

granulocytes, were usually either barely detectable or detectable at low levels. In contrast, rather limited number of studies have been conducted on sEVs in SCD patients. Two reports from the same group described higher plasma concentration of sEVs in children and young adults with SCD at steady state level compared to controls (106, 107). The cellular origins of sEVs detected in SCD patients show a wider distribution than those of m/lEVs, being originated from RBC precursors, endothelial cells, lymphocytes, monocytes and platelets. All these plasma EV subtypes exhibit high concentrations in SCD patients, compared to controls, except for those originated from platelets. To our best knowledge, no study dedicated to apoptotic bodies has been performed in SCD yet.

The clinical severity of the disease has been linked to plasma EV levels measured at steady state. Severe vaso-occlusive phenotype (98, 104) and positive history of acute chest syndrome, pulmonary hypertension (98), osteonecrosis of the femoral head (108) and leg ulcers (102) have been associated with high concentration of m/lEVs released from various blood cell types. In contrast, lower concentration of reticulocyte-derived and RBC-derived m/lEVs were detected in SCA patients with a positive history of priapism and retinopathy, respectively, compared to SCA patients without these complications (102).

Few studies have specifically addressed the relationship between sEVs and the previous clinical course of SCD. Based on a cohort of 22 SCA children followed since birth and classified according to the painful vaso-occlusive rate, higher counts of sEVs originated from endothelial cells, progenitor cells, monocytes and lymphocytes were detected in the most severe patients compared to the milder ones (106). In addition, comparison of sEVs miRNA content between these two groups lead to the identification of miRNA expression patterns specific of the disease severity. A classification based on acute chest syndrome rate failed to detect difference in sEV levels except for those derived from monocytes (107).

Overall, if high levels of EVs have been repeatedly detected in SCD patients at steady-state, few of the associations with the severity of the clinical course of the disease have been reproduced and the usefulness of both EV subtypes as biomarkers of previous occurrence of specific SCD complication needs to be confirmed.

During Acute Complication

It has been shown that the clinical status of the SCD patients may impact m/lEVs concentration. Higher levels of m/lEVs have been reproducibly reported during vaso-occlusive crisis compared to steady state condition (97, 98, 100, 101, 109). However, the cellular origins of these vesicles, for which an increase had been detected, varied from one study to another. Several parameters may explain these discrepancies such as the study design, clinical definition of sickle cell crisis, the delay between blood sampling and hospital admission, and pre-analytical and analytical procedures used (110, 111). In the larger longitudinal survey published so far, in which 32 SCA patients were analyzed both at steady state and during painful vaso-occlusive crisis, a 2-fold increase in blood concentration of RBC-derived m/lEVs has been detected (112), in agreement with previous reports (98, 100, 109), as well as an increase of PS-externalization by these m/lEVs. Further studies are warranted to confirm and better describe these qualitative changes which impact on the biological properties of these vesicles, as described in subsequent paragraphs. To our knowledge, analysis of sEVs during the occurrence of SCD complications has not been performed yet.

In Patients Treated With Hydroxyurea

The impact of hydroxyurea (HU) treatment on the concentration of m/lEVs, the only EV subtype analyzed so far in relation to HU treatment, is still controversial. Indeed, decreased (98, 113, 114), increased (109, 115) and unchanged (96, 105) m/lEV concentrations have been reported in HU-treated SCA patients, compared to untreated patients. Knowing the wide distribution of m/lEV concentration in SCA patients (103, 112, 113), these contradictory results could be related to the cross-sectional design of these studies. In order to reduce the inter variability, we have implemented a longitudinal follow-up of SCA patients before and after 2 years of HU treatment (116). While no change in m/lEV concentration was detected, two qualitative parameters of m/lEVs originated from RBCs were modified during the course of HU treatment: EVs size was increased and their PS exposure was decreased. Such HU-related changes could affect their biological properties and need to be confirmed by further longitudinal studies.

Triggering Pathways of EV Release in SCD

Several triggers and pathways of EV biogenesis in SCD pathophysiological context have been identified for two blood cell types: RBCs and platelets.

After the initial observation that repeated RBC sickling/unsickling induce the shedding of EVs (117), it has been shown that oxidative stress resulting from accelerated denaturation of HbS, leads to RBC membrane protein oxidation, weaker interactions between the membrane skeleton and lipid bilayer, destabilization of RBC cytoplasmic membrane and ultimately to EVs shedding (118–120). More recently, the involvement of hyperphosphorylation of Band 3, induced by the inhibition of SCD RBC tyrosine phosphatase (121, 122), in the release of EVs, has been documented. Indeed, direct relationship between tyrosine phosphorylation of Band 3 and the concentration of RBC-derived m/lEVs has been detected in SCD patients while *in vitro* inhibition of Syk kinases, the kinases responsible for Band 3 phosphorylation, was associated with lower shedding of these vesicles (123). Phosphorylation of Band 3 has also been linked to storage lesions of RBCs and RBC vesiculation (124). In addition, eryptosis, a condition associated with clustering of Band 3, induced by Band 3 phosphorylation, and characterized by increased RBC calcium level, PS externalization, RBC shrinkage, energy depletion and membrane blebbing, has been associated with the release of m/lEVs (125). Since Band 3, the most abundant protein of the RBC cytoplasmic membrane, plays a key role in membrane stability and deformability by linking the lipid bilayer to the cytoskeleton, it is not surprising that plasma level of m/lEVs produced by RBCs were reproducibly associated with both anemia level and hemolytic markers levels in SCD patients (103–105, 114, 126, 127).

Another identified biogenesis pathway of RBC-derived EVs relies on the dysregulation of the metabolism of sphingolipids. Sphingomyelinase, one of the key enzymes of this metabolic pathway, hydrolyzes sphingomyelin, a lipid representing 10% of the total lipid of the plasma membrane. It is activated by membrane curvature and increased mechanical bending stress in RBCs (128). The increased activity of sphingomyelinase in sickle RBCs from sickle mice compared to control mice, was linked to the generation of both sEVs and m/lEVs. Moreover, *in vivo* and *in vitro* pharmacologic inhibition of sphingomyelinase reduces their release from RBCs (129).

It has also been shown that infusion of thrombospondin-1, a protein stored in platelet α-granules and released upon activation, in mouse models, induces the shedding of RBC-m/lEVs through CD47 signaling pathway, a process exacerbated in sickle mice (130).

As previously described, several hemolysis end-products like cell-free hemoglobin and heme may activate platelets. The binding of HbS to GP1bα at the platelet membrane level induces a signaling process through Lyn, PI3K, Akt and ERK pathways and the shedding of m/lEVs (131). The binding of heme to TLR4, expressed not only by platelets but also by monocytes/macrophages, neutrophils and endothelial cells, activates these cells and presumably induces the release of EVs. Activation of these cells by inflammatory mediators such as cytokines, for which high levels are detected in SCD patients, may also lead to EVs shedding. In addition, *in vitro* experiments have shown that decreasing RBC oxidative stress, using the anti-oxidant N-acetylcysteine, was associated with lower shedding of m/lEVs by sickle RBCs (125). It is therefore tempting to hypothesize that ROS may also be involved in the release of EVs by these cells. However, no report has yet tested these hypotheses in SCD clinical context to our knowledge.

EXTRACELLULAR VESICLES AS BIO-EFFECTORS OF SCD

Since EVs exhibit biological properties, they could modulate, negatively or positively, pathophysiological SCD pathways. Some data suggest that vesiculation of cells could be a self-protective mechanism. Indeed, it has been shown that half of RBC-derived m/lEVs circulating in the plasma of healthy individuals were linked with natural antibodies directed against antigen-associated band 3 protein, a well-known senescence marker of RBCs, while a much lower fraction of RBCs was positive for this senescent marker (132). Based on these observations, it has been hypothesized that vesiculation may be a mechanism of removing damaged proteins from otherwise healthy cells and thereby increasing their lifespan. However, this phenomenon was described only for RBCs and may not be effective for the other blood cell types.

Besides this potential beneficial aspect of vesiculation, the involvement of EVs in several abnormal pathophysiological pathways of SCD has been documented, based on either association studies and/or direct testing of their biological properties in sickle mouse models or using *in vitro* experiments. These biological properties are summarized in **Figure 2**.

Impact on Coagulation

EVs generated *in vitro* from stimulated monocytes, RBCs and platelets using calcium ionophore are able to trigger thrombin generation through TF-dependent or TF-independent mechanisms, when they are generated from the former and from the two latter cell types, respectively (133, 134). Accumulating evidence indicated that both activation pathways of coagulation are supported by EVs in SCD. The initial relationship detected by Setty et al. (68) in SCD patients between RBC-derived m/lEVs and plasma prothrombin fragment F1.2 has since then been confirmed and extended using other markers of coagulation activation (99, 126). In addition, RBC-m/lEVs of SCD patients were positively associated with acceleration in the propagation phase of thrombin generation while *in vitro* thrombin generation induced by these EVs was partly inhibited by anti-human factor XI (99). Activation of intrinsic coagulation pathway driven by RBC- and platelet-derived m/lEVs relies to the exposure of PS at their outer membrane leaflet which provides a suitable surface for the assembly of tenase and prothrombinase complexes (78). While platelet-derived m/lEVs are usually described as the most abundant m/lEVs detected in SCD patients, most of the studies detected association between RBC-EVs and coagulation activation. It is therefore temping to hypothesize that these unexpected results could be related to the higher density of externalized PS in RBC-m/lEVs than that of PLT-EVs (103) and therefore could activate coagulation more efficiently. In addition, it has been recently shown that m/lEVs released during red cell storage, can trigger coagulation activation not only through the canonical intrinsic pathway but also through the activation of a non-canonical pathway in which Kalikrein directly activates factor IX leading to thrombin generation (135). This observation also suggests that RBC-derived m/lEVs could be more efficient than platelet-derived m/lEVs in coagulation activation, but whether m/lEVs produced in the plasma of SCD patients exhibit similar biological properties remains unknown. In contrast to these studies, Shet et al. (97) reported positive relationship between TF-positive m/lEVS originated from monocytes and coagulation markers as well as the partial inhibition of the pro-coagulant activity of sickle m/lEVs by TF-neutralizing antibody in *in vitro* assays (97). Despite these discrepancies, these data clearly documented the involvement of m/lEVs in the hyper-coagulation and prothrombotic states, known to be significant contributors to vaso-occlusion in SCD (16).

Impact on Pro-inflammatory Status

The capacity of EVs to interact with and to induce an inflammatory phenotype of several vascular cell types has been documented by several studies in the SCD context.

sEVs and m/lEVs produced *in vitro* from sickle RBCs, can be internalized by monocytes/macrophages leading to the secretion of several pro-inflammatory cytokines (129). In addition, peripheral blood mononuclear cells (PBMC) incubated with these EVs exhibited an increased adhesion to endothelial cells. Using m/lEVs produced *in vitro* by sickle RBCs, Camus

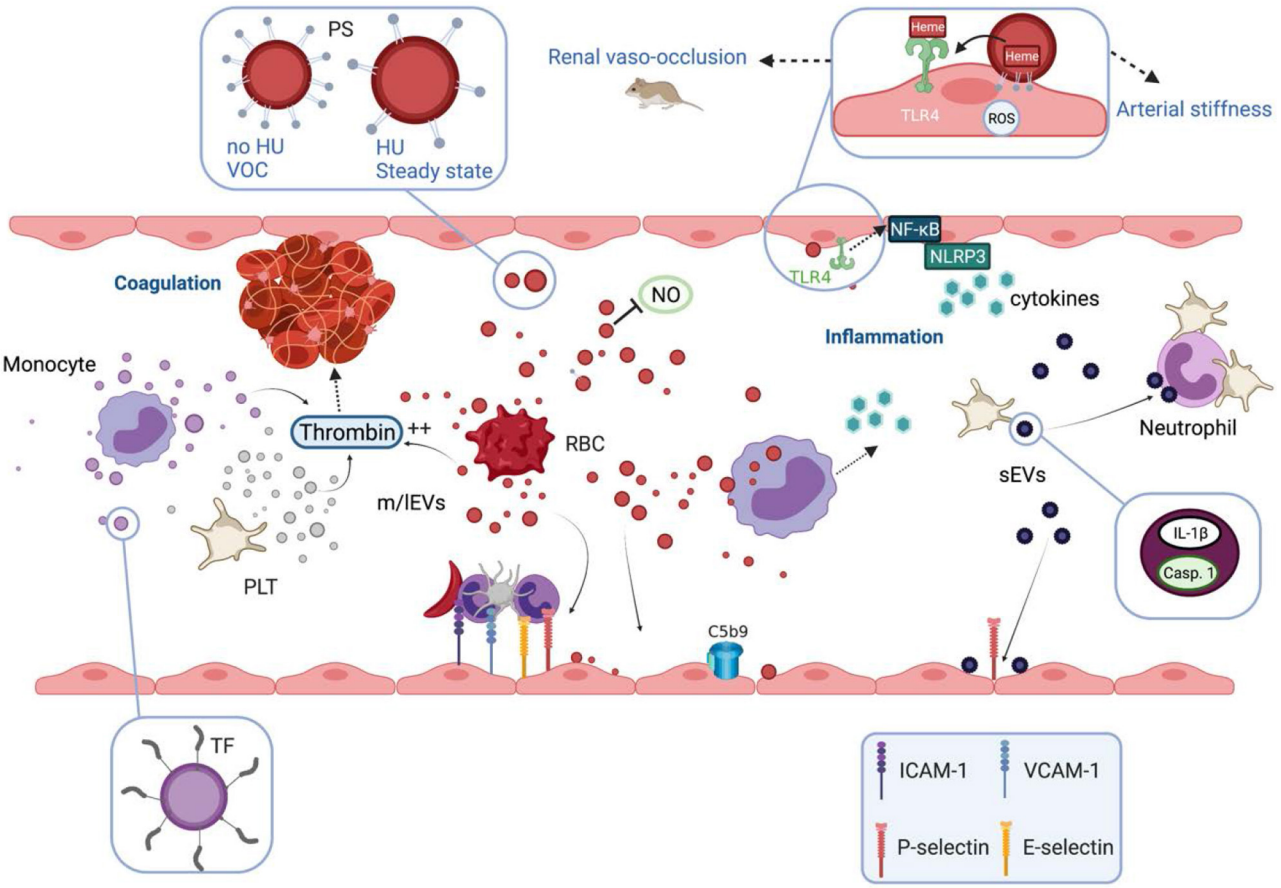

FIGURE 2 | Biological properties and pathophysiological consequences of extracellular vesicles in sickle cell disease. Extracellular vesicles (EVs) partly cause the hypercoagulant and prothrombotic state known in sickle cell disease (SCD). m/lEVs generated *in vitro* from stimulated monocytes, RBCs or platelets are able to trigger thrombin generation through TF-dependent and TF-independent mechanisms. Intrinsic coagulation pathway activation by RBC- and platelet-derived m/lEVs relies on the exposure of phosphatidylserine at their outer membrane leaflet. EVs also contribute to the inflammatory state of SCD patients. m/lEVs produced *in vitro* from sickle RBCs can be internalized by monocytes leading to the secretion of several proinflammatory cytokines and can increase the adhesion of monocytes to the endothelium. m/lEVs generated *in vitro* by sickle RBCs have been shown to promote renal vaso-occlusion in sickle cell mice and to induce endothelial cell apoptosis and ROS production. The high level of PS exposed at the surface of these vesicles, as well as their content in heme, could play a role in their deleterious effects on the vascular function. RBC-m/lEVs directly isolated from SCD patients' blood samples, promote the expression of adhesion molecules (ICAM-1, E-Selectin) and the production of pro-inflammatory cytokines by cultured endothelial cells. The endothelial activation mediated by these EVs involves the TLR4 signaling pathway. These proinflammatory properties are considerably reduced for m/lEVs obtained from patients treated with HU, which exhibit low PS externalization. In contrast, m/lEVs collected from patients during vaso-occlusive crisis exhibit high PS exposure and have deleterious effects on endothelial cells. RBC-m/lEVs could decrease NO bioavailability through their scavenging effects. In addition, both externalized PS and heme exposed by RBC-derived m/lEVs obtained using a calcium ionophore, have been shown to activate complement system on endothelial cell membranes. In humanized SCD mice, the stimulation of platelets leads to the release of sEVs highly loaded with IL-1β and caspase-1, which bind to neutrophils and promote platelet-neutrophil aggregation. VOC, vaso-occlusive crisis; NO, nitric oxide; ROS, reactive oxygen species; HU, hydroxyurea; PS, phosphatidylserine; TF, tissue factor; TLR4, toll like receptor 4; Casp.1, caspase 1; PLT, platelets; RBC, red blood cells.

et al. (130, 136) showed that their infusion in sickle mice promoted renal vaso-occlusion, reduced vasodilation of *ex vivo* isolated micro-vessels and induced endothelial cell apoptosis as well as ROS production (130, 136). The high level of PS externalized at the surface of these vesicles, as well as the fact that they contain a large amount of heme, would play a role in the impaired vascular function (130, 136). Both externalized PS and heme exposed by RBC-derived m/lEVs obtained using calcium ionophore, promoted alternative and terminal complement activation pathway in serum and on endothelial cell membrane (137).

Since content, structural characteristics and biological properties of EVs vary according to triggering factors (138, 139), we designed studies aiming at analyzing the biological properties of m/lEVs directly isolated from SCD patients in various clinical conditions. In these more pathophysiological relevant conditions, we have shown that m/lEVs isolated from patients at steady state induced ICAM-1 expression in cultured endothelial cells and thereby increased the adhesion of neutrophils (140). To decipher which blood cell type-derived m/lEVs are responsible for these biological effects, we used immuno-depletion to select vesicles according to their cellular origins and identified those

released by RBCs as the main contributors (140). While pre-incubation of m/lEVs with annexin-V, a PS blocker, abolished the induced endothelial ICAM-1 overexpression, we have shown that the proinflammatory properties of m/lEVs collected during a vaso-occlusive crisis, a condition associated with high PS externalization of m/lEVs (114), were exacerbated. In contrast, these proinflammatory properties were considerably reduced for m/lEVs obtained from patients treated with HU, which exhibited low PS externalization (116). In addition, we presented evidence that the endothelial activation mediated by these EVs also involved TLR4 signaling pathway (125). In this study, we also detected a direct relationship between arterial stiffness in SCA patients and plasma concentration of RBC-m/lEVs. EVs could also disturb NO bioavailability through their NO scavenging effects (141). Altogether, these data strongly suggest that externalized PS, alone or associated with heme, or hemoglobin retained by these EVs, play a significant role in the induced endothelial cell dysfunction. However, the involved signaling pathways partly remain to be deciphered.

It has also been documented that sEVs, purified from SCD patients, could modify the phenotype of several vascular cell types. Indeed, Khalyfa et al. have shown that sEVs obtained from SCD patients exhibiting severe vaso-occlusive phenotype, decreased endothelial permeability, promoted P-selectin expression in endothelial cells, and induced a pro-adhesive phenotype of monocytes (106). However, the cellular origin of the vesicles responsible for these biological effects remains unknown. sEVs released from platelets may also play a significant role in the occurrence of acute chest injuries. In SCD humanized mice, a specific activation of platelets by LPS, an agonist of TLR4, leads to the activation of the NLRP3 inflammasome and to the release of sEVs highly loaded with IL-1β and caspase-1, which bind to neutrophils and promote platelet-neutrophil aggregation in lung arterioles (142). Such heterotypic aggregates may cause arteriolar microthrombi and mimic chest injuries observed in SCD patients.

FUTURE DIRECTIONS

A recently described feature of EVs may be significantly relevant in SCD etiology and clearly deserves further studies. Fitzgerald et al. demonstrated that cytokines are not only released in soluble form, but also encapsulated in EVs in numerous biological fluids (143). They also showed that the relative fractions of free and EV-associated forms of cytokines are regulated and modulated upon cellular activation. EV-retained cytokines remained biologically active and could be released to their targeted cells by a yet uncharacterized mechanism. Since cytokines inside the vesicles are not detected by standard target cell-free assays and multiplexed immunoassays (143), the blood content of these inflammatory mediators has been underestimated in SCD patients so far, and the relationships between SCD clinical complications and cytokine levels are clearly issues which need to be reanalyzed. The quantifications of both free cytokines and those conveyed by EVs in SCD patients in various clinical conditions, could reduce this caveat and provide a better view of the inflammatory processes involved. Besides their cytokine contents, the other bio-active molecules contained in EVs, and specifically those produced *in vivo*, remain to be better characterized and/or to be identified. Indeed, very few studies have addressed this issue and the content of EVs reported so far has been mostly obtained from vesicles generated *in-vitro* using artificial experimental conditions. Future studies focusing on the description of *in-vivo* generated EVs characteristics are still pending. At last, the capacity of these EVs to modulate the phenotype of blood-cell types not yet investigated such as neutrophils, needs to be analyzed.

CONCLUSION

In this review, we described the current knowledge regarding the quantitative and qualitative profiles of EVs in SCD patients, the clinical conditions modulating these plasma concentrations, the mechanisms involved in their genesis and their biological properties. While high plasma levels of EVs have been reproducibly described, uncertainties remain about their cellular origins. Nevertheless, these vesicles, both sEVs and m/lEVs, are able to modulate key processes of SCD pathophysiology and are therefore bio-effectors in SCD.

AUTHOR CONTRIBUTIONS

PC and MR conceived the concept and wrote the article. EN and YG edited and significantly improved the manuscript. EN drawn the figures. All authors contributed to the article and approved the submitted version.

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Iron Overload in Patients With Heavily Transfused Sickle Cell Disease—Correlation of Serum Ferritin With Cardiac T2* MRI (CMRTools), Liver T2* MRI, and R2-MRI (Ferriscan®)

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The treatment of sickle cell disease (SCD) is mainly supportive, except for a minority, who receive bone marrow transplantation (BMT). Serum ferritin (SF) is routinely available but is notoriously unreliable as a tool for iron-overload assessment since it is an acute-phase reactant. Although blood transfusion is one of the most effective ways to deal with specific acute and chronic complications of SCD, this strategy is often associated with alloimmunization, iron overload, and hemolytic reactions. This study, thus, aims to evaluate iron overload in patients with SCD on chronic blood transfusions and specifically, correlate SF with the current standard of care of iron-overload assessment using MRI-based imaging techniques. Amongst a historic cohort of 58 chronically transfused patients with SCD, we were able to evaluate 44 patients who are currently alive and had multiple follow-up testing. Their mean age (±SD) was 35 (9) years and comprised of 68.2% of women. The studied iron-overload parameters included cardiac T2* MRI, liver iron concentration (LIC) by Liver T2* MRI, and serial SF levels. Additionally, in a smaller cohort, we also studied LIC by FerriScan© R2-MRI. Chronic blood transfusions were necessary for severe vaso-occlusive crisis (VOC) (38.6%), severe symptomatic anemia (38.6%), past history of stroke (15.9%), and recurrent acute chest syndrome (6.9%). About 14 (24%) patients among the original cohort died following SCD-related complications. Among the patients currently receiving chelation, 26 (96%) are on Deferasirox (DFX) [Jadenu® (24) or Exjade® (2)], with good compliance and tolerance. However, one patient is still receiving IV deferoxamine (DFO), in view of the significantly high systemic iron burden. In this evaluable cohort of 44 patients, the mean SF (±SD) reduced marginally from 4,311 to 4,230 ng/ml, mean Liver T2* MRI dropped from 12 to 10.3 mg/gm dry weight, while the mean cardiac T2*MRI improved from 36.8 to 39.5 ms. There was a mild to moderate correlation between the baseline and final values of SF ng/ml, $r = 0.33$, $p = 0.01$; Cardiac T2* MRI ms, $r = 0.3$, $p = 0.02$ and Liver T2* MRI mg/kg dry weight, $r = 0.6$, $p < 0.001$. Overall, there was a positive

correlation between SF and Liver T2* MRI (Pearson's $r = 0.78$, $p < 0.001$). Cardiac T2*MRI increased with the decreasing SF concentration, showing a negative correlation which was statistically significant (Pearson's $r = -0.6$, $p < 0.001$). Furthermore, there was an excellent correlation between SF ng/ml and LIC by FerriScan© R2-MRI mg/g or mmol/kg (Spearmen's rho $= -0.723$, $p < 0.008$) in a small subset of patients ($n = 14$) who underwent the procedure. In conclusion, our study demonstrated a good correlation between serial SF and LIC by either Liver MRI T2* or by FerriScan© R2-MRI, even though SF is an acute-phase reactant. It also confirms the cardiac sparing effect in patients with SCD, even with the significant transfusion-related iron burden. About 14 (24%) patients of the original cohort died over the past 15 years, indicative of a negative impact of iron overload on disease morbidity and mortality.

Keywords: sickle cell disease, blood transfusion, iron overload, serum ferritin, cardiac T2*, FerriScan®, MRI

INTRODUCTION

Sickle cell disease (SCD) refers to a group of inherited conditions, characterized by sickle-shaped red blood cells that precipitate recurrent episodes of vaso-occlusive episodes (VOC). The WHO published a global prevalence map of SCD and other data (http://www.who.int/genomics/public/Maphaemoglobin.pdf), revealing that about 20–25 million individuals of health organizations, worldwide, have homozygous SCD; about 12–15 million in sub-Saharan Africa, 5–10 million in India and about 3 million distributed in other parts of the world. It is estimated that 312,000 people with sickle hemoglobin (Hb) are born each year throughout the world, with the majority of these births (236,000) in sub-Saharan Africa (1), whilst, in Oman, the sickle cell gene has an overall prevalence of 6% (2). A yearly increase as the result of newborn cases is about 120–150 (3). Thus, SCD is a major public health problem in the Sultanate of Oman, with a high rate of morbidity and mortality (4–12).

Blood vessel occlusion is a fundamental pathological process in SCD (13, 14). During VOC, the vessel lumen is blocked by cells interrupting the capillary blood flow to various organs and other parts of the body. This precipitates an inflammatory process that leads to painful crises and damage to the brain, the liver, the kidneys, the lungs, the spleen, and other vital organs (15, 16). Painful crises affect virtually all patients with SCD, often beginning in late infancy and recurring throughout life (17, 18).

High-risk patients with SCD, particularly children, in accordance with the local and international guidelines, are treated with periodic on demand or chronic blood/exchange transfusions (19, 20). This reduces recurrent (21, 22) and initial stroke by over 80% (23, 24). Unfortunately, it is also associated with a high rate of complications; including the transmission of infective agents, iron overload, alloimmunization, and transfusion reactions (9, 25–27).

Alloimmunization of SCD remains a serious consequence of blood transfusions that often leads to life-threatening, acute and delayed transfusion reactions (28–30). Although allo-immunization in patients with SCD arises mostly due to RBC antigenic differences, only a subset of these patients develops RBC allo- or auto-antibodies, in spite of a similar transfusional background, indicating an underlying inherent genetic susceptibility. Genome-wide sequencing studies have shown single nucleotide polymorphisms (SNPs) on Chromosomes 2 and 5 approaching statistical significance with SNPs in CD81 gene, that encodes signal modulation of B lymphocytes, showing a strong association with alloimmunization and, thus, could serve as predictive biomarkers for alloimmunization (31).

Current guidelines recommend blood transfusions for primary and secondary prophylaxis (i.e., stroke) and therapy (i.e., acute chest syndrome and stroke) (19, 20), with less conclusive data, for other complications, such as priapism, VOCs, leg ulcers, pulmonary hypertension, and during complicated pregnancies (25, 32). Classically, serum ferritin (SF) and liver biopsy have been used to monitor patients with iron overload and assess their response to chelation therapy. SF has the advantage of being widely available, but is an acute-phase reactant, and does not always correlate with body iron stores (33). MRI has now emerged as the standard of care for effective detection and quantification of iron in the heart and the liver. T2 and T2* are two approaches utilized to assess hepatic iron, based on T2 spine-echo sequences and gradient-echo sequences, respectively. Due to the increased tissue iron, the inverse of T2 and T2* relaxation rates (R2 and R2*) are used for the quantifications of liver iron, as they are reciprocals of T2 and T2*, respectively and increases as iron stores increase (34). R relaxometry FerriScan® is now an FDA-approved, clinically validated, and commercially available technique for this purpose, with multiple T2 echo readings being utilized to calculate R2. R2* provides a more linear correlation with liver iron concentrations (LICs). Iron deposition in patients with SCD occurs predominantly in the liver and less so in the heart and the endocrine organs (35). Hepatic deposition initially accumulates preferentially in the sinusoidal spaces; however, it generally follows the traditional pattern of transfusional iron overload, with parenchymal hepatocyte deposition also occurring early and even at low LICs (36). It is worth noting that chelation preferentially removes iron from the reticuloendotheliam (37). Nevertheless, iron-overload monitoring not only includes cardiac T2*MRI but also Liver T2*MRI as both can be obtained at the same time.

TABLE 1 | Patient characteristics.

	Baseline ($n = 58$)		Current ($n = 44$)
Age, mean (±SD), yrs.	30 (9)		35 (9)
Male	21 (36.2)		14 (31.8)
Female	37 (63.8)		30 (68.2)
Genotype, *n* (%)			
SS	53 (91.4)		40 (91)
Sβ$^+$ Thal	5 (8.6)		4 (9)
Transfusions, *n* (%)			
Simple	43 (74)		31 (70)
Exchange	15 (26)		13 (30)
Indication for Transfusions, *n* (%)			
Severe Crisis	24 (41.4)		17 (38.6)
Symptomatic anemia	19 (32.8)		17 (38.6)
H/o of Stroke	9 (15.5)		7 (15.9)
Recurrent ACS	6 (10.3)		3 (6.9)
Chelation, n (%)			
DFO	3 (5.1)		1 (2.3)
DFP	2 (6.9)		0 (0)
DFX	44 (75.8)		26 (59)
No chelation	9 (12.2)		17 (38.6)
Chelation dosing, mg/Kg/day			
DFO	40 mg/kg/day IV infusion		
DFP	75 mg/kg/day in divided doses		
DFX	20–40 mg/kg/day, PO		
Jadenu	12–28 mg/kg/day, PO		
Iron Assessment in the evaluable current patients ($n = 44$)			
Ferritin[a], ng/ml, *n* (%)	44 (100)		44 (100)
Mean (±SD),	4,311 (4,030)		4,230 (3,059)
p-value[$]		0.9	
LIC[b], mg/gm Dry wt, n (%)	44 (100)		44 (100)
Mean (+SD),	12 (9)		10.3 (7)
p-value[$]		0.1	
Cardiac T2*MRI[c], msec, *n* (%)	44 (100)		44 (100)
Mean (+SD),	36.8 (17)		39.5 (6)
p-value[$]		0.1	
FerriScan R2 MRI[d], mg/g dry tissue, *n* (%)	14 (100)		
Median (IQR)	36.5 (6.3–43)		
FerriScan R2 MRI[e], mmol/kg dry tissue, *n* (%)	14 (100)		
Median (IQR)	658.5 (113–770)		

[$] *Paired student's t-test.*
[a] *SF Ref. Range (10–28).*
[b] *LIC T2* MRI Ref. Range (0.17–1.8).*
[c] *Cardiac T2* MRI Ref. Range (>20 msec).*
[d] *FerriScan© R2 MRI mg/g dry tissue Ref. Range (0.17–1.8).*
[e] *FerriScan© R2 MRI, mmol/kg dry tissue Ref. Range (3.0–33).*
DFX, Deferasirox; DFP, Deferiprone; DFO, Deferoxamine (Desferal); IQR, Interquartile range; msec, milliseconds.

Our study aims to evaluate iron overload in patients with SCD on chronic blood transfusions and specifically correlate SF with cardiac T2* MRI (CMRTools), Liver T2* MRI, as well as LIC by R2-MRI (FerriScan®), with its probable impact on mortality.

MATERIALS AND METHODS

In this retrospective cohort study, transfused patients with sickle cell disease (SCD) were monitored for their iron-overload status and chelation. The study was initiated after approval from the institutional medical ethics and review committee. The indications for transfusions include acute sickle cell-related complications, or as a part of a chronic exchange transfusion program, as per local and international guidelines.

Their demographic, clinical, and laboratory data were obtained from the electronic medical records of the hospital. Baseline and current demographic information comprised of SCD diagnosis and subtype, age and gender, and indications for chronic red cell transfusion.

Serum ferritin (SF) estimations were obtained every 3 months, whereas, Liver T2* MRI and Cardiac T2* MRI were performed yearly, in addition to viral activity parameters (i.e., Hepatitis viruses, HIV). Almost all of these patients are currently on Deferasirox (DFX), although a few had earlier received Deferiprone (DFP) at 75 mg/kg/day in three divided doses and IV deferoxamine (DFO), especially during their intra-hospital admissions.

Assessment of iron-overload monitoring parameters included serial SF, LIC by T2*MRI, and Cardiac T2* MRI, with data analyzed by the CMRTools software (Cardiovascular Imaging Solutions, Ltd., London) (38). The LIC was computed by Liver T2* MRI (38), as well as by spin density projection-assisted R2-MRI (FerriScan, Resonance Health, Australia) (39) in a smaller cohort, as per their standardized methodologies. Normal FerriScan© R2-MRI reference range was 0.17–1.8 mg/g dry tissue, or 3–33 mmol/kg dry tissue (40).

Statistical Analysis

Descriptive analyses including mean, SD, median, interquartile range (IQR), and 95% confidence intervals (CIs) were used to describe patient characteristics. Continuous variables were compared between groups using *t*-tests or Mann–Whitney U tests, as appropriate. The relationship of LIC SF was estimated by Pearson's or Spearman's correlation coefficient, as appropriate. All statistical analyses were performed with Stata12 software (Stata Corp, College Station, TX) and a $P < 0.05$ was considered statistically significant.

RESULTS

Our original cohort included 58 patients with a mean age (±SD) of 30 (9) years, 21 (36.2%) men, and 37 (63.8%) women. The majority, 53 (91.4%), had HbSS genotype, whereas, 5 (8.6%) had Hb Sβ$^+$ Thal (**Table 1**). Chronic blood transfusions were indicated for severe vaso-occlusive crisis (VOC) in 24 (41.4%), severe symptomatic anemia in 19 (32.8%), history of stroke in nine (15.5%), and recurrent acute chest syndrome in six (10.3%) patients. Among these 58 patients, the baseline mean serum ferritin (SF) (±SD) was 4,092 (3,579) ng/ml, mean cardiac T2*MRI (±SD) was 39 ms (18), whereas mean liver T2* MRI (±SD) was 12 mg/gm dry (9).

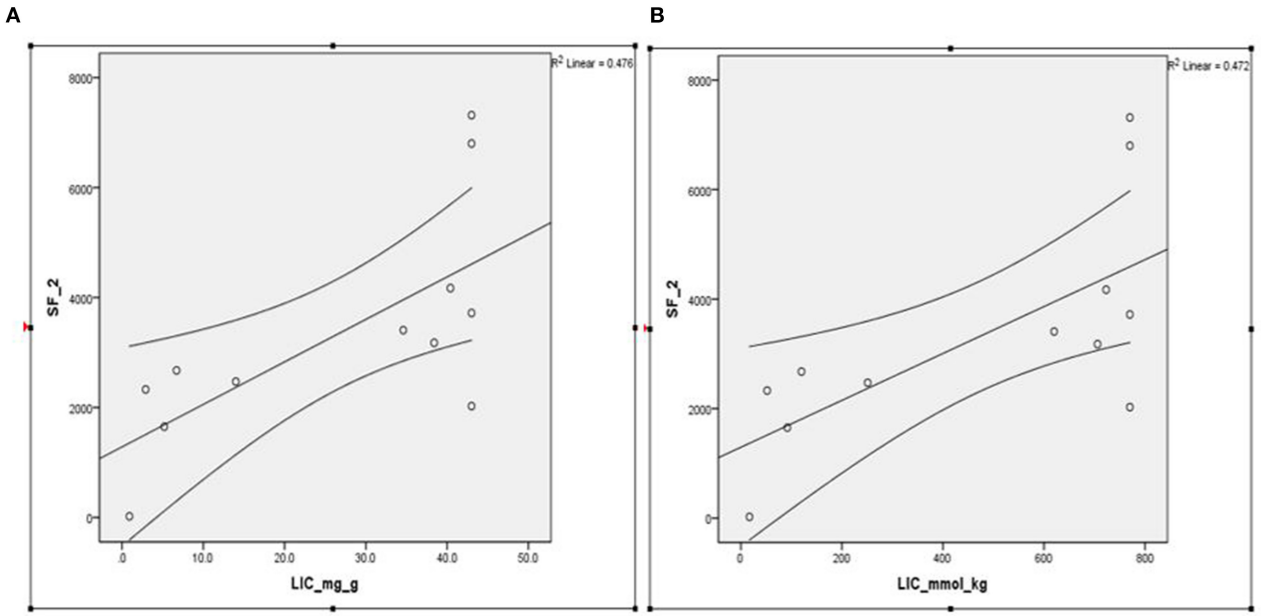

FIGURE 1 | (A,B) Spearmen's correlation between SF and LIC R2 by FerriScan© as either **(A)** mg/kg or **(B)** mmol/kg dry tissue showing that LIC by R2 MRI (FerriScan©) improves with decreasing SF concentrations (Spearmen's rho = −0.723, $p < 0.008$). SF, serum ferritin; LIC, liver iron concentration.

TABLE 2 | Effect of chelation: progressive change in iron burden with overall, on demand, and regular transfusion subgroups.

	Initial serial SF, ng/ml mean (±SD)		Final serial SF, ng/ml mean (±SD)		Initial LIC T2* MRI, mg/kg dry wt. mean (±SD)		Final LIC T2* MRI, mg/kg dry wt. mean (±SD)		Initial Cardiac T2* MRI, ms mean (±SD)		Final Cardiac T2* MRI, ms mean (±SD)	
Overall ($n = 44$)	4,311 (4,030)		4,230 (3,059)		12 (9)		10.3 (7)		36.8 (17)		39.5 (6)	
p-value[$]		0.9				0.1				0.1		
On Demand ($n = 31$)	4,436 (4,273)		4,389 (2,168)		11 (8)		9.5 (4)		37.5 (17)		39.4 (4)	
p-value[$]		0.9				0.4				0.6		
Regular ($n = 13$)	4,030 (2,429)		3,875 (3,183)		16 (9)		12.5 (8)		37 (26–47)		40.1 (7)	
p-value[$]		0.9				0.2				0.1		

[$] *Paired Student's t-test.*

In the current evaluable cohort of 44 patients with sickle cell disease (SCD) (**Table 1**), the mean SF (±SD) reduced marginally from a baseline value of 4,311 (4,030) ng/ml to 4,230 (3,059) ng/ml. Mean LIC (±SD) by Liver T2* MRI dropped marginally from 12 (9) to 10.3 (7) mg/gm dry wt. Mean Cardiac T2* MRI (±SD) improved marginally from 36.8 (17) to 39.5 (6) ms. The mean time (±SD yrs) between the first and the last examinations was 3 (±2) years.

Serial SF data analyzed at the time of performing FerriScan® iron study on a subset of these patients ($n = 14$) revealed a median SF of 2,926 ng/ml, with an interquartile range (IQR) between 2,119 and 3,676 and showed an excellent correlation with LIC mmol/kg as well as mg/kg dry tissue (Spearmen's rho = 0.723, $p < 0.008$; **Figures 1A,B**). Further, the median LIC by FerriScan© R2-MRI analysis was 36.5 mg/gm dry tissue weight, with an IQR between 6.3 and 43. Alternatively, in terms of mmol/kg of dry tissue weight, the median LIC was 658.5 with an IQR between 113 and 770.

44 patients are alive and had multiple follow-up investigations, whereas 14 (24%) among the original cohort, have died owing to SCD-related complications. The mean age (±SD) of this cohort is 35 (9) years, with a range between 20 and 64 years, comprising of 30 (68.2%) women (**Table 1**). Currently, 27 (61.4%) patients are receiving iron chelation therapy. Among those on chelation, 26 (96%) are receiving Deferasirox (DFX) [Jadenu® (24), or Exjade® (2)], with good compliance and tolerance. However, one patient still needs parenteral Deferoxamine (DFP) (Desferal®) in view of the very high systemic iron burden. The remaining 17 patients currently are not on any chelation therapy but are followed and monitored regularly, to check the need for the introduction of chelation. It is our policy to stop chelation once the SF levels reach below 500 ng/ml on two consecutive quarterly follow-ups.

Table 2 shows the effect of chelation when analyzing the progressive change in iron burden characterized, as overall, on demand and regular transfusion subgroups. There were no statistically significant differences in the serial SF (ng/ml), Liver T2* MRI (mg/kg dry wt.), and cardiac T2* MRI (ms), in the subgroups receiving on demand blood transfusions ($n = 31$) or regular transfusions ($n = 13$).

Table 3 shows the correlation of LIC with serial SF in specific subgroups characterized by the initial baseline and final current values when correlated with the three different levels of LICs. There was no statistically relevant trend in the Pearson's correlations between Liver T2* MRI (mg/kg dry wt.) and SF (mg/ml) subgroups characterized by LIC < 7 with SF $<$ 1,500, LIC between 7 and 15 with SF between 1,500 and 2,500, and LIC > 15 with SF $>$ 2,500 among the initial and final values. However, the only subset that showed a good correlation was with the lowest SF and LIC levels, where Pearson's correlation improved from $r = 0.2$ to $r = 0.86$ ($p = 0.002$).

Figures 2A–C shows a mild to moderate Pearson's correlation between the baseline and final values of (A) SF (ng/ml), $r = 0.33$, $p = 0.01$ (B) Cardiac T2* MRI (ms), $r = 0.3$, $p = 0.02$ and (C) Liver T2* MRI (mg/kg dry weight), $r = 0.6$, $p < 0.001$.

Figure 3A shows the percentage distribution of serum SF (ng/ml) subgroups <1,500, between 1,500 and 2,500, and >2,500, with respect to the three Liver T2* MRI subgroups (mg/kg dry wt.) <7, between 7 and 15, and >5, respectively.

Figure 3B shows the percentage distribution of Liver T2* MRI (mg/kg dry wt.) subgroups <7, between 7 and 15, and >15 among the initial and final values.

Figure 4A shows a positive correlation between the serial SF and Liver T2* MRI showing that Liver T2*MRI reduced with decreasing SF concentration, and this change was statistically significant (Pearson's $r = 0.78$, $p < 0.001$).

Figure 4B shows that Cardiac T2*MRI increased with the decreasing SF concentration, showing a negative correlation, which was also statistically significant (Pearson's $r = -0.6$, $p < 0.001$). Importantly, none of these patients had any

TABLE 3 | Effect of chelation: correlation of liver iron concentration (LIC) with serial serum ferritins in specific subgroups, initial baseline values, and final current values.

Initial baseline	Median (95%CI)	Pearson's correlation, *r*	*p*-value
Serial Serum Ferritin subgroups, <1,500 ng/ml	1,206 (540–1,464)	0.2	0.32
LIC by T2* MRI subgrouping, <7 mg/kg dry wt.	4 (2–6.9)		
Serial Serum Ferritin subgroups, 1,500–2,500 ng/ml	1,990 (1,776–2,077)	0.4	0.08
LIC by T2* MRI subgrouping, 7–15 mg/kg dry wt.	11 (8–13)		
Serial Serum Ferritin subgroups, >2,500 ng/ml	4,573 (2,878–22,244)	0.5	0.06
LIC by T2* MRI subgrouping, >15 mg/kg dry wt.	22 (16–39)		
Current/Final			
Serial Serum Ferritin subgroups, <1,500 ng/ml	553 (27–1,445)	0.86	0.002
LIC by T2* MRI subgrouping, <7 mg/kg dry wt.	2.1 (1.5–3.8)		
Serial Serum Ferritin subgroups, 1,500–2,500 ng/ml	1,674 (1,637–2,256)	0.1	0.6
LIC by T2* MRI subgrouping, <7–15 mg/kg dry wt.	10 (7.8–14.4)		
Serial Serum Ferritin subgroups, >2,500 ng/ml	5,365 (2,691–15,083)	0.3	0.2
LIC by T2* MRI subgrouping, >15 mg/kg dry wt.	19 (15.5–28)		

CI, Confidence Interval.

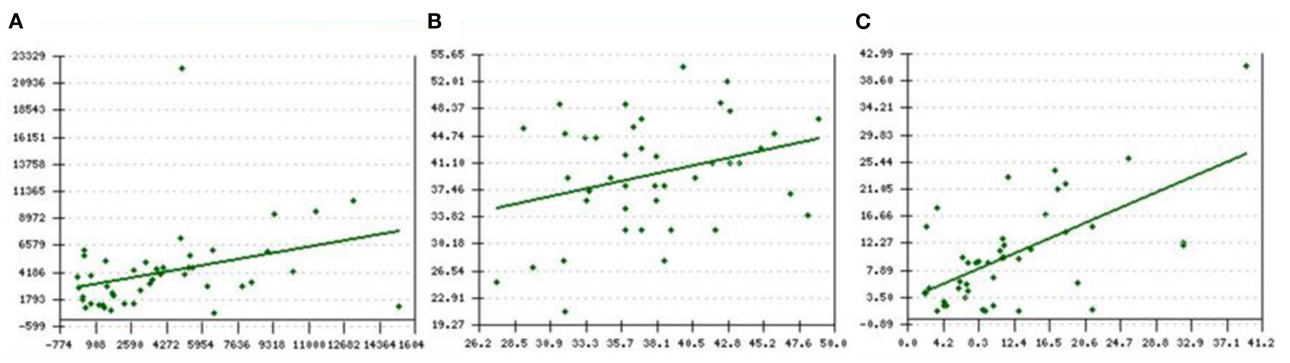

FIGURE 2 | (A–C) Pearson's correlation between baseline and final values of **(A)** SF ng/ml, $r = 0.33$, $p = 0.01$ **(B)** Cardiac T2* MRI ms, $r = 0.3$, $p = 0.02$ and **(C)** LIC T2* MRI mg/kg dry weight, $r = 0.6$, $p < 0.001$. X-axis represents the baseline levels of respective parameters vs. y-axis which represents the final/current levels of the same parameter. SF, serum ferritin; LIC: liver iron concentration.

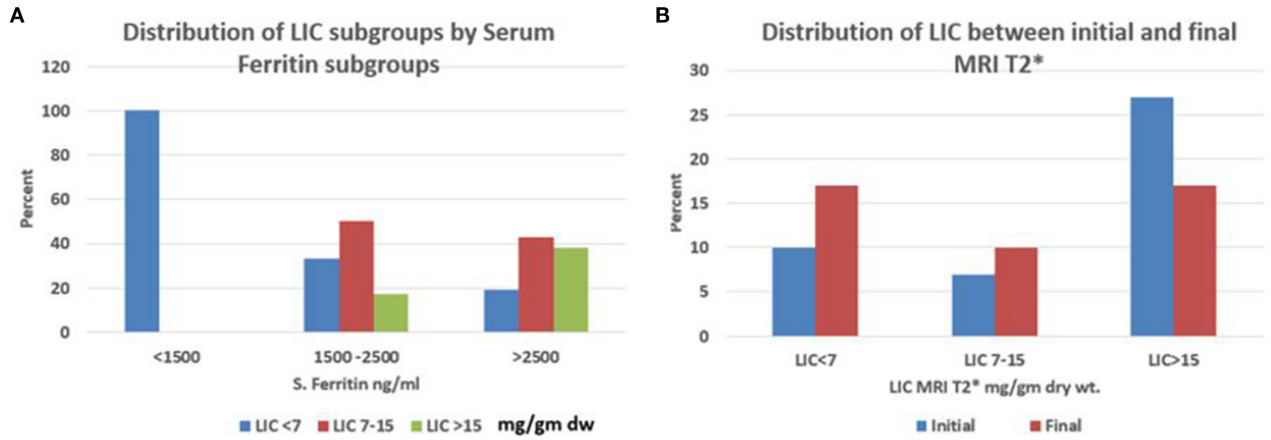

FIGURE 3 | (A,B) Percentage distribution of **(A)** LIC subgroups ≤7, 7–15, ≥15, mg/kg dry tissue by SF subgroups ≤1,500, 1,500–2,500 and ≥2,500 ng/ml and; **(B)** LIC subgroups ≤7, 7–15, ≥15, mg/kg dry tissue between the initial and final MRI T2*. SF, serum ferritin; LIC: liver iron concentration.

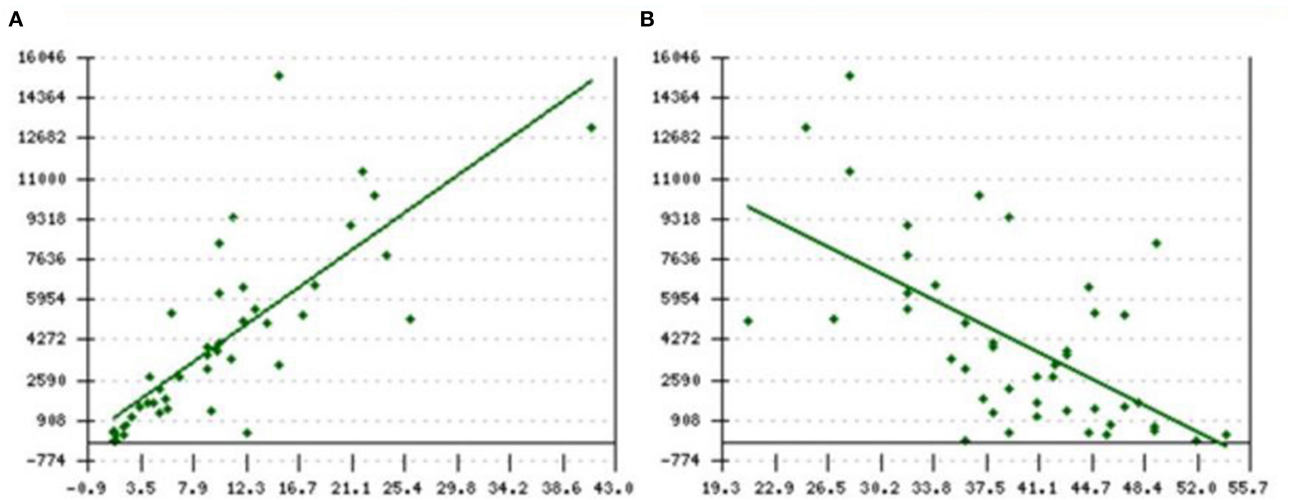

FIGURE 4 | (A,B) Pearson's correlation between current **(A)** SF and Liver T2*MRI (positive) $r = 0.78$; $p < 0.001$ as well as **(B)** SF and Cardiac T2*MRI (negative) $r = -0.6$; $p < 0.001$. X-axis represents final/current LIC levels whereas SF and T2* are represented on Y-axis in **(A,B)**, respectively. SF, serum ferritin; LIC, liver iron concentration.

evidence of cardiac iron overload with the mean cardiac T2* MRI levels of 36.8 ms at the initial examination which improved to 39.5 ms in the final examination. Further, the range of cardiac T2* MRI varied from 21 to 54 ms, which was always above the normal reference range of >20 ms (CMRTools software).

Supplementary Figures 1, 2 illustrate the sample images of FerriScan© R2-MRI and Cardiac T2* MRI of a putative patient showing liver iron overload, but sparing the heart.

Supplementary Figures 3, 4 show sample images of Liver T2* MRI and Cardiac MRI T2* of a putative patient showing liver iron overload, but sparing the heart.

DISCUSSION

In 2006, the WHO recognized hemoglobinopathies, including sickle cell disease (SCD), as a global public health problem and Oman has a high prevalence of hemoglobin (Hb) disorders (3) (http://apps.who.int/iris/bitstream/handle/10665/20890/A59_9-en.pdf?sequence=1&isAllowed=y).

In SCD, blood transfusions remain a critical therapeutic intervention as it improves the blood flow by reducing the proportion of red blood cells capable of sickling (25). Further, the chronic hemolytic state that is characteristic of SCD, with the release of free heme that quenches nitrous oxide and

the activation of inflammatory cascades, ultimately results in hemolysis and endothelial damage (14, 41). Blood transfusions will not only limit the amount of hemolysis but also prevent the endothelial damage that results in the high proportions of sickle polymer-containing red blood cells. Additionally, blood transfusions will also increase the blood oxygen-carrying capacity in severe chronic anemia or with severe vaso-occlusive episodes (VOC). Therefore, blood transfusions are established not only as a preventive strategy for stroke, especially in patients with high intracranial blood flow velocity revealed by transcranial Doppler studies, but also for their therapeutic benefits in a wide variety of complications, such as VOC, priapism, pulmonary hypertension, and during complicated pregnancies (25). However, the major and unavoidable complication of blood transfusions in SCD is systemic iron overload. In our setup, on demand blood transfusions accounted for almost two-thirds (70–74%) of these patients, whereas, regular transfusions were given in less than a third of these patients (26–30%). This proportion is significant since it represents the current real-life situation in patients with SCD who receive chronic blood transfusions.

Repeated transfusions of packed red blood cells (PRBCs) are currently the simplest and the primary method employed in chronic transfusion programs (42). However, although apheresis of RBC (erythrapheresis) is currently the safest and the most efficient method, it is costly, complicated, and cannot be implemented everywhere, nor is it suitable for all patients (42). Therapeutic phlebotomy is an alternate technique that is safe and well-tolerated, with net iron removal but is currently used only in patients with SCD who have iron overload and have undergone bone marrow transplantation (BMT) for SCD (43). We have used this method in eight of the 58 patients from the original cohort who underwent BMT. It has given us good results in terms of managing iron overload in this setting, but three patients in this cohort expired due to post-BMT complications, while the remaining five patients are doing well with regular monitoring of their iron-overload status. Manual exchange transfusions combined with one or more manual phlebotomies with a PRBC transfusion is, thus, what we have been practicing in the majority of our patients on chronic blood transfusions. However, iron overload is the principal side effect of this therapy.

The utility of the SF alone, while monitoring iron overload in the chronically transfused patients with SCD is disputable. This is so because of the propensity of the inflammatory stimulus that would invariably, falsely elevate SF. However, using serial SF estimations, performed during steady-state, will help in the assessment of the true systemic iron-load status as was seen in our current practice. Routinely performing SF estimations every quarter, enabled us to ascertain the average SF levels over the year, and avoided the spikes seen during acute inflammatory states like VOCs. It, thus, became a reliable tool in the monitoring of iron-overload status in this cohort as it showed a fairly good correlation with Liver T2* MRI results (**Figure 4A**). There was a progressive fall in the percentage of LIC > 15 with a rise in the LIC < 7 indicative of the progressive improvement in the iron-overload status over the follow-up years (**Figure 3B**). Further, in this cohort, SF levels below 1,500 ng/ml and LIC levels below <7 mg/kg dry wt. showed a good correlation, but SF levels above 1,500 and >2,500 ng/ml were associated with wide variability in the Liver T2* MRI (**Table 3**, **Figure 3A**). These findings are not only consistent with other prior studies (44–46), but also point to the progress in patient management that historically did not receive optimal monitoring iron overload (47). Thus, with the long-term aim of getting the Liver T2* MRI below 7 mg/kg, the utility of annual LIC measurement with T2* MRI or FerriScan© R2-MRI, where available, needs to be emphasized, especially when the SF levels are above 2,500 ng/ml. A significant reduction in Liver T2* MRI between the first and final liver MRI studies with a greater proportion of LIC below 7 mg/g dry wt. supports the putative benefit from Liver T2* MRI monitoring (**Figure 3B**). Further, a clear positive correlation between SF and Liver T2* MRI consolidates this point (**Figure 4A**, Pearson's $r = 0.78$; $p < 0.001$). However, ferritin is a poor indicator and although ferritin is a convenient measure of iron status, ferritin trends are unable to predict changes in LIC in individual patients (48). Ferritin trends need to be interpreted in conjunction with the direct measurement of LIC.

Tissue iron is paramagnetic and increases the MRI relaxation rates, R2 and T2* in a quantifiable manner (32, 39). These non-invasive iron estimation techniques by MRI have been validated at several centers (32, 38, 39, 46). Our liver FerriScan© R2-MRI study on a small subset of patients gave us a comparative view of the two currently available liver tissue iron estimation techniques. Further, since the FerriScan© R2-MRI platform was not available locally, over the years, we have been relying consistently on the 1.5 Tesla MRI T2* technique, after purchasing a license for the use of the CMRTools software. This certainly has positively affected the clinical care in our patient population, with Liver T2* MRI helping us to make the necessary adjustments in chelation therapy. The results also served to reinforce the continuation of the current assessment tools to optimize patient care and monitor the iron overload to address adherence to chelation therapy and improve management decisions with increasing familiarity with this technology. Moreover, we have discontinued iron chelation therapy in 12 patients, as their SF levels were consistently lower than 500 ng/ml, and they are meticulously monitored by serial SF levels and Liver T2*MRI results. The other five post-BMT patients who are not on chelation therapy are on periodic phlebotomies with a progressive decline in the SF and Liver T2* MRI levels with regular follow-up monitoring.

Patients with transfusion-dependent anemia develop fatal cardiac and endocrine toxicities from iron overload (32, 49). Further, although blood transfusion therapy is life-saving for patients with SCD and thalassemia, iron overload (especially cardiac) has impacted significantly on survival, especially before the era and availability of chelation therapy (50). However, there are intrinsic differences between patients with SCD and thalassaemia in terms of the sites of parenchymal organ involvement. This is because it is believed that patients with thalassemia have increased plasma malondialdehyde and circulating non-transferrin bound iron (NTBI) relative to patients with SCD, and lower levels of some cytokines (interleukin 5 and interleukin 10) and γ-tocopherol (36). Thus, in contrast to hemolytic anemias like Thalassaemia and SCD,

in patients with Diamond Blackfan syndrome, there is variable ineffective erythropoiesis, with little or no RBC production. Thus, iron-related toxicity in this situation results from unutilized iron from senescent red cells that are not reused and much of it becomes labile plasma iron and then labile cellular iron, causing toxic damage (51).

These significant differences also support the hypothesis that the biology of SCD predominantly shows an increased inflammation, with elevated interleukin-6 and increased levels of protective antioxidants compared to patients with thalassemia, in whom NTBI-related parenchymal damage results in organ failure (36, 37). Thus, although iron deposition in patients with SCD generally follows the traditional pattern of transfusional iron overload, with parenchymal hepatocyte iron overload, optimal chelation therapy is desirable to offset the continuous iron deposition in the liver parenchyma. Our study confirmed the role of iron chelation and monitoring showing the cardiac sparing effect in patients with SCD, even with significant transfusion of burden-related systemic tissue iron overload (**Table 1** and **Supplementary Figures 1–4**). This is further substantiated with a clear negative correlation between SF and cardiac T2*MRI (Pearson's $r = -0.6$; $p < 0.001$, **Figure 4B**). However, to further optimize iron chelation, progressive reduction in SF and LIC are the desirable goals of therapy to bring down the total body iron burden. We need to pursue this objective in order to reduce the risk of long-term complications of liver iron overload, namely liver cirrhosis and carcinoma, which are known to occur in the long term. Thus, as in the management of Thalassaemia Major, our aim is to try to further optimize chelation and to achieve not only a clear heart but also try to get and maintain the LIC below 3 mg/kg dry wt.

Although deferoxamine (DFO) has been historically the major iron-chelating therapy of transfusional iron overload, compliance is a major hindrance in achieving the optimal therapeutic goal. Further, the availability of oral iron chelation with Deferiprone (DFP) since 1987 was useful, but showed poor efficacy, when used alone as compared to DFO. However, Deferasirox (DFX) became clinically available in 2006 and has been the preferred method that was adopted in our hospital for chelation therapy. DFX is the predominately prescribed chelating agent in our cohort, with occasional use of DFO during hospital admissions. Due to the small numbers of subjects receiving DFP and DFO, a direct comparison of the change in the iron burden on different chelating agents is not possible. Moreover, currently, no patient is on DFP therapy. Nevertheless, good adherence with the oral chelator agent correlated with better SF and Liver T2* MRI results. This supports the need for routine assessment of adherence and barricades to it. The success of chelation therapy is significantly impacted by patient adherence to the prescribed treatment, and consequently, adjustment of drug schedules for increasing the adherence to treatment becomes critical (52). Our patients were fortunate to have been able to receive oral iron chelation with DFX, as although this is a relatively expensive treatment, and the patients do need to continue the same over long periods or indefinitely, they also continue to receive chronic blood transfusions. This oral iron chelation therapy is completely free of cost to Omani patients since the treatment cost is borne by the government healthcare providers. Thus, almost all the current patients on oral iron chelator are receiving DFX therapy with good tolerance and compliance. It has been owing to the good compliance that we were able to take 12 patients off chelation therapy and they are currently being monitored with periodic SF levels and T2* MRI imaging results.

Limitations of this study include the retrospective data analysis in this study cohort, who received chronic blood transfusions for various indications. LIC by FerriScan© R2-MRI is not widely available, which limits its use as an assessment platform, and since we did not have it locally, we had to develop Liver and Cardiac T2* MRI technique and get the license for the use of the CMR tools software to compute LIC T2* MRI and Cardiac T2* MRI. The effectiveness of erythrocytapheresis in preventing or reducing systemic iron loading in patients with SCD could not be assessed as it was not routinely available at our center, but it is a technique that can be effectively used in patients who are on chronic blood transfusion therapy. Phlebotomy has been useful in the eight patients with SCD who were transplanted, but its use was restricted only to this category of patients. Finally, mortality was seen in 14 patients (24%) being multifactorial, could not be assessed to see a direct cause and effect relationship to the iron overload. Three patients died due to post-BMT complications while two died with chronic renal failure. The remaining nine patients died due to multiorgan failure following sepsis. Certainly, there was a significant background for iron overload in these patients at the time of death which could have precipitated the complications that ultimately culminated in the death of these patients.

In summary, the availability of oral chelation paralleled with the assessment of iron overload by MRI imaging has improved the management of iron overload in our population with SCD. Further, although SF has significant limitations in the assessment of iron burden when performed repeatedly and in steady-state, it showed a good statistically significant correlation with LIC and cardiac iron in patients with SCD. Moreover, it further needs to be emphasized that these assessment tools are essential for the optimal management of iron overload. Additionally, judicious limitation of unnecessary simple transfusion therapy, wherever possible, and encouraging adherence to chelation therapy are also important strategies to control iron overload and its associated clinical consequences.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

SA, VP, SA-R, and KA-S were fully involved in the conception and design of the study, recruitment and care of patients, and acquisition of data. AP and SA have been fully involved in the analysis and interpretation of data and were instrumental in the drafting the article and critical appraisal before submission. All authors have made substantial contributions and have seen and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.731102/full#supplementary-material

Supplementary Figures 1–4 | Sample Images (1 & 2) of LIC by FerriScan© and LIC and Cardiac T2* MRI of a patient showing liver iron overload but sparing the heart. Sample Images (3 & 4) of LIC by MRI T2* and Cardiac MRI T2* of a patient showing liver iron overload but sparing the heart. SF, serum ferritin; LIC, liver iron concentration.

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MINI REVIEW
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Odisha Revisited: A Personal Account

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In 1986, a paper in the Lancet was the first to collate hematology, molecular findings, and clinical features of homozygous sickle cell (SS) disease in India. The paper came from the group organized by Professor Bimal Kar in Burla Medical College, Sambalpur University, in western Odisha. Although widely quoted, few readers will be aware of the history of this work that is now attached in an informal summary.

Keywords: sickle cell, India, Jamaica, cohort study, haplotype comparison

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BACKGROUND

Following the first report of the sickle cell gene in India (1), many surveys established the distribution of the sickle hemoglobin gene recently summarized by Hockham et al. (2). Latterly, much has been learnt on its molecular characteristics from Dr. Roshan Colah and colleagues at the Indian Council for Medical Research (ICMR) Immuno-hematology Unit in Mumbai. In the mid-1980's, Professor Kar and his pediatric colleague, Professor Satapathy had noted the frequency of children with jaundice, joint pains, and a positive sickle test, but although SS disease was suspected, confirmatory hemoglobin electrophoresis was not available at that time at Burla Medical College. Professor Kar sought help from the British Council in Kolkata who responded by sending a statistician, Richard Hayes from the London School of Hygiene and Tropical Medicine. It was a happy choice since although Professor Kar and his colleagues, did not at that time have data for analysis, Richard had worked with the Sickle Cell Unit of the Medical Research Council at the University of the West Indies in Kingston, Jamaica, and recommended that staff from there should visit Burla. Professor Graham Serjeant (Director of the MRC Laboratories in Jamaica) and Beryl Serjeant (chief technologist) were therefore invited by the British Council to visit India in February 1985. The Council arranged a lecture tour that included visits to the ICMR Unit in Mumbai, The University of Pune, the Medical College at Kolkata, and the School of Tropical Medicine where Professor Serjeant gave the 13th JB Chatterji, Memorial Oration entitled "The Broad Spectrum of Sickle Cell Disease." Finally, after an overnight train journey from Kolkata, they reached Burla Medical College where they saw many children, subsequently confirmed to have SS disease in samples carried back to Jamaica.

To learn more about the patients of Professor Kar, it was proposed to carry a laboratory of equipment and consumables generously loaned/donated for the study by companies in the United Kingdom. The British Medical Research Council's base in Mill Hill, London, oversaw the shipping of six large crates weighing 334 kilograms, to Kolkata where they were transferred to a British Council's Land Rover and traveled with Graham Serjeant and the Nepali driver of the British Council for the 2-day journey to Burla. Meanwhile the visiting team of colleagues that included

TABLE 1 | Features of patients with HbSS from Jamaica and western Odisha.

Feature	Jamaica *n* = 311	Odisha *n* = 130*	Comments/references
Molecular features			
Alpha thalassaemia	*n* = 276	*n* = 122	
α-/α-	9 (3.3%)	13 (10.7%)	(5)
α-/αα	91 (33.0%)	54 (44.3%)	
αα/αα	172 (62.3%)	55 (45.1%)	
Others	4 (1.4%)	–	
Beta haplotype			
Benin	80%	None	(6)
Asian	None	97%	
Red cell indices (mean, SD)**			
Fetal hemoglobin (%)	6.1, 4.2	16.6, 5.2	All differences significant at 1% level (3)
Total hemoglobin (g/dl)	8.04, 1.12	8.73, 1.69	
MCHC (g/dl)	0.34, 0.02	0.32, 0.03	
MCV (fl)	87.3, 8.1	83.6, 9.4	
MCH (pg)	29.7, 3.4	26.6, 3.6	
Reticulocytes (%)	10.5, 4.0	6.5, 4.7	
Clinical features			
Splenomegaly	55% in first year	Occurs later	Jamaican/Odisha data (7)
Dactylitis	45% by age 2 yrs	52% by age 5 yrs	Jamaican data (8)
Bone pain crisis	++	++	
ANFH	10–15%	15%	Jamaican data (unpublished)
Stroke	8% by 14 yrs	? 2 strokes	Jamaican data (9)
Leg ulcers	30%	<1%	Jamaican data (10)
Priapism	33%	None	Jamaican data (11)

*One patient did not have full hematology. Jamaican data derived from the Cohort except for red cell indices** which were derived from non-Cohort patients to allow better age matching.

Dr. Andreas Kulozik, a German research fellow working with the Department of Hematology in Oxford, England, his wife Martina, a dermatologist, who was invaluable in taking blood samples, Su Sirr, an English nurse from the University Hospital in Kingston, Jamaica, who volunteered to work with us, and Beryl, traveled by overnight train to the station at Sambalpur Road, where they were collected by our hosts. There were no hotels in Burla, and we stayed in the Hirakud Dam Rest House First Class built to accommodate the German engineers involved in the construction of the 27 km Hirakud Dam across the Mahanadi River, the largest earth dam in the world. Entertainment was limited and the focus tended to be the European wine shop and the café in the market that had recently installed an Italian espresso coffee machine.

In the medical college, the laboratory was set up in a lecture theater where we had electricity most of the time and cleaning of the glassware was performed by Beryl in the buckets of water of increasing cleanliness until the last bucket contained distilled water. The work in earnest began on Monday, February 24, 1986, when the local radio station announced that a team of international specialists in sickle cell disease was visiting Burla. Each morning, we arrived at the laboratory to find families who had traveled during the night and saw 18 patients on the 1st day, 48 on the next, and a total of 445 patients and family members were investigated by March 4, when we ran out of the reagents for the blood cell analyzer. A further 80 subjects were screened by the electrophoresis alone.

FINDINGS IN ODISHA PATIENTS

The study described 131 patients with homozygous SS disease (3) and 17 with sickle cell-beta$^+$ thalassemia with the mutation IVSI-5G>C (4). Compared with Jamaica, where most data were derived from the cohort study, patients from Odisha were almost entirely of the Asian haplotype, had higher total hemoglobin and lower reticulocyte counts, lower mean cell volume and mean cell hemoglobin, and much higher fetal hemoglobin (HbF) levels, and over half of the patients had alpha thalassemia (**Table 1**).

Clinically, there were many similarities between patients of Jamaica and India, but Indian patients differed in the rarity of chronic leg ulceration, the absence of priapism and the later appearance of splenomegaly that persisted for the longer, pitted red blood cells counts being consistent with persisting splenic function. Since the age-specificity of pneumococcal infection declines sharply after 3 years, the persistence of splenic function beyond this age could contribute to the apparent lack of pneumococcal septicemia in Indian pediatric experience. Alpha thalassemia and HbF level both inhibit sickling and their increased frequency in Odisha was consistent with a lower hemolytic rate. The impression of a milder disease in Odisha was also supported by the incidental detection of 15 patients by family study including five parents.

Dactylitis, in which avascular necrosis of the bone marrow affects small bones of the hands and feet, has clear epidemiology

FIGURE 1 | Professor Bimal Kar (right) with the patients at his post-retirement clinic in Burla Market, Odisha, India.

in Jamaican patients, commencing as early as 3 months of age, frequently recurring, usually resolving without permanent sequelae, and becoming uncommon after the age of 5 years. Recollection is likely to be unreliable but confining inquiry to the mothers of children aged 2–9 years found a prevalence similar to that among Jamaican patients. Furthermore, three Indian subjects had permanently shortened metacarpals consistent with premature fusion that occurs after an infection of the avascular bone marrow. The prevalence of avascular necrosis of the femoral head was similar between the groups although the numbers were small and influenced by symptomatic presentation. Precipitating factors for the bone pain in Odisha were similar to those in Jamaica, with almost all the patients reporting an association with skin cooling and improvement with advancing age after 30 years. It was of interest that the more frequent alpha thalassemia and high HbF levels that might be expected to inhibit intravascular sickling, did not appear to ameliorate bone pain crises, casting doubt on the relevance of these factors to this complication, but bone pain is a complex phenomenon influenced by the social, environmental, and psychological factors.

COMPARISON OF ODISHA WITH OTHER INDIAN STUDIES

Compared to that observed in Odisha, the disease in central India has been claimed to be more severe (12, 13), but it is currently unclear whether such differences are intrinsic to the disease or different ascertainment biases in the studied populations. Geographic comparisons are fraught with difficulties in interpretation and comparing data between Nagpur and Odisha was confounded by the different age structures and ascertainment biases which, in Nagpur involved high-default rates and in Odisha included referral from existing clinics and also the chance event of hearing and responding to the radio announcements of the study. Marked elevations in HbF have been universal in Indian subjects, compared with that in Jamaica, and this may be responsible for the later appearance of splenomegaly and persistence of splenic function. The prevalence of the alpha thalassemia has varied between 16 and 55% in the Indian patients from different locations and this variation may allow assessment of the relevance of alpha thalassemia to the clinical features. Detecting populations at birth is the best method of avoiding bias, but only if the high-default rates reported from two Indian studies (13, 14) can be avoided.

COMPARISON OF DISEASE IN NAGPUR WITH JAMAICAN DATA

Comparison of birth cohorts from Nagpur and Jamaica found that patients from Nagpur had higher HbF levels, less alpha thalassemia, few hematological differences, and later development of the splenomegaly (15).

RETURN TO BURLA

It was 11 years before Graham and Beryl could visit Burla again but Professor Kar continued his research interests with support from the ICMR. By 1997, Professor Kar had retired from the Burla Medical College and continued his active interest in sickle cell disease from his office in Burla market entitled MARC (Medical Aid and Research Centre) Sickle Cell Clinic (**Figure 1**).

In his typical dynamic and enthusiastic fashion, he had trained a local lady to take blood samples and his driver to prepare sickle tests. He passed away on September 7, 2009 having made a monumental contribution toward the clinical understanding of SS disease in India.

AUTHOR CONTRIBUTIONS

GS performed the histories and clinical examinations. AK did the DNA analysis. BS oversaw the hematological investigations. All authors contributed to the article and approved the submitted version.

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REVIEW
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Sickle Cell Disease: Thoughts for India From the Jamaican Cohort Study

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The sickle cell gene in India represents a separate occurrence of the HbS mutation (the Asian haplotype), which has occurred against a genetic background characterised by high levels of fetal haemoglobin and widely varying frequencies of alpha thalassaemia. These features, which tend to inhibit sickling, change the expression of the disease, which, in India, may be further modified by poor nutrition, malaria and other infections, and limited public health resources. Sickle cell disease in Jamaica is predominantly of African origin (the Benin haplotype) and faces some similar challenges. This review assesses similarities and differences between disease expression in the two countries and seeks to explore lessons from Jamaica, which may be relevant to Indian health care. In particular, it addresses common causes of hospital admission as detailed from Indian clinical experience: anemia, bone pain crisis, and infections.

Keywords: sickle cell disease, Jamaica, cohort study, India, geographic comparison

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INTRODUCTION

The sickle cell gene in India was first reported among tribal people in the Nilgiri Hills of Tamil Nadu in the south of the country (1). Since then, the distribution of HbS has been well documented across Gujarat, Maharashtra, Madhya Pradesh, Andhra Pradesh, Chhattisgarh, and western Odisha with a smaller focus in the southern states of Tamil Nadu and Kerala (2).

In peoples of African origin, there are three major beta globin haplotypes (Benin, Bantu, and Senegal), and the clinical features of the Benin haplotype, which dominates in North America and the Caribbean, have been well documented, but only limited information is available on the Asian haplotype, which accounts for the disease in India and the Eastern Province of Saudi Arabia. For brevity in the text, patients of African origin are referred to as African HbSS and those with the Asian haplotype as Asian HbSS. An early study of 131 patients with Asian HbSS from Burla Medical College in Western Odisha (3) found a disease relatively mild compared to Jamaica with little chronic leg ulceration and priapism, although hypersplenism appeared common. This impression of mildness was supported by the finding of 15 cases of unsuspected SS disease among parents and older relatives. Later studies from central India (4–6) reported a more severe clinical course but the extent to which these differences are influenced by varying ascertainment biases is uncertain. Newborn screening should avoid a symptomatic bias but only if complete follow-up is possible and two major programmes based on newborn screening in south Gujarat (7) and Nagpur in central India (6) were marred by default rates of 30%−36% within the first year. The current review seeks to clarify this rather confusing picture, assessing the evidence and, where possible, comparing Indian findings with those in the Jamaican Cohort Study (8). In particular, it focuses on the most common causes of hospital admission in Nagpur (4): anemia, bone pain crises, and infections.

ANAEMIA

The Haematology of HbSS in Jamaica and Odisha

All patients with homozygous sickle cell (HbSS) disease have a shortened red cell survival due, in part, to the effect of sickle haemoglobin (HbS) in reducing the deformability of red cells. HbS within the red cell has a low oxygen affinity becoming nearly fully saturated in the lungs but releasing more oxygen per gram on haemoglobin in the periphery. Both these factors should be borne in mind when assessing haemoglobin levels in HbSS. In Jamaican patients, the mean red cell survival was 12.6 days, which ranged between 4.2 and 25.0 days (9) (compared to approximately 120 days in normal people). For comparison of Jamaica and Odisha, Jamaican data were derived from the non-Cohort clinic of 1,389 subjects, since this population provided more appropriate age matching with Odisha patients (3). The results showed that Odisha patients had significantly higher haemoglobin levels (mean 8.7 vs. 8.0 g/dl), lower reticulocyte counts (mean 6.5 vs. 10.5%), and much higher fetal haemoglobin (HbF) levels (16.6 vs. 6.1%) (**Figure 1**).

Molecular, haematological, and selected clinical features show many differences between Odisha and Jamaica (10).

Anaemia Due to Impaired Bone Marrow Response

Aplastic Crisis

In Jamaica, acute anaemic events defined by haemoglobin levels of 3–4 g/dl and absent reticulocytes show seroconversion to parvovirus B19 in 95%, (11) and seroconversion in the cohort occurred in 60% of SS subjects by age 15 years (12). Parvovirus attacks red cell precursors in the bone marrow, and the development of parvovirus antibodies usually allows bone marrow recovery within 7–8 days, but this may exceed the mean red cell survival causing death in some patients with African HbSS. In Jamaica, treatment consists of outpatient transfusion of a single unit of blood with follow-up after 3–4 days to confirm the reticulocytosis of spontaneous bone marrow recovery. Parvovirus B19 is highly infectious and approximately 80% of susceptible siblings become aplastic either simultaneously or within 3 weeks so susceptible siblings should be closely monitored. The good news is that immunity to parvovirus appears to be lifelong and recurrence of parvovirus-induced aplasia has never been reported in SS disease.

In India, the frequent lack of reticulocyte counts in the investigation of acute anaemia does not allow a clinical definition of aplastic crisis, so comparable data are not available but the virus is common as seroconversion to parvovirus B19 occurred in 70% of healthy adult blood donors in India (13). Red cell survival in Asian HbSS is unknown but it would be expected that the commonly associated high levels of HbF may prolong red cell survival, diminishing the fall in haemoglobin level and the clinical severity of aplasia.

Hypoplasia From Nutritional Factors

Folate Deficiency

A falling haemoglobin associated with lower reticulocytes (1–4%) may reflect relative bone marrow hypoplasia from nutritional deficiency, infections, or metabolic problems such as renal failure. The mean cell volume (MCV) is useful in distinguishing iron deficiency (MCV low), or folate deficiency (MCV high), compared to the patients' steady-state values. The expanded bone marrow in SS disease increases the requirements for folic acid, and folate deficiency may lead to megaloblastic change with falling haemoglobin and increasing MCV. Observations from West Africa on the frequency of folate-deficient megaloblastic change (14) led to the widespread use of folate supplementation for SS disease elsewhere but the low dietary folate levels in countries such as Nigeria may render patients especially prone to this complication. The metabolic demands for folic acid are increased in SS disease but the crucial question is whether or not these are met by the diet.

In Jamaica, the dietary content of folic acid is relatively high and folate supplementation had little demonstrable effect on the clinical or haematological features of HbSS (15) so routine folate supplementation seems unnecessary although changing economic circumstances may influence policies (16). In India, the situation is largely unknown although dietary limitation for economic, religious, or cultural reasons may contribute to bone marrow hypoplasia. Folate deficiency, suspected on the basis of >3 lobes in neutrophils, occurred in over 80% of some populations in a National Multi-centric ICMR study of tribal populations (17).

Iron Deficiency

Although it is commonly assumed that iron from haemolysis is available for recycling in HbSS and that iron deficiency is unusual, the ICMR multi-centric study found evidence of iron deficiency in two-thirds of patients with Asian HbSS and mean haemoglobin levels increased by 2 g/dl after 3 months of iron supplementation (18).

Hypoplasia From Renal Failure

In Jamaica, renal function declines in most SS patients beyond the age of 40 years (19) with an associated fall in total haemoglobin (20) presumed to be consequent on reduced erythropoietin. Such patients are treated by top-up transfusions indicated by patient symptoms, and it is remarkable how low haemoglobin levels are tolerated by some elderly patients. In India, chronic renal impairment occurs in some elderly SS patients, but its prevalence and natural history are unknown.

Red Cell Sequestration

Acute or chronic sequestration, most commonly in the spleen, may contribute to lower haemoglobin and higher reticulocyte counts.

Acute Splenic Sequestration (ASS)

In the Jamaican cohort, this was a major cause of early death, (21) and events characterised by acute splenic enlargement of 4 cm or more below the costal margin, a lowered haemoglobin level

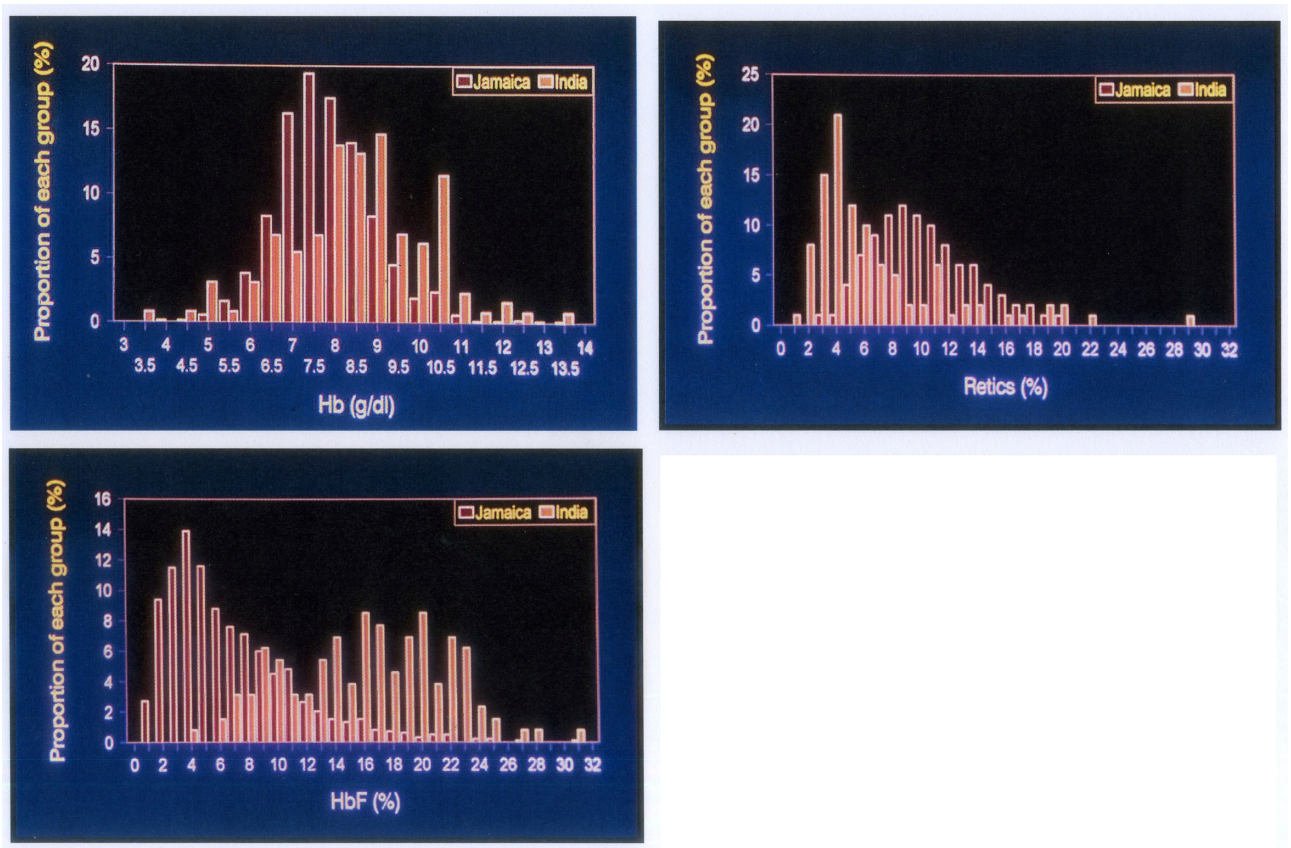

FIGURE 1 | Comparison of haematological indices in SS disease of African origin (red columns) and Indian patients from Odish. **Top left:** haemoglobin levels, **Top right:** reticulocyte counts, **Lower left:** fetal haemoglobin levels. All differences are highly significant $p < 0.001$.

and increased reticulocytes, occurred in a quarter of children by 2 years of age. Most events resolved after blood transfusion and the spleen diminished in size. Recurrence was common, often at shorter intervals with continuing mortality leading to a policy of splenectomy after two attacks. Parents were taught to feel for the spleen every day when bathing their child and to contact the clinic immediately if they suspected enlargement. Analysis of 5 years before, and after, this education programme showed an apparent increased incidence from 4.6 to 11.3 per 100 patient/years, indicating events detected by the mother and confirmed by doctors at the clinic so families can be taught to detect this complication. The death rate fell from 29.4 to 3.1 per 100 events (22).

In India, this complication is known to occur (6), but its natural history and the role of splenectomy in its management are unknown.

Chronic Hypersplenism (CHS)

Sometimes splenomegaly is sustained with prolonged red cell sequestration and a new haematological equilibrium with haemoglobin levels (usually 4–6 g/dl), and higher reticulocyte counts (usually over 15%) and red cell survival may be as short as 1–3 days. The spleen is usually 4 cm or more below the costal margin and often much larger and the metabolic demands of the expanded bone marrow compete with those for growth and height velocity may fall (23). The essential difference between the pathology of ASS and CHS is currently unclear.

In the Jamaican cohort, this complication occurred in approximately 10% and cases are monitored at monthly intervals with haemoglobin and reticulocyte counts and measurement of height, and if there is no evidence of spontaneous resolution within 6 months or if height velocity crosses growth centiles, then splenectomy is performed. An alternative approach is chronic transfusion, but this has been found to be impractical under Jamaican conditions. Two Jamaican patients died from superimposed ASS while awaiting splenectomy for CHS.

In India, CHS occurs and may be more common than in Jamaica. In the Odisha study, splenomegaly of 4 cm or more occurred in 33/122 (27%) patients but splenomegaly does not necessarily imply increased sequestration. Local awareness of an enlarged spleen may lead to branding over the splenic area (**Figure 2**), and others are treated by multiple transfusions. A 12-year-old patient attending the clinic in Valsad, Gujarat, had had 69 transfusions with a spleen measuring 16 cm below the left costal margin extending into the right iliac fossa (**Figure 2**), and following splenectomy, he has required no transfusions over the

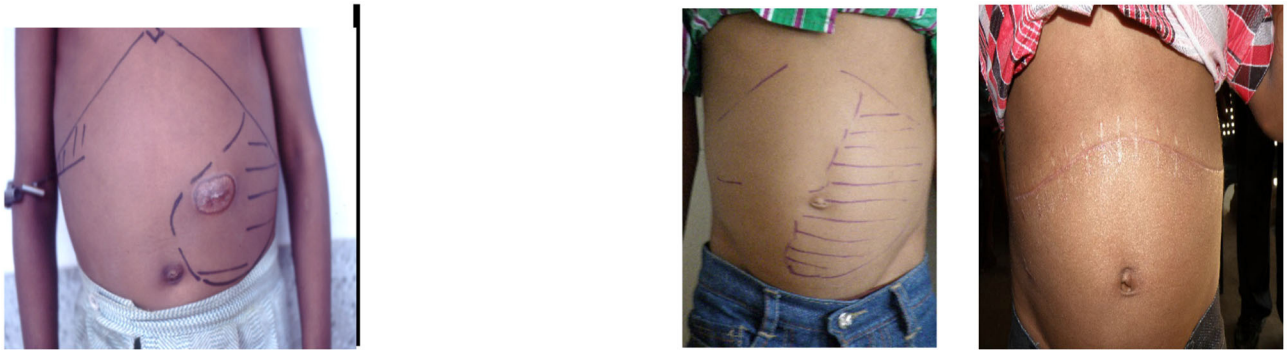

FIGURE 2 | Left, 13-year-old patient with 12 cm spleen and splenic burn 4 months earlier. **Right**, 12-year-old patient from Valsad with 16 cm spleen and 3 months after splenectomy.

past 8 years. Splenectomy in the treatment of CHS has worked well in Jamaica, which is malaria free, but the role of malaria may influence the decision for splenectomy and requires independent assessment in India.

Comment on Anaemia

Most patients with African HbSS, while in the steady state, maintain haemoglobin levels of 6–9 g/dl with reticulocyte counts of 8–12%, well below maximal bone marrow activity, and because HbS has a decreased oxygen affinity, oxygen delivery is near normal. When haemoglobin levels deviate from steady-state values characteristic of that patient, there is always a reason and, where possible, this should be investigated and addressed rather than the "blanket" treatment of blood transfusion. Indeed in some Indian clinics, patients are transfused at steady-state levels simply to raise their haemoglobin to what the doctor considers a "normal" level. Then, as the haemoglobin returns to the steady-state levels, they are transfused again because of the falling haemoglobin. This is unnecessary and poor practise exposing the patient to cost and potentially serious complications.

BONE PAIN

Avascular necrosis of the bone marrow results in a spectrum of clinical conditions, including dactylitis (hand-foot syndrome), bone pain crisis of adolescence and early adult life, and avascular necrosis of the femoral head (ANFH), each of which has a characteristic epidemiology.

Dactylitis

In the Jamaican cohort, dactylitis commenced at 3–4 months of age, affected 45% by 2 years of age (24), and became rare after the age of 5 years when the susceptible bone marrow no longer occupies the small bones of the hands and feet. The only comparable Indian data derive from the cohort study in Nagpur in which dactylitis occurred in 9% by the age of 5 years (6), although a history of dactylitis was given in 52% in the Burla study (3). Dactylitis commonly recurs and usually resolves completely although transient bacteraemias may cause premature fusion and a permanent shortening of the affected small bones (**Figure 3**).

Bone Pain Crisis

In Jamaica, the bone pain crisis affects principally the juxta-articular areas of the long bones, ribs, and spine commencing in later childhood, increasing in frequency during adolescence, especially in males and tapering in frequency and severity after the age of 30 years (25). Risk factors include a high total haemoglobin (26, 27) and low HbF (27), and common precipitating factors are skin cooling from exposure to cold water, baths, and rain. When associated with high fever or chest signs, hospital investigation may be necessary, but in their absence, this complication is essentially pathologically benign and home management is chosen in over 90% of cases admitted to the day-care centre in Jamaica (28) and elsewhere (29). Jamaica uses the WHO analgesic ladder commencing with paracetamol and paracetamol/codeine mixtures, the latter available without prescription and providing more adequate pain relief than paracetamol alone. Analgesic policy may vary between countries, and it is understood that narcotic agents cannot be given for home use in France, but in Jamaica, this has worked well.

In India, the limited data suggest a similar clinical pattern, and this complication is a common cause of hospital admission. However, only weak analgesics such as paracetamol are available for home use forcing patients requiring stronger pain relief to attend hospitals with the associated costs, difficulties, and disruption – indeed, the journey to hospital or a local clinic for stronger pain relief is only likely to increase anxiety and the degree of pain. Although some tablets combining paracetamol and codeine are available in India, these are not widely used and home analgesic policy for SS patients in India requires urgent revision.

Avascular Necrosis of the Femoral Head

In Jamaica, this occurred in about 10% of the cohort usually developing in adolescence or early adult life. The outcome depends upon the age at which avascular necrosis occurs, involvement of the immature capital epiphysis often allowing retention of the joint space, and remoulding of the hip, whereas

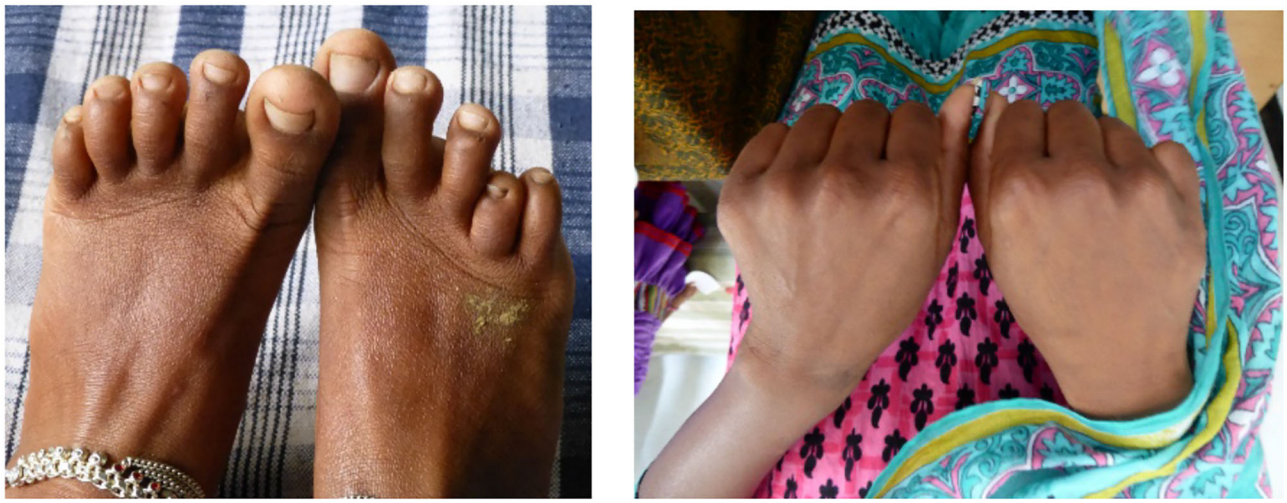

FIGURE 3 | Subjects attending sickle cell camps in Chhattisgarh, India. **Left**, shortened fourth metatarsal of the right foot. **Right**, shortened fifth metacarpal of the right hand. Both almost certainly the sequel of infection superimposed on dactylitis.

the involvement of the mature capital epiphysis causes secondary osteoarthritis with persistent pain and limitation of movement. Early diagnosis and avoidance of weight bearing may retard progression but eventually many patients require remodelling of the femoral head or total hip replacement.

In India, the prevalence is unknown but it is common, and requires study of its own risk factors, natural history, and protocols for optimal management.

INFECTIONS

Spectrum of Bacteria

In Jamaica, review of 93 initial septicaemic events in the cohort found 28 (30%) due to *Streptococcus pneumoniae*, 17 to *Salmonella*, 12 to *Staphylococci*, 11 to *Haemophilus*, 8 to *Klebsiella*, 6 to *Escherichia coli*, 5 to *Enterobacter*, and 6 others (Rankine-Mullings – unpublished).

Streptococcus Pneumoniae

Invasive pneumococcal disease (IPD) affected approximately 10% by the age of 12 years in the Jamaican cohort (30), and pneumococcal prophylaxis has become routine (31, 32) in African HbSS. In India, paediatric studies from Nagpur found that febrile events were common causes of hospital admission (4, 6), and organisms isolated on culture included *Staphylococci*, *E. coli*, and *Klebsiella*, with a notable absence of *S. pneumoniae*.

In African HbSS, splenomegaly precedes the loss of splenic function, which was abnormal as early as the first year of life (33, 34). There is then an age-related decline in frequency of splenomegaly (35). In Asian HbSS, splenomegaly develops later and persists for longer (3, 36). Pitted red blood cells, which correlate with other indices of splenic function, such as Howell-Jolly bodies and technetium (^{99m}Tc) sulphur colloid scans (37), increased later in Asian HbSS in eastern Saudi Arabia (38), and in western Odisha (3, 39), compared to Jamaica (40). Since the age specificity of IPD declines after the age of 3 years (41), persistence of splenic function beyond 3 years could explain the apparent absence of IPD in Indian experience and casts doubt on the need for pneumococcal prophylaxis.

Salmonella

In the Jamaican cohort, the second most common organism was *Salmonella*. Although it is well known to be associated with osteomyelitis in HbSS, the absence of clinical bone involvement in a septicaemic child led to the assumption of IPD and antibiotic therapy inappropriate for *Salmonella* resulted in a mortality rate of 23% (42). Salmonella carriage is likely to be more common in India, and more extensive blood cultures are needed to define the spectrum of septicaemic disease in Indian patients.

Other Infections

Malaria no longer exists in Jamaica but *Plasmodium vivax* has been shown to be common among febrile HbSS patients in Gujarat (43).

DIFFERENCES BETWEEN AFRICAN HbSS AND ASIAN HbSS

There are many similarities between African HbSS, as observed in Jamaica, and Asian HbSS but also some major differences. In the Jamaican cohort, chronic leg ulceration, defined by a minimal duration of 6 months, occurred in 30% (44) and priapism in 33% (45), but in Indian patients, both complications are rare and probably occur in less than 1%. The persistence of splenic function beyond the high-risk period for IPD may be vitally important casting doubt on the need for pneumococcal prophylaxis. A further difference relates to the use of hydroxyurea, which in African HbSS requires near toxic doses of 35 mg/kg requiring regular monitoring of haematology, whereas in Asian HbSS, a substantial elevation of HbF may be

achieved by much smaller doses of 10 mg/kg (46, 47), implying less risk and less-intensive haematological monitoring.

A direct comparison between newborn cohorts in Jamaica and Nagpur, India, confirmed that Indian patients had higher levels of HbF, later appearance of splenomegaly, and absence of IPD (48).

Comparison of HbSS Within India

Current claims of differences between HbSS in different parts of India must be treated with caution because of the different methods of patient ascertainment. The patients from Western Odisha (3, 10) represented survivors and also the chance event of awareness of the study. The supposedly more severe disease described in central India and south Gujarat is based on newborn screening but compromised by biases of selective default where failure to attend may be due to the lack of symptoms or death with their different implications. In India, HbF levels are consistently elevated compared to Jamaican patients but the prevalence of alpha thalassaemia defined by at least one alpha globin gene deletion varied widely in India, occurring in 16% in Akola (5), 28% in Nagpur (6), 53% in western Odisha (3), and 92% in South Gujarat (7) compared to 37% in Jamaica. This may contribute to possible regional clinical variation in India but the necessary clinical data are not yet available.

CONCLUSIONS

This review has focused on the most common causes of hospital admission in children with Asian HbSS in Nagpur, India, and allowed some comparisons with Jamaican data. Haemolysis is increased in both populations but a lower haemolytic rate may occur in Indian disease consequent on the higher HbF levels. In both Jamaica and India, bone pain continues to be a major cause of hospital admission, and the wider use of stronger analgesics may allow more Indian patients to be treated at home. There is an impression that ANFH may be more common in India although this may reflect symptomatics selection. In Indian patients, splenic function persists beyond the high risk period for IPD with profound implications for clinical management. These differences also provide insights into the pathological mechanisms of the disease, which in India occurs against a genetic background of universally elevated HbF levels but variable frequencies of alpha thalassaemia. Some of the apparent variations between Jamaican and Indian disease may reflect social, cultural, and economic differences in the societies. Indian colleagues may learn from protocols developed for African disease, but these may not always be appropriate for Indian practise. Careful documentation of the disease in India, preferably by following cases diagnosed at birth, will be essential in evolving locally appropriate models of care.

AUTHOR CONTRIBUTIONS

GS has worked in Jamaica and India on sickle cell disease for many years and is the sole author of this review.

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Clinical Utility of the Addition of Molecular Genetic Testing to Newborn Screening for Sickle Cell Anemia

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Sickle cell disease (SCD) is a group of related yet genetically complex hemoglobinopathies. Universal newborn screening (NBS) for SCD is performed in the United States and many other nations. Classical, protein-based laboratory methods are often adequate for the diagnosis of SCD but have specific limitations in the context of NBS. A particular challenge is the differentiation of sickle cell anemia (SCA) from the benign condition, compound heterozygosity for HbS and gene-deletion hereditary persistence of fetal hemoglobin (HbS/HPFH). We describe a sequential cohort of 44 newborns identified over 4.5 years who had molecular genetic testing incorporated into NBS for presumed SCA (an "FS" pattern). The final diagnosis was something other than SCA in six newborns (12%). Three (7%) had HbS/HPFH. All had a final, correct diagnosis at the time of their first scheduled clinic visit in our center (median 8 weeks of age). None received initial counseling for an incorrect diagnosis. In summary, genetic testing as a component of NBS for SCD is necessary to provide correct genetic counseling and education for all newborns' families at their first visit to a sickle cell center. Genetic testing also permits the use of early, pre-symptomatic hydroxyurea therapy by preventing infants with HbS/HPFH from receiving unnecessary therapy. We argue that genetic testing should be incorporated into contemporary NBS for SCD.

Keywords: hemoglobinopathies, newborn screening, sickle cell anemia, electrophoresis, genetic testing, HPFH, hydroxyurea

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INTRODUCTION

Sickle cell disease (SCD) is the name for a group of related hemoglobinopathies that affects numerous populations worldwide. Although it is a monogenic disease caused by mutations of the β-globin gene (*HBB*), there are a number of distinct genotypes of SCD that require differentiation to inform clinical care. The most common and severe form of SCD is the homozygous state for the sickle hemoglobin (HbS) mutation, called sickle cell anemia (SCA) or HbSS. There are also compound heterozygous forms of SCD, such as sickle-hemoglobin C disease (HbSC), sickle-β^+-thalassemia (HbSβ^+), and sickle-β^0-thalassemia (HbSβ^0). In contrast, compound heterozygosity for the HbS mutation and gene-deletion hereditary persistence of fetal hemoglobin (HbS/HPFH) is essentially benign.

Accurate NBS for SCD is needed to permit the prompt initiation of penicillin prophylaxis and referral to a specialized SCD center for ongoing comprehensive care, which has been shown to reduce morbidity and mortality (1–4). Most NBS programs use protein-based, Hb separation techniques that include electrophoresis (gel- or liquid-based), isoelectric focusing (IEF), and high-performance liquid chromatography (5). Although protein-based methods are the mainstay of Hb diagnostics (5), they may be inadequate in the context of NBS to detect β-thalassemia mutations (6) and unable to differentiate between HbSS and HbS/HPFH (7).

Without parental studies, the clinical differentiation of HbSS from HbS/HPFH can take several years to be correctly realized, given the prolonged postnatal decline in HbF concentration in children SCD (8). In the past, a watch-and-wait approach was reasonable given the limited and usually delayed initiation of treatment options. The hazards of this approach include unnecessary venipunctures, clinic visits, and prophylactic antibiotics for the children with HbS/HPFH and anxiety and fear about a serious disease, incorrectly diagnosed, for their parents and family. Now, the increasing use of early, pre-symptomatic disease-modifying therapy (e.g., initiation of hydroxyurea by 6 months of age for SCA) has made this critical diagnostic distinction time-sensitive (9). Treating children who have HbS/HPFH with hydroxyurea is not indicated. Here we describe a sequential cohort of newborns who had a presumed diagnosis of SCA by NBS (an "FS" pattern), several of whom actually had HbS/HPFH or other forms of SCD that were correctly and rapidly diagnosed by genetic testing before the child's first visit to our center, thereby preventing incorrect counseling and unnecessary medical interventions.

PATIENTS AND METHODS

Newborn screening in the state of Ohio begins with an initial dried blood spot that is analyzed at a central laboratory using a combination of HPLC and IEF. Newborns with Hb variants detected by this initial phase of testing have a second, confirmatory panel of testing performed using new blood specimens obtained by venipuncture in the first 2–4 weeks of life at a Regional Sickle Cell Services Program (RSCP). The Ohio Department of Health Region 1 RSCP is located at the Cincinnati Comprehensive Sickle Cell Center at Cincinnati Children's Hospital Medical Center (CCHMC). Region 1 comprises eight counties in southwestern Ohio in which approximately 500 newborns undergo confirmatory testing for suspected hemoglobinopathies each year. For newborns with suspected SCD, protein-based confirmatory testing includes capillary zone electrophoresis, acid hemoglobin electrophoresis, and IEF. Additionally, beginning in July 2015, newborns with an "FS" pattern indicating suspected SCA (here defined to comprise HbSS and HbSβ^0), also had *HBB* sequence analysis and copy number variation analysis of the α-globin and β-globin gene clusters performed simultaneously using the same blood specimen. The overall goal of this NBS-based genetic testing is to differentiate HbSS from HbS/HPFH before affected newborns have their first visits in our comprehensive sickle cell center. No *a priori* sample sized was calculated, rather all consecutive patients were included in this arbitrary 4.5-year sample (**Figure 1**). Medians were compared by the 2-tailed Mann Whitney test. $P < 0.05$ was considered statistically significant.

RESULTS

We analyzed 3,392 consecutive newborns with abnormal NBS results between July 2015 and December 2020 and identified 44 who had a presumed diagnosis of SCA (**Figure 1**). Genetic testing was not performed for 1 newborn at the time of the second, confirmatory NBS test in error (only protein-based testing was done); genetic testing later confirmed that she had HbSS. Of the remaining 43 newborns who had genetic testing as part of NBS (**Table 1**), 37 (86%) had a final diagnosis of HbSS. The other six newborns (14%) had different SCD genotypes. Three (7%) had HbS/HPFH, two (5%) had sickle-β^+-thalassemia (HbSβ^+), and one (2%) had sickle-β^0-thalassemia (Hb Sβ^0).

Of the newborns 3 who had HbS/HPFH, 2 had the HPFH-2 (Ghanaian) deletion and 1 had the HPFH-1 (Black) deletion. These three newborns had a final genetic diagnosis of HbS/HPFH by the time of their first clinic visits in our center (ages at first visits: 6, 8, and 11 weeks). None was started on hydroxyurea. If prophylactic penicillin was initiated before the first clinic visit it was discontinued, and each is now followed only yearly in our center. The two with HbSβ^+ also had a final genetic diagnosis of by the time of their first clinic visit.

Of the patients with SCA, 37/38 (97.3%) were prescribed hydroxyurea starting at a median of 8.0 months of age (interquartile range: 7.1–10.0). The single patient with SCA not yet prescribed hydroxyurea was last seen in our clinic at 4 months of age, and he has a scheduled visit at 7 months of age to start this medication. Comparing the first and second halves of this cohort, the median age at prescription of hydroxyurea decreased from 9.6 to 7.8 months ($P = 0.023$).

DISCUSSION

In this consecutive cohort of newborns with suspected SCA, the final diagnosis was something other than HbSS or HbSβ^0 in about 12%. Three (7%) had HbS/HPFH and 2 (5%) had HbSβ^+. The distinction between HbSS and HbSβ^0 by NBS is not critical, because both diseases are clinically similar and managed identically, and these two genotypes are often both called sickle cell anemia (10, 11). Nevertheless, this genetic distinction can be clinically helpful to explain microcytosis, provide accurate genetic counseling, and determine eligibility for genetic therapies.

A diagnosis of HbSβ^+ will become clear within a few months of age by the detection of a small amount of HbA by Hb analysis, but a diagnosis of HbS/HPFH might take several years to be correctly realized. In children with SCA, the postnatal decline in HbF concentration may take 5–6 years to be completed (8). In the past, a "watch-and-wait" approach to distinguish HbSS from HbS/HPFH with serial measurements of HbF and blood

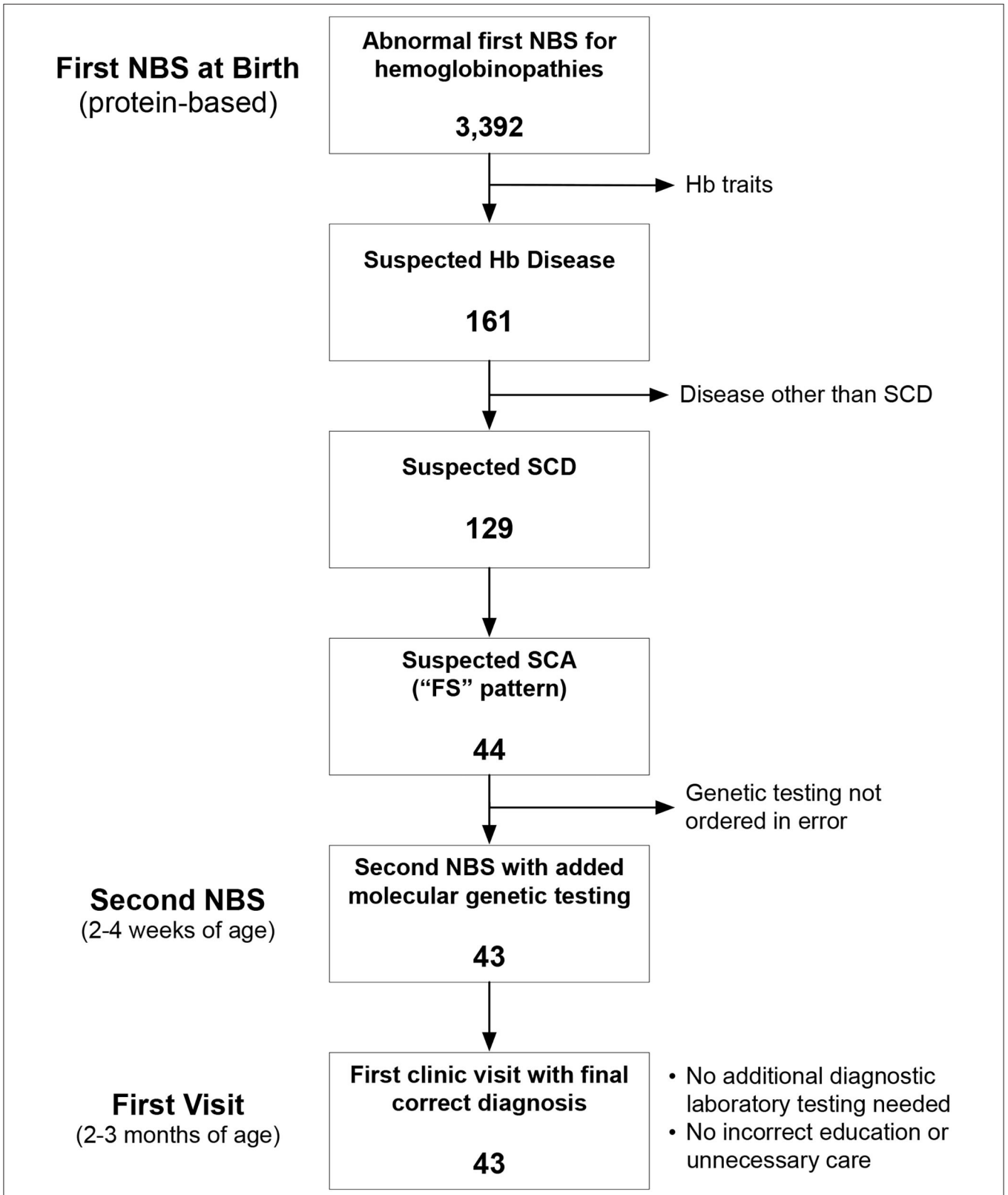

FIGURE 1 | Identification of the study cohort. Between July 2015 and December 2020, there were 3,392 abnormal newborn screening (NBS) tests for hemoglobinopathies. We excluded newborns with hemoglobin (Hb) trait states and hemoglobinopathies other than sickle cell disease (SCD). We identified 44 newborns during this time with an "FS" pattern, indicating suspected sickle cell anemia (SCA), which we define here to include both homozygous sickle cell anemia (HbSS) and sickle-β^0-thalassemia (HbSβ^0).

TABLE 1 | Results of molecular genetic testing as a component of newborn screening (NBS) for suspected sickle cell anemia (SCA).

Final diagnosis (β-globin genotype)	*N* (%)
Sickle cell anemia (HbSS)	37 (86%)
Sickle-β^0-thalassemia (HbSβ^0)[a]	1 (2%)
Sickle-β^+-thalassemia (HbSβ^+)	2 (5%)
HbS/HPFH[b]	3 (7%)

Forty-three consecutive newborns with an "FS" pattern on the first NBS test, indicating a likely diagnosis of SCA, had genetic testing performed as a component of the second, confirmatory NBS testing panel. In addition to protein-based testing, molecular genetic testing included direct sequencing of the β-globin gene (HBB) and copy number variation analysis of the β-globin gene cluster.

[a]HbSS and HbSβ^0 are often both referred to as sickle cell anemia (SCA).

[b]Compound heterozygosity for the HbS mutation and gene-deletion forms of hereditary persistence of fetal hemoglobin [$(\delta\beta)^0$-HPFH].

counts was reasonable given the formerly limited and delayed initiation of disease-modifying therapy. Now, the increasing use of early, pre-symptomatic disease-modifying therapy (e.g., hydroxyurea initiation at 6 months of age) has made this critical diagnostic distinction time-sensitive (9, 12). For infants with SCA, hydroxyurea should be started to prevent and reverse this early decline in HbF rather than waiting for the HbF level to decline to some arbitrary level (13). However, treating children who have HbS/HPFH with hydroxyurea is not indicated and exposes them to unnecessary risks, expenses, laboratory monitoring, and inconvenience. Genetic testing incorporated into NBS can facilitate this new standard (6).

We also determine α-globin gene copy number (data not shown), because it provides supplemental prognostic information in SCA, and copy number variation analysis of the β-globin gene cluster is already being performed. We do not test for common non-gene-deletion HPFH determinants at the time of NBS, because these polymorphisms are not likely to inform early clinical management, but practice may change with the decreasing costs of broader genetic testing over time (e.g., next generation sequencing panels). Currently, the cost of genetic testing is offset by the elimination of both parental testing and additional diagnostic laboratory testing in the infant.

Molecular genetic testing is not required to determine if an infant has HbS/HPFH if parental studies can be performed. However, both parents may not be available or willing to be tested. The time needed to perform this testing may also take longer than needed to permit the earliest initiation of hydroxyurea. In this 4.5-year study period, the median age of initiation of hydroxyurea decreased from 9.6 to 7.8 months of age, highlighting the need for a timely correct diagnosis. Genetic testing added to NBS overcomes these obstacles and can be performed on a blood sample that had already been obtained for protein-based testing. A recent plea has been published for the diagnosis of HbS/HPFH in newborns (7), which we have answered here. Larger studies are needed to demonstrate the generalizability and cost-effectiveness of our approach.

In summary, molecular genetic testing incorporated into NBS for SCD allows correct education and counseling for the newborn's family to be given at the first visit in a sickle cell center and eliminates additional diagnostic testing (no additional venipuncture). It prevents unnecessary grief and anxiety from incorrectly diagnosing a newborn with SCA, only later to "revise" the diagnosis to a benign condition. Moreover, a timely correct diagnosis can prevent infants with HbS/HPFH from receiving unnecessary medical interventions and treatments.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

LS and CQ: conceptualization, visualization, and funding acquisition. CQ: formal analysis. DH: investigation and data curation. LS: writing—original draft preparation. DH and CQ: writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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Priming With Red Blood Cells Allows Red Blood Cell Exchange for Sickle Cell Disease in Low-Weight Children

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Red blood cell exchanges are frequently used to treat and prevent cerebrovascular complications in patients with sickle cell anemia (SCA). However, the low weight of young children represents serious concerns for this procedure. The Spectra Optia device can perform automatic priming using red blood cells (RBCs) (RCE/RBC-primed) which could allow RBC exchanges (RCE) to be performed in young children without hypovolemic complications, but this method requires evaluation. We prospectively analyzed the clinical safety of the RCE/RBC-primed procedure in 12 SCA low-weight children under either a chronic RCE program or emergency treatment over 65 sessions. We monitored grade 2 adverse events (AEs) such as a decrease in blood pressure, increase in heart rate, fainting sensation, or transfusion reactions and identified the critical times during the sessions in which AEs could occur. Post-apheresis hematocrit (Hct) and a fraction of cell remaining (FCR) values were compared to the expected values. We also compared the impact of automatic RCE ($n = 7$) vs. RCE/RBC-primed ($n = 8$) on blood viscosity and RBC rheology. A low incidence of complications was observed in the 65 RCE sessions with only seven episodes of transient grade 2 AEs. Post-apheresis Hct and FCR reached expected values with the RCE/RBC-primed method. Both the automatic and priming procedures improved RBC deformability and decreased the sickling tendency during deoxygenation. Blood rheological features improved in both RCE/RBC-primed and automatic RCE without priming conditions. The RCE/RBC-primed procedure provides blood rheological benefits, and is safe and efficient to treat, notably in young children with SCA in prophylactic programs or curatively when a SCA complication occurs.

Keywords: red blood cell exchange, sickle cell anemia, low weight children, priming, safety, performances, blood rheology

INTRODUCTION

Sickle cell anemia is caused by a point mutation in the seventh position of the β-globin gene leading to the production of abnormal hemoglobin called HbS. When deoxygenated, polymerization of HbS may occur, leading to mechanical distortion of red blood cells (RBC), i.e., sickling (1). Decreased deformability and increased fragility of sickled RBCs are the cause of frequent painful vaso-occlusive events and enhanced hemolysis in patients with sickle cell anemia (SCA), respectively (1, 2). Moreover, patients with the highest blood viscosity are at high risk for frequent vaso-occlusive crises and other complications (2, 3). Recurrent vaso-occlusive events additionally cause endothelial dysfunction and inflammation, resulting in progressive tissue and organ damage (3–5).

Replacement of sickle RBCs containing HbS with healthy RBCs containing normal hemoglobin improves the outcome of patients with SCA. This replacement can be achieved by transfusions associated with manual bloodletting. However, this methodology allows a limited exchange of abnormal RBCs and may generate blood hyperviscosity, which in turn may result in vascular complications, and does not prevent iron overload (6). RBC exchange (RCE) using an apheresis device can replace a greater number of RBCs, is more efficient in preventing the occurrence of SCA complications when regularly performed, decreases whole blood viscosity, and limits the risk of iron overload (6–10).

However, the extracorporeal volume (EV) (160–185 ml depending on the device) of the RCE circuit limits its use in low-weight children (11–13). Transfusion experts suggest performing RCE without priming only in children with a bodyweight higher than 20 kg (with total blood volume [TBV] around 1,500 ml and EV corresponding to 12%) (14). When EV is greater than 15% of the total blood volume (TBV), they recommend performing blood priming before RCE (15). In addition to weight, hematocrit (Hct) must be considered when performing RCE since low weight associated with low Hct predisposes to hypovolemic complications during RCE (16).

A few years ago, the Cobe Spectra® device was replaced by Spectra Optia®, which was the first device relying on a completely automatic priming procedure using RBC during RCE (RCE/RBC-primed). RCE/RBC-primed could allow RCE to be performed in young children without hypovolemic complications but this method requires evaluation. The main goal of this study was to assess the feasibility and safety of RCE/RBC-primed in low-weight children with SCA. In addition, we evaluated the impact of RCE/RBC-primed on RBC rheology and blood viscosity and compared the responses to the ones obtained with RCE without RBC priming.

Abbreviations: RBC, Red blood cell; RCE, Red blood cell exchange; SCA, Sickle cell anemia; AE, Adverse events; Hct, Hematocrit; FCR, Fraction of cell remaining; EV, Extracorporeal volume; TBV, Total blood volume; sBP, systolic blood pressure; RBCU, Red blood cell units; aFCR, actual FCR; pFCR, predictive FCR; fFCR, final FCR; nHct, native Hct; pHct, predictive Hct; fHct, final Hct; PoS, Point of sickling; ACD-A, acid citrate dextrose A.

MATERIALS AND METHODS

Patient Selection

This prospective study was conducted in accordance with the Declaration of Helsinki and approved by the Hospices Civils de Lyon ethics committee (L14-127). Twelve children with SCA were included after obtaining parental written informed consent. The inclusion criterion was a body weight < 20 kg and the exclusion criterion was hemodynamic instability with mean systolic blood pressure (sBP) < 50 mmHg.

Description of the RCE With Personalized Priming Procedure

In chronic programs, RCE sessions were performed every 6 weeks. Otherwise, RCE sessions were performed when an acute SCA complication occurred. Venous access was short-term central venous access in all cases.

Irregular agglutinin research was performed before RCE sessions. Transfused RBC units (RBCUs) were sickle-negative, leuco-reduced, crossmatch-compatible, and phenotypically matched for the C, E, c, e, and K (JK2 and MNS3 when possible) antigens. All the RBCUs transfused were heated to 37°C in a water bath.

Considering the low weight of the children, the TBV was calculated using the initial and simplified formulae of Glicher: TBV (ml) = weight (kg) × 75 (17). The number of RBCUs needed can easily be deduced since the Spectra Optia® software (version 7.2) calculates the volume of transfused RBCs required to reach the predictive fraction of cell remaining (pFCR) and the predictive Hct (pHct). To perform RBC priming, a supplementary compatible RBCU (called reconstituted RBCU) was ordered and completed with ABO-compatible fresh frozen plasma to obtain a reconstituted RBCU. To prevent hemoconcentration during the critical session stage, i.e., after infusion of 185 ml of concentrated RBCs while the device has not removed significant amounts of abnormal RBCs, we primed the circuit with RBCU diluted with ABO-compatible fresh frozen plasma targeting an Hct close to the blood level of the patient, i.e., with an Hct of 28%, based on our clinical experience.

After discontinuation of the priming circuit (volume 200 ml and flow 60 ml/min), the reconstituted RBCU was removed, and the RBC-primed circuit was connected to the central venous access. Depending on the degree of filling of the infusion chamber, the EV of the RCE for the Spectra Optia® (Terumo BCT) device circuit ranges from 141 to 185 ml (14), and the latter value was chosen for further calculations. Since rinse-back is not recommended at the end of the RCE in children (15), 15 ml/kg of 4% albumin was continuously perfused to prevent hypovolemia at the end of the procedure.

Anticoagulation of the circuit was performed using acid citrate dextrose solution A (ACD-A), and a significant decrease in blood calcium concentration was prevented by continuous intravenous calcium infusion.

Clinical Tolerance, a Fraction of Cell Remaining, Hematocrit, and Factors Prone to Influence Hemostasis

The clinical tolerance of RCE using RCE/RBC-primed in low-weight children was divided into three periods: during the first 10 min of the RCE/RBC-primed sessions, corresponding to the time between the blood intake of the patient and the infusion of normal RBCs (called the critical time of priming reinfusion), and during two consecutive 40-min periods. Systolic BP and heart rate (HR) were monitored every 2 min during the critical time of priming reinfusion and every 10 min during the remaining session. According to the National Cancer Institute Common Terminology Criteria for Adverse Events guidelines (18), grade 2 AEs or higher correspond to a significant decrease in sBP (>20 mmHg) and a significant increase in HR (>20 pulses per minute) associated or not with a significant fainting/asthenia sensation. The number of RCE/RBC-primed sessions with grade 2 AEs or higher were recorded, as well as the symptoms of transfusion-related AEs (pruritus, urticarial erythematous wheals, skin edema, and wheezing).

At the end of the RCE sessions, post-apheresis Hct was assessed to obtain actual Hct (aHct). In parallel, the post-apheresis percentage of HbS (actual Hbs or aHbs) was assessed to calculate actual FCR (aFCR). The aFCR was calculated as follows: aFCR = aHbS)/pre-apheresis HbS (also called native HbS or nHbS). We also noted the predictive FCR (pFCR) which is recorded on the device before the sessions and the final FCR (fFCR), which corresponds to the FCR recorded by the device at the end of the sessions. In parallel, we noted pre-apheresis or native Hct (nHct), predictive Hct (pHct, recorded on the device before the session), and final Hct (fHct, recorded on the device at the end of the sessions).

Changes in factors prone to influence hemostasis parameters were assessed, i.e., ratio of ACD-A/calcium infused and decrease in platelet blood levels (calculated as follows: decrease in platelet level = [pre-apheresis blood level – post-apheresis blood level]/pre-apheresis blood level).

Blood Rheology

We also analyzed the impact of RBC-priming by comparing RCE/RBC-primed (n = 8 sessions in 5 of the 12 patients followed) vs. automatic RCE (i.e., without priming RBCs; n = 7 sessions in 7 other patients) on blood viscosity and RBC rheology. The two groups of patients were assessed during RCE programs. Measurements were performed before RCE/RBC-primed, at the end of the critical time of priming reinfusion (10–15 first min) and the end of the sessions (**Figure 1A**). The same blood rheological parameters were measured at the same time points in patients having the RCE/RBC-primed and automatic classical RCE. Blood viscosity was measured at native Hct and shear rates of 45 and 90 s^{-1} using a cone-plate viscometer (Brookfield DVII+ with CPE40 spindle, Brookfield Engineering Labs, Natick, MA, USA) and expressed in centipoises (cP). RBC deformability was determined at 37°C, in isotonic conditions and 3 Pa by laser diffraction analysis (ektacytometry), using the Laser-assisted Optical Rotational Cell Analyzer (LORRCA Maxsis, RR Mechatronics, Hoorn, The Netherlands). In addition, ektacytometry was carried out with the oxygenscan module (LORRCA Maxsis, RR Mechatronics, Hoorn, The Netherlands) to measure RBC deformability over an oxygen gradient as previously described (19–22). The suspension was sheared at 30 Pa and 37°C into the Couette system of the ektacytometer. The oxygen partial pressure (pO_2) was gradually decreased from 160 to 20 mmHg (deoxygenation) and then increased to normoxic values. The diffraction pattern obtained by ektacytometry was analyzed with a camera and a computer and the elongation index (EI), which reflects RBC deformability, was also calculated. The Point of Sickling (PoS) was obtained during deoxygenation and corresponds to the pO_2 at which RBC deformability decreases below 5% of the maximal deformability reached during normoxia (i.e., before deoxygenation). All measurements were standardized as recommended (21, 22). RBC aggregation was determined at 37°C *via* syllectometry, (i.e., laser backscatter vs. time, using the LORRCA) after adjustment of the Hct to 40%.

Statistical Analyses

Values are expressed as mean ± *SD*. A one-way ANOVA with repeated measurements was used to compare aHct, pHct, and fHCT; aFCR, pFCR, and fFCR. A two way-ANOVA was used to compare the effects of RCE/RBC-primed and RCE without RBC priming on blood rheology. The Tukey *post-hoc* test was used to locate differences when appropriate. $p < 0.05$ was considered significant.

RESULTS

Feasibility and Safety of the Priming Protocol and the Whole RCE

Twelve children were treated with RCE/RBC-primed (**Table 1**). In most children (10/12), EV of the circuit represented a high volume related to their TBV (EV/TBV ratio greater than 15%) while lower EV/TBV was associated with low nHct (21 and 22% for patients 6 and 12, respectively) were found in two of them.

During all sessions, it took approximately ten min (9 to 15 min) for the device to reinfuse the volume of RBC-primed while the blood of the patient was still being removed. No significant grade 2 AEs (no decrease in sBP, no increase in HR, no fainting sensation) were observed during that time in the 65 RCE/RBC-primed sessions performed (**Table 2**).

The mean pFCR was 19 ± 4% (**Table 3**), which corresponded to a significant RBC replacement (68 ± 8 ml/kg) of at least 80% of the initial RBC volume. As recommended (15), the target Hct after RCEs in children was expected to be 27–30%. Mean Hct levels in children after RCEs (aHct) were not significantly higher than nHct (27 ± 2 vs. 26 ± 3%, respectively), reducing the risk for developing hemodynamic complications during and after RCEs (**Table 3**).

The exchange step lasted 80 ± 11 min. All complications occurred during the last 30 min or in the first 5 min after the end of the session (**Table 2**). Systolic blood pressure (sBP) decreased in two patients just after the end of the sessions (**Table 2**). One episode of a significant increase in heart rate occurred in two patients and a fainting sensation occurred during three sessions

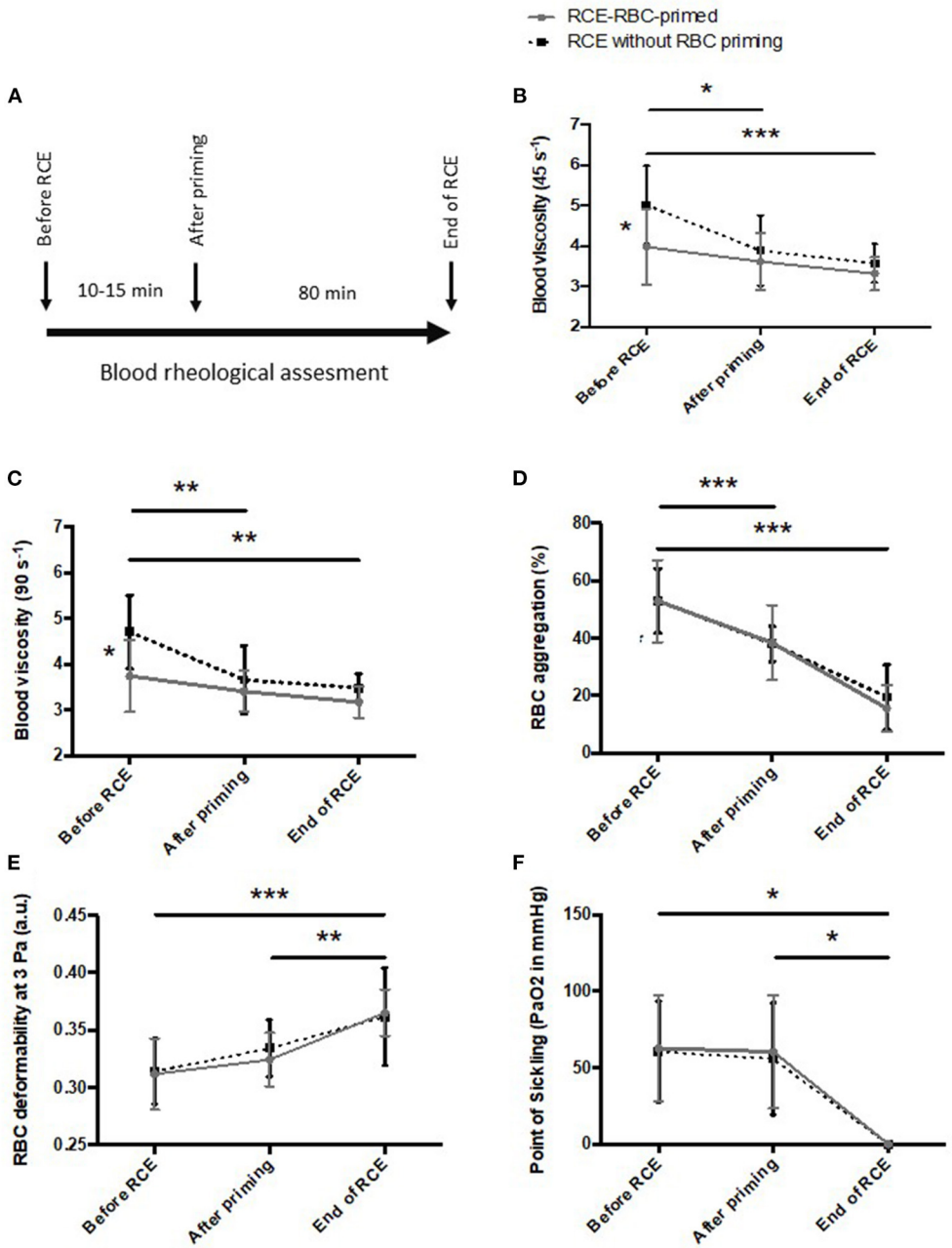

FIGURE 1 | Changes in blood rheological properties during red blood cell exchange/red blood cell (RCE/RBC)-primed vs. RCE without priming. **(A)** protocol; **(B)** blood viscosity at 45 s^{-1}; **(C)** blood viscosity at 90 s^{-1}; **(D)** RBC aggregation; **(E)** RBC deformability at 3 Pa; **(F)** Point of sickling. Statistical difference: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

in two children, all during the last 30 min. Saline and 4% albumin were infused, inducing a decrease in HR or fainting sensation (while RCE continued) and a rapid increase in sBP. All the hemodynamic complications occurred at the end of the RCE sessions. The occurrence of AEs at this time suggested a role of the large volume of RBC exchanged (more than 80% of RBC exchanged i.e., aFCR < 20%) and we could speculate that exchanging lower amounts of RBC may decrease the incidence of AEs. No pruritus or urticarial wheals and no wheezing were observed thereafter during the entire protocol (**Table 2**).

Performances of RCE/RBC-Primed

We analyzed the ability of the RCE/RBC-primed method to obtain the Hct required, and the ability to replace sufficient amounts of RBC in the blood of patients. No difference was observed between aHct,

TABLE 1 | Clinical characteristics of the 12 patients with sickle cell anemia (SCA) at the beginning of red blood cell exchange/red blood cell (RCE/RBC)-primed.

Parameters	Pt 1	Pt 2	Pt 3	Pt 4	Pt 5	Pt 6	Pt 7	Pt 8	Pt 9	Pt 10	Pt 11	Pt 12
Gender	F	F	F	M	M	M	F	M	F	M	F	F
Age (year)	3	3.5	3.5	3	3	6	3	4.5	4.5	3	5.5	6
Weight (kg)	11	15	13	12	13	18	13	16	17	13	17	19
TBV (mL)	825	1,050	975	900	975	1,350	975	1,200	1,190	952	1,270	1,425
EC/TBV (%)	22	17	19	20	19	14	19	15	16	19	15	13
nHct (%)	28	31	21	28	26	21	22	23	12	30	22	22
RCE Program	Yes	yes	yes	no	yes	yes	yes	no	no	no	no	yes
Indications for RCE	ACS+ Stroke	ACS	ACS+ VOC	ACS	ACS+ VOC	CVO+ Surgery	Stroke	ACS	Meningitis	Surgery	Surgery	Cerebral vasculopathy
nHbS (%)	31	46	84	81	83	75	88	84	92	61	87	91

Pt, patient; F, female; M, male; TBV, Total blood volume; VOC, vaso-occlusive crisis; ACS, acute chest syndrome; RBCP, packed red blood cells; RCE, red blood cell exchange; nHct, native hematocrit (blood hematocrit before RCE); nHbs, native HbS (blood HbS before RCE).

TABLE 2 | Clinical complications during RCE/RBC-primed.

Parameters	Pt 1	Pt 2	Pt 3	Pt 4	Pt 5	Pt 6	Pt 7	Pt 8	Pt 9	Pt 10	Pt 11	Pt 12	All pts
Number of RCE/RBC-primed sessions	28	10	8	2	5	3	4	1	1	1	1	1	65
Decrease in sBPS													
1st period (0–10 min)	0	0	0	0	0	0	0	0	0	0	0	0	0
2nd period (11–50 min)	0	0	0	0	0	0	0	0	0	0	0	0	0
3rd period (51–90 min)	1 epd*	0	0	0	1 epd*	0	0	0	0	0	0	0	2
Increase in pulses													
1st period (0–10 min)	0	0	0	0	0	0	0	0	0	0	0	0	0
2nd period (11–50 min)	0	0	0	0	0	0	0	0	0	0	0	0	0
3rd period (51–90 min)	0	1 epd	0	0	1 epd	0	0	0	0	0	0	0	2
Fainting sensation													
1st period (0–10 min)	0	0	0	0	0	0	0	0	0	0	0	0	0
2nd period (11–50 min)	0	0	0	0	0	0	0	0	0	0	0	0	0
3rd period (51–90 min)	2 epd	0	0	0	0	0	1 epd	0	0	0	0	0	3
Transfusion reactions (0–90 min)	0	0	0	0	0	0	0	0	0	0	0	0	0

*Pt, patient; sBP, systolic blood pressure; epd, episode. *occurrence during 5 min after the end of the sessions.*

pHct, and fHct, or aFCR, pFCR, and fFCR (**Table 3**), indicating that the expected Hct and FCR values are reached.

The ratio ACD-A/calcium infused was around 10 in most of the patients (**Table 3**), as observed in our experience in adults treated with RCE (data not shown). The mean decrease in platelet levels was 61 ± 13%, which needs to be considered in some situations when RCE is performed.

Blood Viscosity and RBC Rheology: Comparison of RCE/RBC-Primed vs. RCE Without Priming

The use of an RBCU during the priming raised the question of transient blood hyperviscosity during the priming and at the end of the sessions that could promote complications in SCA. Indeed, blood rheological parameters were investigated in 8 young low-weight children with SCA (19 ± 1 kg; 4 ± 1.2 years) having the RCE/RBC-primed procedure and compared to the blood rheological responses of seven older children with SCA (33 ± 13 kg; 12.4 ± 3.6 years) who were having the classical automatic RCE procedure without RBC-priming (**Figure 1**). Of note, the children of both groups were under chronic RCE programs and none of the sessions assessed was performed in an emergency.

Initial blood viscosity was lower in the RCE-RBC-primed group compared to the group having RCE without RBC priming. Blood viscosity remained unchanged in the patients having RCE/RBC-primed (**Figures 1B,C**). However, during automatic (classic) RCE, blood viscosity decreased after the first RBCU to reach similar blood viscosity to the other group up until the end of the procedure. RBC deformability increased and RBC aggregation decreased in the two groups over the procedures, with no difference between them (**Figures 1D,E**). The PoS decreased in the two groups to reach very low values at the end of the procedure and no difference was observed between the two groups (**Figure 1F**).

TABLE 3 | Biological results to assess performances of RCE/RBC-primed.

Parameters	Pt 1	Pt 2	Pt 3	Pt 4	Pt 5	Pt 6	Pt 7	Pt 8	Pt 9	Pt 10	Pt 11	Pt 12	All pts
nHct (%)	28 ± 1	25 ± 3	22 ± 1	26 ± 2	26 ± 3	26 ± 5	23 ± 1	23	12	30	30	12	26 ± 3
pHct (%)	27 ± 1	27 ± 1	26 ± 1	27 ± 1	27 ± 1	27 ± 1	26 ± 1	28	25	29	28	26	27 ± 1
fHct (%)	28 ± 1	27 ± 5	25 ± 1	26 ± 2	27 ± 1	29 ± 1	26 ± 1	25	25	29	30	26	27 ± 1
aHct (%)	27 ± 2	28 ± 3	26 ± 2	27 ± 1	28 ± 1	29 ± 1	25 ± 1	25	26	29	29	26	27 ± 2
pFCR (%)	17 ± 4	18 ± 3	19 ± 5	17 ± 3	18 ± 3	20 ± 0	21 ± 2	20	20	20	18	20	19 ± 4
fFCR (%)	16 ± 4	16 ± 2	17 ± 7	17 ± 4	17 ± 5	19 ± 0	23 ± 2	20	20	20	19	26	18 ± 5
aFCR (%)	14 ± 6	17 ± 4	19 ± 7	12 ± 2	15 ± 6	17 ± 6	23 ± 4	19	23	19	18	22	17 ± 6
Number of RBCUs/session	3 to 4	3	2 to 3	3	2 to 3	3 to 4	2 to 3	2	3	2	3	3	2 to 4
ACD-A infused to Pt (mL)	116 ± 22	101 ± 8	94 ± 18	91 ± 10	99 ± 11	108 ± 12	87 ± 19	124	44	88	103	152	106 ± 22
Calcium injected (mL)	11 ± 3	13 ± 3	10 ± 3	9 ± 0	10 ± 3	15 ± 3	10 ± 2	11	9	11	16	15	11 ± 3
Ratio ACD-A/calcium used	11 ± 2	9 ± 2	10 ± 1	11 ± 1	10 ± 3	7 ± 1	8 ± 1	11	5	8	6	10	10 ± 2
PBL before RCE/RBC-primed	183 ± 62	252 ± 62	304 ± 50	432 ± 179	297 ± 98	394±154	398 ± 53	151	186	269	353	304	277± 103
PBL after RCE/RBC-primed	79 ± 13	79 ± 24	75 ± 12	92 ± 15	54 ± 27	210 ± 57	152 ± 48	69	106	119	150	167	97 ± 46
Decrease in PBL (%)	52 ± 10	69 ± 11	76 ± 4	76 ± 14	79 ± 3	56 ± 15	61 ± 14	54	43	56	57	45	61 ± 13

nHct, native (pre-apheresis Hct); pHCt, predictive Hct (expected Hct registered on device before session); fHct, post-apheresis Hct given by device after session; aHct, actual Hct (post-apheresis peripheral blood Hct); pFCR, predictive FCR; fFCR, final FCR (expected FCR given by device after session); aFCR, actual FCR (post-apheresis Hbs divided by pre-apheresis HbS); Pt, patient; PBL, platelet blood levels.

DISCUSSION

The indications of RCE during curative and prophylactic treatments of SCA are well-known (23). The principle of RCE is to prevent the occurrence of SCA complications by replacing abnormal RBCs with normal RBCs so that the HbS level does not exceed a defined threshold between two sessions (15, 23). Since prophylactic treatment has become more routine among apheresis teams who treat more and more Patients with SCA, the RCE method has to be improved to optimize this treatment in all patients, including the youngest ones. In very young children, two main issues hamper the use of RCE on a routine basis: 1) the venous access series [this is why we used temporary central venous catheter access for which we demonstrated tolerance and efficiency (24)] and 2) the risk of hypovolemia. The availability of a device combining RCE and an automatic priming step (RCE/RBC-primed) led us to evaluate its feasibility, safety, and performance in this population.

Our work showed that the RCE/RBC-primed method was well tolerated both during the priming and during the RCE step itself. The incidence of complications was thus very low and rapidly self-limiting. The method was safe without any risk of increasing blood viscosity during and after the sessions. Moreover, the RCE/RBC-primed method was efficient. i.e., allowed to reach the targeted post-apheresis FCR and Hct.

The main problem when performing RCE in very young children is the risk of hypovolemia due to a high EV compared to a low TBV. Indeed, the study of Dedeken et al. switched from manual exchange transfusion to RCE (using the Optia Spectra system) only in children who weighed 30 kg or more, this threshold having been decided to avoid priming of the circuit (7). RCE techniques with low EV, such as COBE Spectra, were used in low-weight children (20 to 30 kg) without inducing hemodynamic complications (25, 26). In other apheresis techniques, RBC-priming appears to be a therapeutic option to treat children with a bodyweight below 20 kg (27). As mentioned, in our series the EV represented around 20% of the TBV of each child, which could induce severe hypovolemia and hemodynamic complications. Monitoring of the early phase of RCE while infusing the volume in the circuit primed with reconstituted RBCs showed no hemodynamic changes during initial blood withdrawal. This absence of early hemodynamic complications leads us to suggest that RBC-priming may be used more systematically in low-weight children. The complications that occurred in 10% of the sessions involved only hemodynamic events. Since they occurred at the end of the sessions, they were probably the consequence of extended RBC exchanges. Moreover, the occurrence of hemodynamic complications led us to consider the use of a continuous albumin infusion during RCE. However, this procedure with RBC-priming seemed to be necessary, as the frequency of AEs would probably be higher without RBC-priming in these low-weight children (14).

The performances of all types of RCE sessions, and here of RCE/RBC, had to be assessed to verify the efficiency and safety of this treatment in low-weight children. The main objective of RCE is to replace abnormal SCA-RBC with non-sickle RBC, which was evaluated by the FCR; the lower the FCR, the higher the replacement of SCA-RBC. Our results showed that the RCE/RBC-primed procedure was efficient since fFCR was similar to aFCR. Moreover, aHct was similar to both fHct and pHCt, excluding an increased risk for post-RCE stroke. Manual RBC exchange can cause a rise in Hct without improvement in blood viscosity (10, 28). The history of the first patient of this series summarizes the outcome and the rheological conditions after transfusion or manual RBC exchange and RCE. Within 24h after a transfusion for ACS, this child developed a stroke and was successfully treated by RCE. Single transfusion may increase Hct and blood viscosity and cause vaso-occlusive complications (28,

29). More recently it has been clearly shown that automated RCE and manual exchange display opposite changes in post-exchange Hct and blood viscosity (10). Manual exchanges can cause a rise in Hct without any improvement in blood viscosity while automated RCE decreases blood viscosity without any increase in Hct (10). As we added RBC during the infusion of primed-RBCs, we analyzed various parameters of blood rheology during and at the end of RCE, showing no increase in blood viscosity at the beginning of the session and a similar decrease in blood viscosity both in RCE/RBC-priming and RCE without RBC priming. The increase in RBC deformability, the decrease of the tendency of RBC to sickle (normal RBCs do not sickle), and the decrease in RBC aggregation during both RCE procedures may explain why blood viscosity decreased in the two groups, demonstrating no risk of blood hyperviscosity for the priming step.

Other factors need to be analyzed, in particular, hemostasis parameters as another blood product is transfused; this priming could influence the blood changes after sessions. Additional analysis of biological parameters showed a 60% decrease in platelet levels after RCE/RBC-priming. This decrease was in accordance with previous studies investigating the effects of RCE (30, 31). However, particular caution must be paid when performing RCE in patients with very recent stroke or at risk of bleeding, because of liver failure for example. In this last clinical situation, ACD-A infusion must be decreased or discontinued (32). In other cases, ACD-A infusion induces a decrease in calcium blood levels, which needs to be counteracted by calcium infusion. This calcium supplementation is particularly necessary for RCE in which ACD-A infusion is significant. The ratio between ACD-A and calcium (both infused) must be monitored, particularly in low-weight children. Even if no guidelines have yet been developed to consider the usage of calcium according to ACD-A, the ratio between both infusions must be monitored to prevent a bleeding risk associated with a decrease in platelets, especially when RCE is performed after stroke. In our study, the ratio ACD-A infused/calcium infused was monitored and was in the same range in all sessions. In our experience in the field of RCE, the ACD-A/calcium ratio of 10 prevents the symptoms of hypocalcemia and the bleeding risk (data not shown).

In conclusion, the usage of RBC-priming in low-weight children appears necessary and safe when performing RCE. RBC-priming did not modify the performances of RCE. Altogether, these results should encourage apheresis and pediatric hematological teams to perform RCE in these low-weight children while considering many precautions. In patients with higher body weight (20–30 kg) or low body weight (15–20 kg) but low Hct, another possibility would be to perform albumin-priming. However, this therapeutic possibility has yet to be evaluated.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Hospices Civils de Lyon Ethics Commitee (L14-127). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Newborn Screening for Sickle Cell Disease Among Tribal Populations in the States of Gujarat and Madhya Pradesh in India: Evaluation and Outcome Over 6 Years

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Sickle cell disease (SCD) poses considerable public health problems in India. This study was undertaken to understand the clinical course of SCD among children identified during newborn screening programmes in Gujarat and Madhya Pradesh where the frequency of the HbS gene is high. A total of 8,916 newborn babies 8,411 from Gujarat and 505 from Madhya Pradesh were screened over 6 years (2010–2016) using HPLC and the diagnosis was confirmed by molecular analysis in a subset. A total of 128 babies (122 Gujarat, 6 Madhya Pradesh) were identified with sickle cell disease, of whom 87 (69 HbSS, 18 HbS-β thalassemia) from Gujarat were followed for 0.5–6.6 years. Acute painful events, severe anemia and fever with infections were the major complications and 23 babies required hospitalization. Severe to moderate clinical presentation was found in 13.8% babies with SCD whereas, 86.2% babies had a milder presentation. Presence of ameliorating factors (α-thalassemia and Xmn 1 polymorphism) did not have a discernible effect on the clinical severity. Parents of babies with SCD were educated and counseled for home care. Distribution of mobile phones to 44 families having babies with SCD was beneficial as it allowed regular contact with patients and their families. Genetic counseling to the affected families has increased the awareness and acceptance for prenatal diagnosis and 18 couples opted for prenatal diagnosis in subsequent pregnancies. SCD is not always mild among tribal groups in India. Therefore, facilities for early diagnosis and prophylactic treatment in the tertiary care centers should be made available. The difficulties in regular follow up of the babies in remote rural areas have also been highlighted.

Keywords: newborn screening, sickle cell anemia, tribal population, comprehensive care, Gujarat, Madhya Pradesh, India

INTRODUCTION

Sickle cell disease (SCD) is a major public health problem in India with a higher prevalence among the tribal and some non-tribal ethnic groups. The clinical manifestations are extremely variable ranging from a severe to mild condition. Early diagnosis and providing care is critical in SCD because of the possibility of lethal complications in early infancy in pre-symptomatic children (1).

Newborn screening (NBS) enables the identification of babies with sickle cell disease at birth or soon after, within the first few days of their life before they present with any symptoms or complications. These babies can then be regularly followed up with the provision of comprehensive care and timely management to reduce morbidity and mortality. It has been demonstrated in several countries that early diagnosis and providing care is critical in SCD as chances of lethal complications in infancy have been noted (2, 3).

It has been estimated that 50% of sickle cell heterozygous and homozygous neonates are born in Nigeria, India and Democratic Republic of Congo. Among these, India contributes to about 15% of the world's sickle cell anemia neonates. Further it has been estimated that widespread newborn screening and follow up care could save the lives of almost 10 million children by 2050 (4). Thus, newborn screening has great relevance in this country. There is no National neonatal screening program for SCD as yet in India and affected children are generally identified when they become symptomatic. However, few newborn screening programs have been initiated in some regions in the last 5–6 years (5–9).

The present study was undertaken to establish a newborn screening programme in the tribal areas of Gujarat and Madhya Pradesh to raise a cohort of babies with SCD and follow them up along with comprehensive care to understand the early morbidity and mortality of the disease.

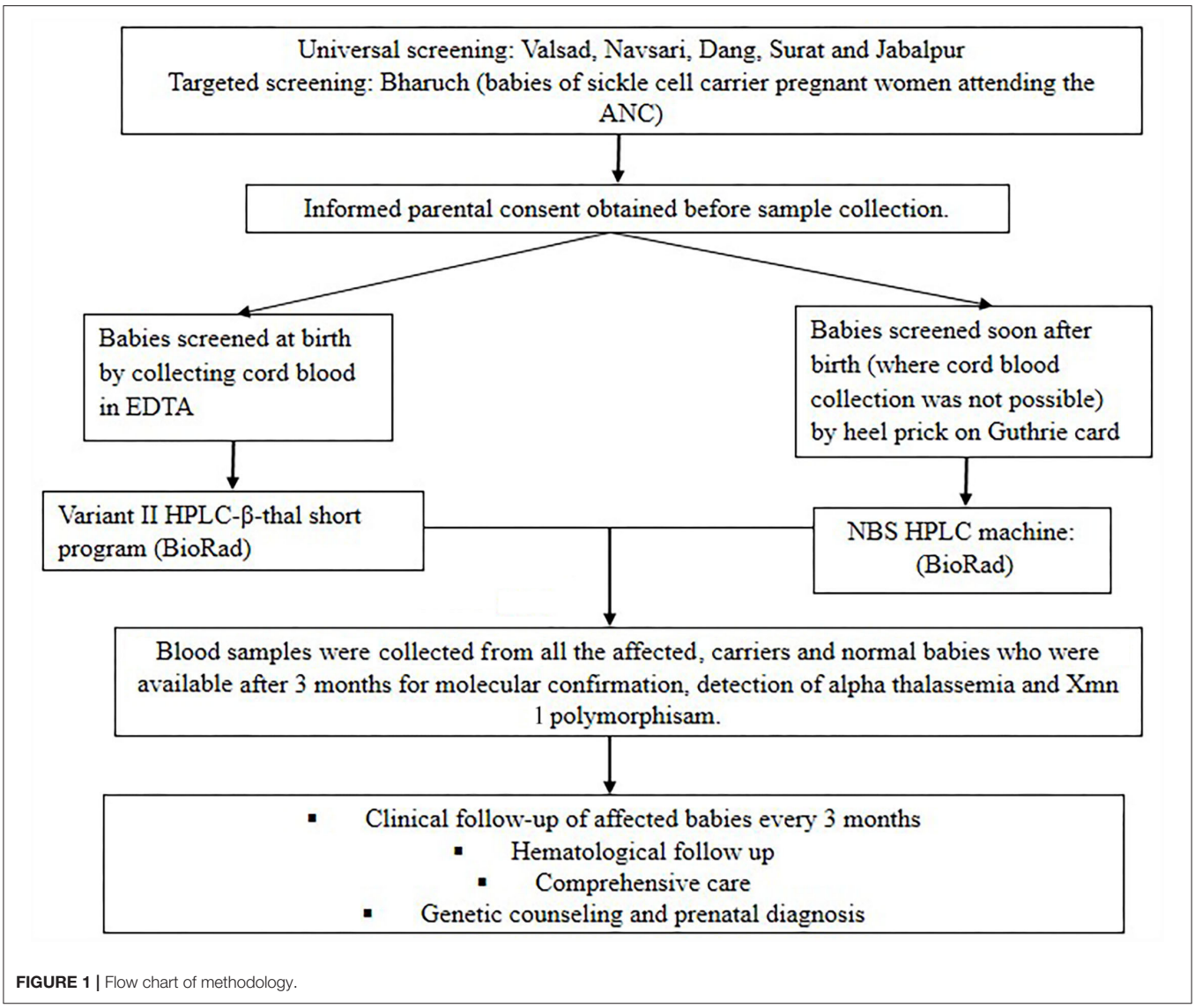

FIGURE 1 | Flow chart of methodology.

MATERIALS AND METHODS

Populations Group Studied

Figure 1 shows the flow diagram of the methodology followed in the present study. A total of 8,916 newborns from different tribal and non-tribal groups were screened from different districts of Gujarat (n = 8,411) and Madhya Pradesh (n = 505) over six years (2010–2016). Five districts were covered in Gujarat viz Valsad, Navsari, Dang, Bharuch and Surat. Newborn screening was largely targeted to tribal women and further targeted to offspring of mothers with an AS genotype at SEWA rural hospital in Bharuch. Babies of all sequential deliveries where the parents gave their consent were screened. In Madhya Pradesh universal newborn screening was carried out mainly from Jabalpur district. Cord blood samples were collected during hospital deliveries while heel prick samples were collected by health care workers on Guthrie cards between days 1 to 7 after birth in those babies where cord blood samples were not available. The study was approved by our institutional ethics committee (IEC).

TABLE 1 | Scoring system for clinical evaluation of children with sickle cell disease.

Complications	Score				
	0	1	2	3	5
Hospitalizations/year	0	1	2	>3	–
Blood transfusions/year	0	1	2	>3	–
Painful events/year	0	1	2	>3	–
Dactylitis events/year	0	1	2	>3	–
Vaso-occlusive crises	No	–	–	Yes	–
Sequestration crises	No	–	–	Yes	–
Acute chest syndrome (ACS)	No	–	–	Yes	–
Sepsis	No	–	–	–	Yes
Stroke	No	–	–	–	Yes

Classification of the patient.
Total score for milder patient: 0-3.
Total score for moderate patient: 4-7.
Total score for severe patient: 8-15.

Hematological Analysis

Complete blood count (CBC) was done on an automated cell counter (SysmexK-1,000, Sysmex Corporation, Kobe, Japan) when cord blood samples were collected. Screening for hemoglobinopathies was done by HPLC on the Variant^{TM} NBS Newborn Hemoglobin System for filter paper samples and on the Variant II HPLC machine using the β-thalassemia short programme for cord blood samples (BioRad Laboratories). Statistical analysis was done using student's t-test to compare the hematological findings in mild vs. moderate/severe cases.

Molecular Analysis

DNA was extracted from the cord blood leucocytes or on follow up using the QIAamp DNA Mini Kit (QIAGEN, Germany). The sickle and β-thalassemia mutations were confirmed by Covalent Reverse Dot Blot hybridization (CRDB) (10) or by Amplification Refractory Mutation System (ARMS) (11). Detection of the common α gene deletions (-$\alpha^{3.7}$ and -$\alpha^{4.2}$) was done by multiplex polymerase chain reaction using Qiagen multiplex master mix (QIAGEN, Germany) (12). The −158(C>T) variation upstream of the Gγgene (Xmn 1 polymorphism) was detected by PCR-RFLP analysis (13).

Clinical Follow-Up

Babies with SCD were followed-up clinically every three months as far as possible. Clinical phenotypes were defined as per the scoring system (**Table 1**) described earlier (14, 15). To define painful crises, mothers were specifically asked about pain and/or swelling of the fingers or toes (defined as dactyilitis) or bone pain elsewhere defined as the bone pain crises. Sepsis was defined clinically based on the integrated management of neonatal and childhood illness (IMNCI) guidelines (16).

RESULTS

Based on the HPLC findings, in Gujarat 122 babies had Sickle cell disease whereas in Madhya Pradesh 6 babies had sickle cell disease. Of the 122 babies with SCD in Gujarat, molecular studies in 87 revealed 69 HbSS and 18 with HbS-β-thalassemia [13-Codon 15 (G→ A), 5-IVS 1–5 (G→ C)].

Clinical and Hematological Follow Up

These 87 babies from Gujarat could be followed for 0.5–6.6 years. This also included 40 babies from our earlier NBS program (8) and 47 babies from the present NBS program. Clinical and hematological evaluation was done. Of the 87 sickle cell disease babies, 63 (72.4%) had one to 4 follow ups while 24 (27.6%) babies had more than 4 follow ups. Of the 87 babies 23 (26.4%) were regularly followed up at the interval of 3 months. CBC and HPLC analysis was only possible in 42 babies due to the unwillingness of parents to allow blood collection of their baby each time when they come for the clinical follow-up.

Table 2 shows the hematological parameters including HbF levels in the mild and moderate to severe cases of sickle cell anemia and sickle-β-thalassemia at the last follow up (age varied from 1 to 6.6 years). Sequential hematology was available in 42 babies 36 classed as mild, 6 moderate to severe. Significantly higher HbF levels ($p < 0.05$) were observed in the mild cases with sickle cell anemia compared to the moderate to severe cases. We did not find any significant difference in HbF levals in different age groups.

The clinical presentation of 87 babies who were followed up is summarized in **Table 3**. Among them, one sickle homozygous baby had Down's syndrome, one had congenital heart disease (CHD) and another presented with intellectual disability and hypotonia. Severe anemia was defined based on the Hb level (<6g/dl). Of the symptoms, dactylitis occurred in 2 (2.3%), acute chest syndrome in 3 (3.4%), bone pain crises in 19 (21.8%), febrile episodes in 26 (29.9%), sepsis in 6 (6.8%), and severe anemia in 11 (12.6%), who were treated with blood transfusion. Twenty three babies (26.4%) required hospitalization for infections, severe painful crises and blood transfusions. On an average period of hospitalization was 2–3 days. Splenomegaly was observed in 10 children (11.5%) with

TABLE 2 | Hematological findings during follow-up of babies with HbSS and HbS-β-thalassemia having mild and moderate to severe clinical presentation.

Hematological parameters	Sickle cell anemia ($n = 35$)		Sickle-β-thalassemia ($n = 7$)	
	Mild ($n = 31$)	Moderate to severe ($n = 4$)	Mild ($n = 5$)	Moderate to severe ($n = 2$)
RBCs ($x10^{12}$/l)	4.76 ± 0.6	3.89 ± 0.9	4.67 ± 0.4	4.50–4.58
Hb (g/dl)	8.8 ± 1.0	7.9 ± 1.7	8.7 ± 0.7	8.80–9.60
MCV (fL)	62.6 ± 8.2	72.7 ± 4.0	62.6 ± 5.9	67.0–69.6
MCH (pg)	18.7 ± 2.3	20.4 ± 0.2	18.8 ± 2.0	20.6–20.0
MCHC (g/dl)	29.8 ± 1.7	28.1 ± 1.2	30.1 ± 1.4	30.0–29.4
RDW (%)	21.6 ± 5.2	19.4 ± 4.4	21.4 ± 6.3	19.0–19.4
HbF (%)	23.5 ± 8.4*	8.6 ± 0.3*	27.5 ± 9.3	18.4–20.7

*Statistically Significant ($p < 0.05$).

TABLE 3 | Clinical presentation of the babies with sickle cell disease during the follow up period (0.5–6.6 years).

Clinical presentation	Sickle homozygous (HbSS) ($n = 69$)		Sickle-β-thalassemia ($n = 18$)	
	No. of babies	No. of episodes	No. of babies	No. of episodes
Painful crises	12 (17.4%)	1–3	7 (38.9%)	1–2
Severe anemia (Hb <6.0 g/dl)	15 (21.8%)	1–2	3 (16.6%)	1–2
Blood transfusion	9 (13.1%)	1–5	2 (11.1%)	1–2
Fever with infection	19 (27.5%)	1–3	7 (38.9%)	1–4
Sepsis	5 (7.2%)	1	1 (5.5%)	1
Hospitalization for infection or severe painful crises	15 (21.8%)	3–6	8 (44.4%)	1–14
Dactylitis	2 (2.9%)	1	0 (0.0%)	0
ACS	2 (2.9%)	1	1 (5.5%)	1
Death	3 (4.3%)	–	0	–

a spleen size of 1 to 7 cm and hepatomegaly in 3 children (3.4%) with a liver size of 1 to 4 cm. There were three deaths due to severe anemia unresponsive to hydroxyurea at age 4 years, Congenital heart disease (CHD) at age 7 years, and Down's syndrome at age 8 years. Most babies were underweight and height was retarded in three babies as compared to the age and sex matched healthy babies from the same regions (unpublished data).

Based on the different clinical events and number of episodes mentioned in **Figure 2**, the babies were classified as clinically mild and moderate to severe. Of the 87 babies, 75 (86.2%) (62 HbS homozygous and 13 HbS- β-thalassemia) were clinically mild whereas remaining 12 (13.8%) (7 HbSS and 5 HbS-β-thalassemia) were clinically moderate to severe. The HbF levels were significantly higher ($p < 0.05$) among the milder babies with SCD (23.5 ± 8.4) compared to the babies with moderate to severe disease (12.3 ± 6.5). Babies with a clinically mild presentation had fewer clinical complications which included occasional painful events, anemia, mild fever with cough and cold, while in the babies with a clinically moderate to severe presentation, clinical complications such as painful events, vaso-occlusive crises, hospitalization, acute chest syndrome, severe anemia requiring blood transfusion, sepsis, and fever with infections were observed (**Figure 2**). The β globin gene mutation in all the moderate to severe sickle—β thalassemia babies was the severe β^0 type of mutation Codon 15 (G→A).

Comprehensive Care

Pneumococcal vaccination (23-valent polysaccharide vaccine-PNEUMOVAX_ 23) was given to all the babies after 2 years of age while folic acid supplementation of 2.5 mg was given after 1 year of age and the dose was increased to 5 mg after 2 years of age. Penicillin was not given. Analgesics were given for symptomatic pain relief. Mobile phones packed with jingles as well as all relevant information on sickle cell disease in the local language were distributed to 44 families having babies with SCD. Due to budgetary restrictions, mobile phones could not be given to all families and were restricted to those who were living in the most remote areas and those who could not visit the clinic regularly. A special questionnaire had been prepared for telephonic follow-up which was filled up during each call. A total of 33 calls were received from 14 parents to inform the center about any major life events or sickle cell related problems in the child and to inquire about the next follow-up date as well as future preventive options. Immediate advice was given by the medical officer. At the same time the genetic counselors and medical officers were in constant touch with these 44 families (**Table 4**). Majority of the babies had

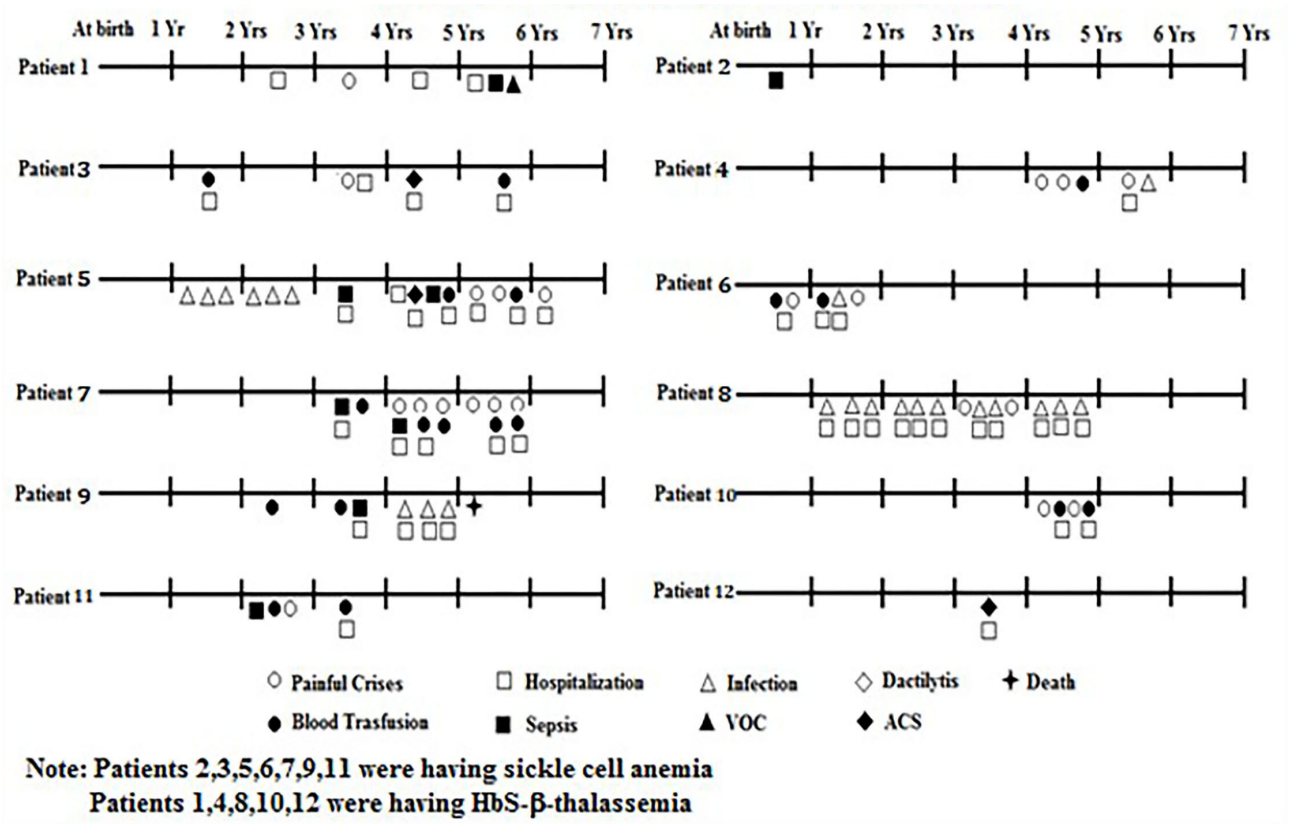

FIGURE 2 | Follow Up of babies with SCD having a moderate to severe clinical presentation.

TABLE 4 | Number of phone calls received from the parents of the affected babies for different complaints.

Complaints	Number of phone calls
Generalized weakness and loss of appetite	3
Fever and pain	6
Anemia and blood transfusion	8
Cold and cough	5
Inquiry about prenatal diagnosis	2
Follow up date inquiry	9
Total	33

generalized weakness, loss of appetite, fatigue, fever, cold and cough. Few had severe sickle cell crises such as painful events, severe anemia requiring blood transfusions. Immediate advice given over the phone by our Medical Officer helped to reassure the parents. Immediate arrangement of blood for transfusion was also done when required for the babies with SCD staying in very remote areas.

Determination of the Presence of α-Thalassemia and Xmn 1 Polymorphism

Presence of α-thalassemia was determined in 83 babies. Alpha—thalassemia was found to be present in 71/73 (97.2%) babies with a mild clinical presentation ($\alpha\alpha/-\alpha^{3.7}-8$, $-\alpha^{3.7}/-\alpha^{4.2}-2$ and $-\alpha^{3.7}/-\alpha^{3.7}-61$) and in 9/10 (90.0%) babies with moderate to severe clinical presentation ($\alpha\alpha/-\alpha^{3.7}-1$, $\alpha\alpha/-\alpha^{4.2}-1$ and $-\alpha^{3.7}/-\alpha^{3.7}-7$). Three babies (2 mild and 1 moderate to severe) had a normal α-genotype (**Table 5**).

The presence of the Xmn 1 polymorphism was determined in 83 (66 HbSS and 17 HbS- β-thalassemia) babies. All the babies with HbSS were homozygous (+/+) for the Xmn 1 polymorphism, a feature of the Arab Indian haplotype whereas all the babies with HbS-β-thalassemia were heterozygous for the Xmn 1 polymorphism (+/-).

Genetic Counseling and Prenatal Diagnosis

The families of the affected children were also called and counseled for home care. Genetic counseling was also given to the parents of all 87 babies (87 couples) for prenatal diagnosis and subsequently 18 couples at- risk of having a HbSS baby where a pregnancy was confirmed opted for prenatal diagnosis. Four fetuses were found to be normal, 8 were sickle heterozygous and 6 were sickle homozygous. As all the couples had a previous child with sickle cell anemia and it is difficult to predict the clinical course of sickle cell homozygous babies, all the 6 couples opted to terminate these pregnancies.

TABLE 5 | α-genotype among the affected babies.

Clinical presentation	αα/αα	$-\alpha^{3.7}/\alpha\alpha$	$-\alpha^{4.2}/\alpha\alpha$	$-\alpha^{3.7}/-\alpha^{3.7}$	$-\alpha^{3.7}/-\alpha^{4.2}$	Total
Mild	2 (2.7%)	8 (10.9%)	0	61 (85.6%)	2 (2.7%)	73
Moderate to severe	1 (10.0%)	1 (10.0%)	1 (10.0%)	7 (70.0%)	0	10
Total	3 (3.6%)	9 (10.8%)	1 (1.2%)	68 (81.9%)	2 (2.4%)	83

DISCUSSION

India had an estimated annual birth of around 42,000 babies with SCA in 2010 mainly among the scheduled tribes and some other economically disadvantaged populations like the scheduled castes and other backward classes (1, 4). Newborn estimates are important to know the precise number of births of babies with SCD to determine the magnitude of the burden of the disease. Recently it was estimated that the number of newborns with SCA in 2020 will be the highest in Madhya Pradesh, followed by Tamil Nadu, Maharashtra, Gujarat, Odisha and Chhattisgarh (17, 18). Few newborn screening programs have been initiated in Maharashtra, Gujarat, Madhya Pradesh, Chhattisgarh, Odisha and Tripura. Most of these were pilot studies which targeted both tribal and non-tribal populations (6–9).

In the present study, defaults rates of 29% occurred in Gujarat despite our best efforts, whereas, in Madhya Pradesh no follow-up was possible due to several reasons such as unwillingness of the parents, incorrect contact details and the distance of the screening centre from their residence. This may have introduced some biases in the analysis of the clinical presentation. Of the 87 affected babies, 40 babies were identified in our earlier NBS program (8) and 47 were from the present screening program. We included the affected babies of our earlier screening program in Gujarat also as they were followed up for a longer duration. The same protocols were followed for screening and follow up in both these cohorts.

The presence of α-thalassemia was also evaluated in these babies and it was found that 63/75 (84.0%) affected babies with a mild clinical presentation and 7/12(58.3%) affected babies with a moderate to severe clinical presentations had two alpha gene deletions. Also all the babies affected with SCD had the Arab Indian haplotype. A significantly higher HbF level was observed in the milder group of HbSS cases than in the moderate to severe group of cases. Earlier study by Upadhye et.al. suggested that the influence of other genetic modifiers like certain SNPs in BCL11A and HBS1L-MYB genes that influence HbF levels were associated with a milder clinical presentation in patients with SCD from Nagpur in central India (19). However, we have not looked at these modifiers in our cohort of affected babies.

The main aim of a newborn screening programme for SCD is to provide comprehensive care to the affected babies and genetic counseling to the couples at risk to avoid further births of affected babies. In the few newborn screening programs conducted earlier it has been seen that many a times the follow–up of the affected babies was not possible because of incorrect contact details, illiteracy of parents and the distance of the health center from the residence of the family (6–9). These problems were particularly faced by us in Madhya Pradesh where the babies with SCD could not be followed up. Unfortunately, in Gujarat too during the first NBS programme there was a high rate of lost to follow up babies (30.4 %) in spite of our best efforts.

To overcome these problems we tried a new concept of providing mobile phones to the parents of the affected babies and also gave them the relevant information on SCD including the guidelines that how the parents should take care of their children at home and recognize any acute events and contact the clinic immediately. The concept of giving mobile phones was interesting especially for patients in remote areas as it allowed regular contact twice a month with patients and their families. At the same time, the parents could contact the social worker and medical officer at the center regarding any major life events or sickle cell related problems in their child. This new concept received an excellent response which helped us to follow-up all the 44 babies affected with SCD regularly. However, more data are required for sufficient evidence to establish the effect of mobile phones in these remote areas. Also, awareness and genetic counseling has led to the acceptance of prenatal diagnosis among the tribal groups. A major limitation of the study was the small number of newborn babies who could be screened in Madhya Pradesh which has the maximum burden of sickle cell disease in India and the absence of follow up of babies with SCD in this state where concentrated efforts would be required to convince the parents on the importance of newborn screening and early care. This was a limitation in Gujarat too where greater efforts would be needed to reduce the number of defaulters for a regular follow up. The target populations in both these states belong to different indigenous socially disadvantaged groups with a low literacy rate often living in rural forested areas where infrastructure is limited and they move to different areas seasonally in search of work. Reaching out to them and convincing them to allow blood collection of their infants and children repeatedly is a challenge.

Yet the main strength of the study is the feasibility of undertaking a newborn screening programme for SCD among large tribal populations in remote areas, evaluating the clinical presentation, providing them care and their acceptability for prenatal diagnosis. Thus, a major focus has to be directed toward educating them, increasing their level of awareness and offering them genetic counseling. This is the only way to move forward for successful implementation of newborn screening programmes in India with long term longitudinal follow ups of cohorts of babies with SCD.

CONCLUSION

The present study demonstrates that unlike earlier belief, the disease is not always mild among the tribal groups in India. In addition, increasing awareness, comprehensive care and regular monitoring of babies with SCD could reduce the morbidity and mortality. Therefore, facilities for early diagnosis and prophylactic treatment in the tertiary care centres should be made available. This would help policy makers to develop facilities where they are most needed and implement newborn screening of SCD in the public health programmes in different states.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by IEC of Indian Council of Medical Research (ICMR) National Institute of Immunohaematology. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

PT and MBM wrote the first draft of the manuscript. PT carried out all the molecular analysis. At Gujarat JP, BR, and KD were involved in HPLC analysis of newborns. VM, AM, and SD clinically evaluated the affected babies at each follow-up and provided necessary interventions. YI and SD supervised the laboratory work and follow-up of the babies. At Madhya Pradesh RS was involved in HPLC analysis of newborns and supervised the laboratory work. RBC and MBM designed the study, helped with the analysis of data and finalized the manuscript. KG provided intellectual inputs and was also involved in clinical evaluation of the affected babies. All the authors have read and approved the manuscript.

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Sickle cell disease chronic joint pain: Clinical assessment based on maladaptive central nervous system plasticity

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Chronic joint pain (CJP) is among the significant musculoskeletal comorbidities in sickle cell disease (SCD) individuals. However, many healthcare professionals have difficulties in understanding and evaluating it. In addition, most musculoskeletal evaluation procedures do not consider central nervous system (CNS) plasticity associated with CJP, which is frequently maladaptive. This review study highlights the potential mechanisms of CNS maladaptive plasticity related to CJP in SCD and proposes reliable instruments and methods for musculoskeletal assessment adapted to those patients. A review was carried out in the PubMed and SciELO databases, searching for information that could help in the understanding of the mechanisms of CNS maladaptive plasticity related to pain in SCD and that presented assessment instruments/methods that could be used in the clinical setting by healthcare professionals who manage chronic pain in SCD individuals. Some maladaptive CNS plasticity mechanisms seem important in CJP, including the impairment of pain endogenous control systems, central sensitization, motor cortex reorganization, motor control modification, and arthrogenic muscle inhibition. Understanding the link between maladaptive CNS plasticity and CJP mechanisms and its assessment through accurate instruments and methods may help healthcare professionals to increase the quality of treatment offered to SCD patients.

KEYWORDS

musculoskeletal pain, symptoms assessment, red cell disorders, practical reasoning, evidence-based medicine

Introduction

Sickle cell disease (SCD) is a set of hereditary diseases caused by substituting glutamine acid for valine at the sixth position of the hemoglobin β chains, which leads to the presence of hemoglobin S (HbS). Conditions such as low oxygen concentration, hypovolemia, and others can precipitate the structure twisting of HbS molecules fibers forming the sickle-shaped red blood cell membrane causing vaso-occlusive crises, which are the main reason for pain complaints in this population throughout life (1). The pain in SCD individuals can be acute or chronic and can emerge from nociceptive, inflammatory, and neuropathic mechanisms (2). SCD pain syndromes are classified as intermittent, persistent pain between vaso-occlusive crises and chronic pain complications (3).

Among the chronic pain complications, chronic joint pain (CJP) is a common condition in SCD that may also be associated with several musculoskeletal problems such as osteomyelitis, dactylitis, arthritis, and osteonecrosis both in adult and pediatric individuals (4–6). These chronic pain complications have a higher incidence in SCD and play an additional role in chronic pain generation (4, 5). The CJP may be focal when involving a single joint or multifocal when involving more than one joint (7). However, to date, few studies demonstrate the influence of maladaptive plasticity in the central nervous system (CNS) in the maintenance of CJP in SCD individuals, although these individuals have chronic pain with nociceptive, neuropathic, and possible nociplastic pain characteristics (8, 9). The presence of central sensitization, for example, is related to more episodes of pain crisis and frequent hospitalizations (10). Of utmost importance, few studies were developed explicitly for CJP in SCD.

The poor correlation between structural lesions, the intensity of self-reported pain (11), and the diffuse nature of the symptoms make CJP assessment a challenge for clinicians and healthcare professionals. In general, healthcare professionals have poor knowledge about pain neuroscience mechanisms (12) and reliable ways of assessing it (13, 14). This poor knowledge goes against the International Association Study of Pain (IASP) recommendation in the declaration of the Montreal meeting, which highlights that all people with pain have the right to have access to appropriate assessment and treatment of the pain by adequately trained healthcare professionals (15). Thus, considering the potential relation between CJP and central maladaptive plasticity in SCD individuals and the deficit in healthcare professionals' knowledge about pain neuroscience mechanisms and pain assessment, this review aims to highlight the mechanisms of CNS maladaptive plasticity that might be related to CJP in SCD and propose a battery for reliable musculoskeletal assessment adapted to those patients.

Method

This review was carried out in the PubMed and SciELO databases, searching for information that could help in the understanding of the mechanisms of CNS maladaptive plasticity related to pain in SCD and that presented assessment instruments/methods that could be used in the clinical setting by healthcare professionals who manage chronic pain in SCD individuals. There was no limit placed on the publication year, and the searching was carried out through a combination of keywords such as Sickle Cell Disease and Joint Pain or Chronic Pain or Pain Assessment or Central Sensitization Evaluation or Painful Movement Assessment, Chronic Joint Pain and Cortical Reorganization or Arthrogenic Muscle Inhibition or Chronic Inventory Central Sensitization or Quantitative Sensory Test or Clinical Evaluation. In addition, the reference list of papers also was searched.

Chronic joint pain in SCD: An overview of the problem

International Association Study of Pain defines pain as "An unpleasant sensory and emotional experience, associated with, or resembling that associated with, actual or tissue damage" (16). Pain plays a vital role in the organism's defense reaction to a hostile environment, and evidence of this is that in individuals with pain insensitivity, injuries are not perceived as such, decreasing life expectancy (17). On the other hand, chronic pain is persistent beyond 3–6 months, has no functional role, and is responsible for rendering dysfunctional several biological systems (18). In SCD, the constant joint tissue injuries secondary to the vaso-occlusive crisis are critical in developing chronic joint pain.

Primary afferent nociceptors richly innervate the joint in their capsule and synovium (19). These fibers are mostly from types Aδ and C and can be classified into two types: (a) True nociceptors; (b) Silent nociceptors. True nociceptors respond to mechanical nociceptive stimuli even in non-pathological conditions. As for the silent nociceptors, to respond to this type of stimuli, they must be primarily sensitized by inflammation-inducing aggressors (19, 20). The primary afferent nociceptors have on their membranes a wide variety of transient receptor potential ion channels that are responsible for the transduction of a wide variety of noxious stimuli arising from high magnitude mechanical, thermic, or chemical origins (21, 22). The nervous system sensitization occurs basically by neurogenic inflammation, mast cell activation, N-methyl-D-aspartate (NMDA) receptors activation, and glial activation (1, 2), which play an important role in the maintenance and subsequent pain chronicity in SCD individuals.

After joint tissue injury, the pro-inflammatory mediators such as bradykinins and prostaglandins interact with receptors or transient receptor potential vanilloid type 1 (TRPV1) of nociceptive fibers and sensibilize them to augment their response to a noxious stimulus (2, 22). Once activated, the nociceptors release peptides and neurotransmitters such as calcitonin gene-related peptide and substance P, which further contribute to the inflammatory response, causing vasodilation, swelling, and mast cell activation. Mast cells act by degranulation of histamine, which further sensibilize nociceptors (23). Interestingly, serum levels of substance P are increased in SCD individuals during the vaso-occlusive crisis and baseline state (24) and have been associated with to use of hydroxyurea (25). This cascade of biochemical events lowers the activation threshold of true nociceptors and recruits previously unresponsive silent nociceptors, which induce hyperalgesia and allodynia in joint pathologies in SCD individuals (26).

N-methyl-D-aspartate receptors are involved in the long-term potentiation process and are a crucial player in the chronicity of pain (27). At the spinal cord level, the constant nociceptive information arrives in the dorsal horn and provoke the release of glutamate neurotransmitter in presynaptic terminals that interacts with NMDA receptors post-synaptic (3). When NMDA receptors are activated, the nitric oxide is synthesized in the presynaptic terminals, increasing the expression of voltage-gated Ca2+ channels mainly responsive to P substance and glutamate (3). Concurrently, glial activation releases pro-inflammatory cytokines and more glutamate in this synaptic environment (1, 3). Thus, these series of intracellular signaling cascades augment and facilitate the transmission of nociceptive information.

These nociceptive information reaches higher encephalic areas, such as Rostral Ventromedial Medulla (RVM), Periaqueductal Gray Matter (PAG), thalamus, amygdala, anterior cingulate cortex, somatosensory, prefrontal, and motor cortices (28, 29) that process and modulates the nociceptive information. However, nociceptive modulation can occur before reaching the thalamus and other brain structures (30). Once that nociceptive information reaches the thalamus, it processes it and redirects it to cortical areas of the primary and secondary somatosensory cortex through thalamocortical and thalamus-amygdala connections (29). The PAG, in turn, receives inputs from these superior centers and sends them to the RVM medulla, which through axonal fibers of "on" and "off" cells, modulate neuronal activity, facilitating or inhibiting the transit of nociceptive information in the dorsal horn of the spinal cord both presynaptic and post-synaptic (30, 31). This complex endogenous mechanism forms a pain processing and control system, often presenting a maladaptive function in chronic joint pain (Figure 1).

Maladaptive CNS plasticity mechanisms and ways to evaluate it

Dysfunction of descending inhibitory control in CJP

Central nervous system has various ways of inhibiting the input of pain information to higher processing centers. Descending inhibitory control is a mechanism of diffuse pain inhibition. Studies with conditions of CJP similar to SCD, such as hip and knee osteoarthritis, showed that the descending inhibitory control dysfunction might be an important triggering factor for central sensitization and chronic pain (32). Although some studies have found no consistent results about dysfunctions of descending inhibitory control in adult SCD individuals (10, 33), neuroimage data from another study with adult SCD individuals showed that there is an increased resting-state functional connectivity between the PAG and cerebellum in SCD individuals (34) which can affect RVM's "on" and "off" cells activity. In pediatric SCD individuals, the dysfunctions of the descending inhibitory control are few explored, but data from non-SCD individuals has shown that deficient endogenous pain inhibition can stem from painful experiences during infancy (35, 36). Therefore, these data make us think that the function of the descending inhibitory control system in SCD still needs to be better understood and evaluated in the clinical context.

One of the traditional ways of assessing descending pain inhibitory system is through the paradigm of Conditioned Pain Modulation (CPM), previously known as "counter-irritation," "pain inhibits pain," and "heterotopic noxious conditioning modulation," and "diffuse noxious inhibitory control"(37). This phenomenon is activated after a set of intense and/or noxious stimuli, making it a protective endogenous response to aggression. The evaluation of descending inhibitory control by the CPM method should be recommended for SCD individuals due to the malfunctioning of this mechanism is closely related to the persistence of joint pain in musculoskeletal conditions such as osteoarthritis and temporomandibular dysfunction (32, 38, 39) (Figure 2). However, clinicians should be aware that the long-term pain, and the use of opioid agents can result in a reduced response of CPM scores (40).

In CPM assessment, a pressure threshold meter for applying painful mechanical stimulus in the thenar region of the non-dominant hand has a good coefficient of intra-session reliability (ICC >0.75). It seems to be a reliable method for performing a Painful Stimulus Test (PST). The pain caused by the mechanical stimulus must be of moderate intensity (41, 42). In turn, the Painful Conditioning Stimulus Test (PCST) can be done with cold or hot water. However, the immersion of the dominant hand in a water vessel with a temperature of 46.5°C has a good Intraclass Correlation Coefficient (ICC = 0.79) (42) and

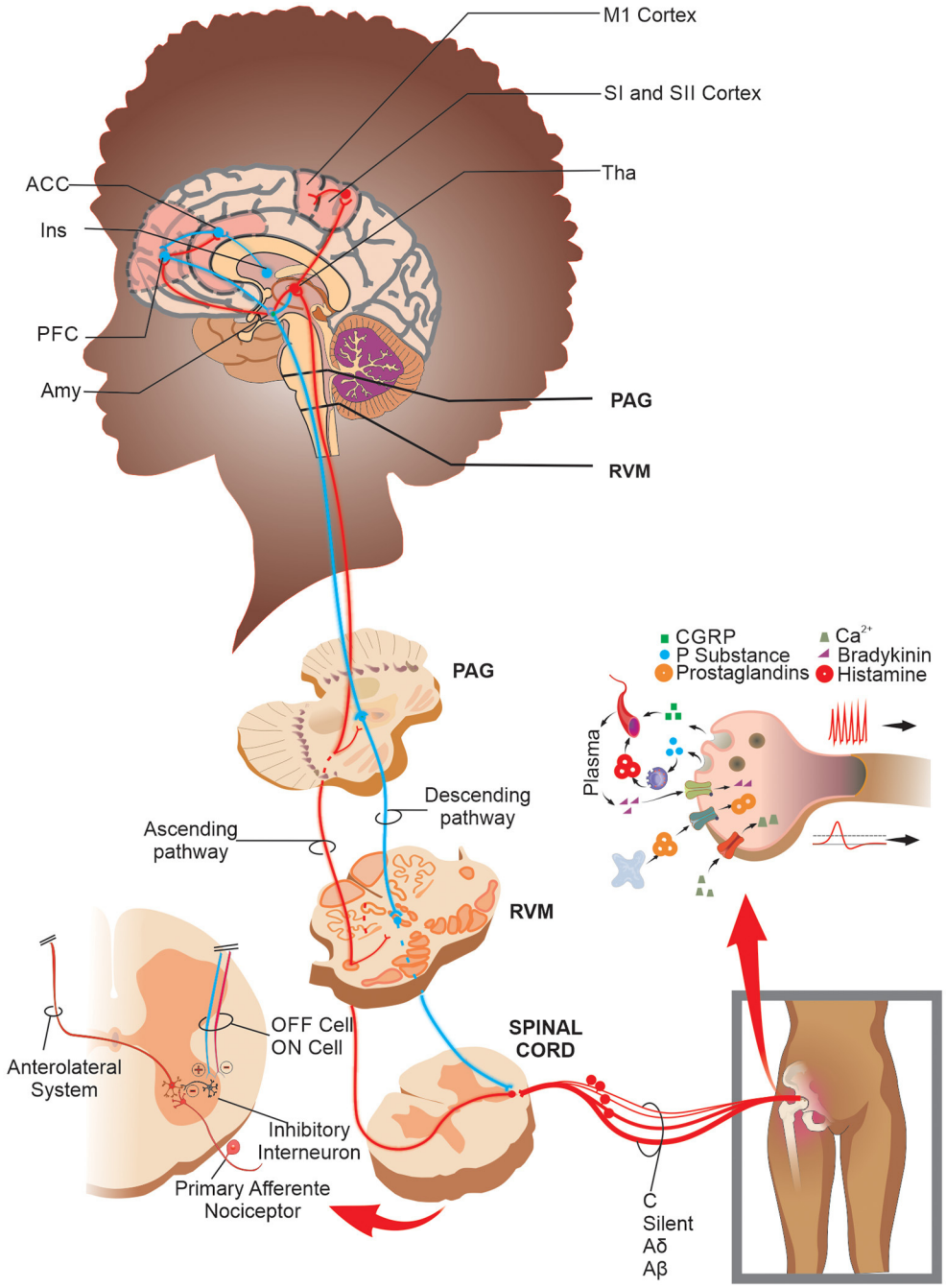

FIGURE 1
Schematic drawing of the peripheral sensitization, processing, and nociceptive modulation in joint pain: After a noxious stimulus in the joint, the TRP channels in true nociceptors transduce the nociceptive information and lead it to second-order neurons in the spinal cord. In addition, when there is a joint injury, a massive release of the pronociceptive chemical substances in/by free nerve endings promotes a depolarization threshold decrease and an increase in firing frequency rate in both true and silent nociceptors and mechanical receptors. The nociceptive information reaches the CNS, which processes and modulates it through brain networks and the PAG-RVM system. Specifically, in SCD patients, there is increased functional connectivity in areas such as PFC, ACC, M1, SI, and SII cortices. Abbreviations: ACC, anterior cingulate cortex; Amy, amygdala; CGRP, calcitonin gene-related peptide; Ins, insula; M1, primary motor cortex; PAG, periaqueductal gray matter; PFC, prefrontal cortex; RVM, rostral ventromedial medulla; SI, primary somatosensory cortex; SII, secondary somatosensory cortex; Tha, thalamus; TRP, transient receptor potential.

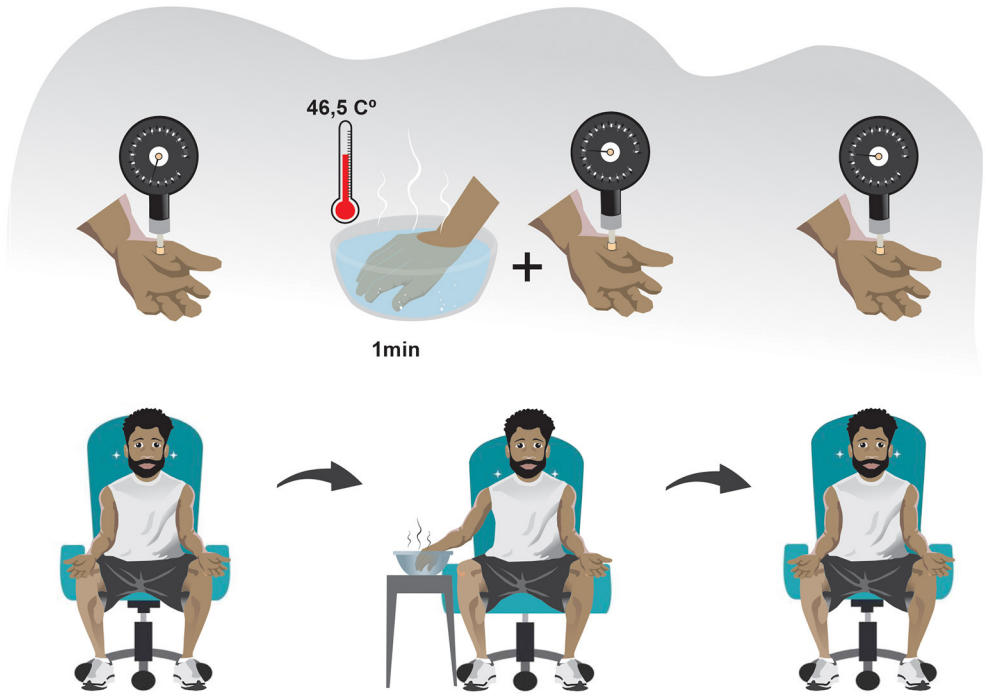

FIGURE 2
Schematic drawing of the conditioned pain modulation assessment: CPM can be assessed in three steps in the clinical setting. (1) A PST is made in the non-dominant side of the body (usually the thenar eminence). (2) After a sufficient period so that the pain caused by the PST has ceased, a PCST is made in a heterotopic region distant from the initially stimulated region and preferably on the contralateral side of the body, lasting 1 minute. (3) The PST is again applied immediately after or concomitantly to a PCST. Abbreviations: CPM, conditioned pain modulation; PST, painful stimulus test; PCST, painful conditioning stimulus test.

is more recommended in the SCD context because it can avoid a vaso-occlusive crisis during the evaluation. Using a thermometer to verify the heat dissipation and ensure the ideal temperature during immersion and using the same kilograms-force generated by the pressure threshold meter during the PST before and after the PCST may decrease potential measurement biases (41, 42).

The quantification of the CPM can be made according to the following equation:

$$CPM = piPST1 - piPST2$$

Where piPST1 corresponds to the pain intensity caused by the first painful stimulus test and piPST2 pain intensity caused by a second painful stimulus test. A positive result indicates the presence of a preserved descending inhibitory control, while a negative result indicates the opposite (42).

Central sensitization in CJP

Previously, the term "centralized pain" was often used to classify the pain experienced by patients with central sensitization. However, this term was not part of recognized by the IASP. Following the proposition of a research group (43), an IASP force task recently added a new pain term called "nociplastic pain" into the list of taxonomic definitions for pain (44), even though it caused a comprehensive discussion related to its real need and the best way for it to be defined (45–47). This new term proposes to differentiate the "pain that arises from altered nociception, despite there is no clear evidence of actual or threatened tissue damage that causes peripheral nociceptor activation or evidence of disease or injury to the somatosensory system that causes pain" from those kinds of pains typically classified only as nociceptive or neuropathic.

The central sensitization mechanisms involve the perpetuation of joint pain that can be favored by poor descending inhibitory control, which over time causes phenotypic alteration of Aβ fibers specialized in conducting non-painful stimuli (30). In addition, nociceptive information is not properly inhibited in the dorsal horn of the spinal cord and advances freely until it reaches higher areas of the nervous system, causing a central sensitization of multiple structures (48). Central sensitization of multiple structures involves a maladaptive change of important anatomic/functional networks that process information in all pain dimensions, i.e., sensory,

emotional, and cognitive (49). Due to the important role in pain processing, these anatomic/functional networks are called the pain connectome (49).

In conditions of chronic non-SCD pain, the default mode network (DMN), the salience network (SLN), the sensorimotor network (SMN), and the antinociceptive system are connectome strongly affected by central sensitization (49, 50). The DMN includes the medial prefrontal cortex, posterior cingulate cortex, precuneus, and lateral parietal cortices and is activated in a resting state of the mind when the individual is instructed not to think about anything specific (49). Next, SLN comprises the bilateral insula cortices, anterior cingulate cortex, and middle cingulate cortex and is activated by salient stimuli that stand out from the environment (e.g., nociceptive stimulation caused by the movement of an inflamed joint) (51). The SMN includes bilateral primary and secondary somatosensory cortices, primary motor (M1) cortex, and the supplementary motor area (SMA) and is involved in the descriptive sensory processing of pain (51). Finally, the antinociceptive system comprises the PAG and RVM, which, as previously discussed, are core structures involved in pain modulation (49). This pain connectome is dynamic due to the capacity to generate connections within and between themselves (49).

In SCD individuals with chronic pain, some studies using functional Magnetic Resonance Image (fMRI) alone or coupled with Electroencephalography (EEG) have found a maladaptive change in the pain connectome (34, 51–53). Their results showed that SCD individuals with high levels of pain and hospitalizations had an increased resting-state functional connectivity between SLN, DMN, and SMN structures (e.g., dorsal anterior cingulate cortex and the right precuneus, secondary somatosensory cortex, and the left precuneus, inferior parietal lobule and the middle cingulate cortex, right posterior cingulate cortex and the right primary somatosensory cortex) when compared with individuals with low levels of pain and hospitalizations (52). SCD individuals also presented hypoconnectivity of SMN structures (i.e., motor cortex) compared to healthy controls and between other regions outside of the SMN, such as the dorsolateral prefrontal and parietal cortices (51). In addition, this same study found that SCD increased functional connectivity between DMN and SLN structures (e.g., precuneus/ posterior cingulate cortex and temporal regions) (51). Finally, studies comparing SCD individuals and healthy controls found changes in functional connectivity of the PAG (a core structure of the antinociceptive system) (34, 53). Functional connectivity between the PAG and the anterior cingulate cortex (a structured core of SLN) is decreased in SCD patients when compared to healthy individuals but increased between the PAG and several cortical regions that play functions of sensory processing, motor processing/executive function, emotion and memory/learning when SCD patients were compared with those without pain (53).

The sensitization of the pain connectome may be associated with multiple musculoskeletal and non-musculoskeletal symptoms found in individuals with severe chronic pain. These include decreased pain threshold, expansion of pain receptive field to further regions unrelated to pain, interpretation of non-painful stimuli as painful, photophobia, bowel diseases, and sleep, attention, and mood-altering (54, 55). The emergence of these phenomena may trigger the change of the clinical status from a musculoskeletal disease to a multi-systems disease. Typically, those symptoms are under-evaluated by clinicians and are not related to the presence of persistent pain. However, these aspects are essential as they help in decision-making and prediction of patient outcomes, as evidenced by a study that showed that individuals with central sensitization due to chronic pain secondary to osteoarthritis of the knee are five times more likely to have pain refractory to surgical treatment of total knee arthroplasty (56). In SCD individuals, central sensitization has been associated with increased vaso-occlusive crises, poor sleep quality, and psychosocial disorders (10). For this reason, this should be considered during the evaluation since this is probably one of the main causes of refractory joint pain (57).

Sensitivity hyperphenomena, such as allodynia or hyperalgesia to thermic and vibratory stimuli, and mechanical and thermal temporal summation, have been associated with central sensitization in non-SCD individuals with chronic pain (57). These sensitivity deficits are also found in pediatric SCD individuals, among lower mechanic pain, cold pain, heat pain, thermal detection thresholds, and heat pain tolerance (58). In addition, studies with adult SCD individuals showed that they also present sensory alterations expressed by a higher intensity of cold pain, heat pain, thermal temporal summation, and mechanic pain is found in compared with healthy controls (33, 59).

Some methods are essential in evaluating central sensitization/nociplastic pain characteristics in clinical and research settings because they can help evaluate whether CJP in SCD is influenced and/or supported by central sensitization. The central sensitization inventory (CSI) is an evaluation instrument that, although non-specific to SCD, is highly recommended to be used in clinical practice in the SCD context (54). The CSI is divided into two parts, A and B. In part A, 25 descriptive alternatives of multidimensional symptoms are associated with central sensitization. Each alternative has a score varying from zero (never) to four (always), with a maximum total score of 100 points. In part B, 10 alternative clinical conditions are recognized as central sensitivity syndromes (CSS) (60). The cut-off at 40 points has excellent levels of sensitivity (81%), specificity (75%), positive predictive (2.93), and negative predictive value (0.52) to recognize central sensitization (60). However, despite these good diagnostic accuracy values, the CSI still needs to be validated in SCD individuals, and its results should be interpreted with caution. Due to the need for severity ratings of central sensitization, a 10-point classification

with severity intervals was created, consisting of the following categories: subclinical (≤29), mild (30–39), moderate (40–49), severe (50–59) and extreme (≥60) (61). This severity rating allows better utilization of CSI in clinical practice and may help as a parameter of the therapeutic response. This instrument has been culturally translated and validated in several languages (62, 63).

Quantitative sensory tests (QST) are another way to assess central sensitization (10, 57, 64). All systematic sensory evaluations that allow quantified responses can be viewed as a QST. However, a set of QST (mechanical, thermal, and vibratory) was standardized to evaluate the integrity of the somatosensory system and to guarantee the accuracy and reproducibility of the findings (65). QST protocols consider several sensory parameters, as well as biological aspects ranging from body temperature to trophic changes in the musculature (64, 65). However, although QST protocols can be performed in both bedridden and non-bedridden individuals, their complete execution is time-consuming and can be impracticable in some clinical contexts. In this context, there are some attempts to validate a bedside QST as a low-cost and time-efficient alternative (66, 67).

The bedside QST can be easily applied in clinical routine, and its execution does not require a large training time. Studies showed that bedside QST protocol using low-cost equipment could be used in each step of the sensory assessment procedure, such as (a) 3 cm^2 metal coin/piece with 22°C or 37°C (cold/warm detection thresholds); (b) cotton wool/Q-tip (mechanical detection threshold); (c) tuning fork (vibration detection threshold); (d) 10-ml syringe sealed or toothpick (mechanical and pressure pain threshold); (e) glass vial filled with hot water 40°C or metal pieces with 45°C (heat pain threshold); (f) ice cubes in a plastic bag or metal piece with 8°C (cold pain threshold); (g) toothpick (temporal summation) (66, 67). However, the correlation between bedside QST and standard QST protocol is variable and impacted by the expertise of a healthcare professional.

A study proposed three steps of a decision tree that helps clinicians to interpret the findings of QST evaluation of mechanical detection threshold (Aβ fibers), cold pain (Aδ fibers), and heat pain (C fibers), specifically in SCD individuals (8). In the clinical setting, QST stimuli should be evaluated in both painful and non-painful sites. In the first step, if all QST findings are negative, the clinical interpretation must be that there is no central or peripheral sensitization. In the second step, if mechanical stimuli findings in the non-painful site are positive, then the clinical interpretation must be that there is central sensitization. In the third step, if cold or heat pain is present in the painful site and these same painful stimuli result negative in the non-painful site, then the clinical interpretation must be that there is peripheral sensitization. Finally, the decision tree proposes that if all three steps result in negative findings, then the interpretation must be that there is mixed pain (8).

The safety of the QST protocol in the clinical setting has been previously tested in subjects with SCD, and there was no perpetuation or worsening of pain after its application (8). However, attention is necessary because data show that after QST testing in SCD patients, there are changes in pro-inflammatory biomarkers such as increased levels of Interleukin 6 (IL-6), substance P, and tumor necrosis Factor-alpha (TNFα) (33). In SCD, the thermal pain threshold (TPT) to cold <17.01°C and heat <43.91°C are indicative of impaired nerve sensitivity, and pressure pain threshold (PPT) <4.42 g is indicative of the existence of altered sensory function (68). Thermal pain threshold (TPT) assessment with temperature in 32°C baselines and an increasing/decreasing temperature at a rate of 1.5°C/s is used in clinical settings (ICC >0.55) (69). In cases of non-SCD pain, specifically osteoarthritis of the knee, the PPT increasing pressure at a rate of 0.5 kgf/s has a good diagnostic reliability value varying according to the evaluated joint site (ICC: 0.64–0.73) (70).

Finally, another way of assessing central sensitization in individuals with SCD uses its typical clinical criteria checklist (71) developed by a consensus of experts. Although this checklist is non-specific to SCD individuals, it is also useful for clinicians and healthcare professionals because it helps identify signs and symptoms characteristic of central sensitization, such as pain disproportionate to injury, disproportionate aggravating/easing factors, and psychosocial symptoms, and diffuse palpation. These discriminative items indicate the presence of central pain sensitization with excellent accuracy values (sensitivity 91.8%, specificity 97.7%, positive predictive value 91.8, and negative predictive value 97.7) (72). Thus, using these instruments during the evaluation of SCD individuals with CJP may help in the more precise knowledge of the mechanism underlying the patient's pain. This clinical criteria checklist provides a basis for better clinical decision-making and possibly less chance of non-adherence to the proposed treatment.

Motor control modifications and cortical reorganization in CJP

In the face of pain, the neuromusculoskeletal system undergoes adaptive motor modifications that affect motor control and joint mechanics. These modifications have been studied over time due to the importance of their understanding for both clinicians and researchers. Therefore, one theoretical model (73) was established to clarify the interaction between pain and motor control changes making the following propositions: Firstly, the adaptation of the motor control to pain is a consequence of the redistribution of the activity within and between muscles. Secondly, the change in mechanical behavior

initially has a protective function of preventing further pain or injury. However, in the long term, it involves changes in various levels of the nervous system, which lead to increased joint load, decreased mobility, and variability of movement and muscle weakness (73).

In the presence of CJP, motor and sensory primary cortical reorganization are associated with motor control impairment. This cortical reorganization has been demonstrated in non-SCD adult individuals with low back pain (74, 75), chronic lateral epicondylalgia (76), osteoarthritis of the knee (77), and chronic patellofemoral pain (78), but there is no study with pediatric individuals. This cortical reorganization is expressed through the overlap (i.e., blurring) or retraction in the areas of somatotopic representation of the motor homunculus. The greater the cortical reorganization, the greater the perpetuation of the pain (77).

The intracortical inhibitory system, modulated by tonic GABAergic activity, plays an important role in the development of cortical somatotopic representations. This specific function is due to mechanisms that differentiate cortical efferent motor actions, either by facilitating muscle activation during a motor task or by inhibiting undesirable muscular activations (79). Although changes in intracortical inhibition are not a consensus (80), intracortical inhibitory dysfunction mediated by GABAergic connections has been demonstrated in individuals with chronic pain (81) through Transcranial Magnetic Stimulation (TMS), a technique that has been often used to assess cortical connectivity.

Transcranial magnetic stimulation also allows the evaluation of muscles' cortical representations through cortical mapping. Briefly, cortical mapping through TMS is made using a set of pulses with intensity fixed in accord with a percentage of the maximal stimulator output (82). This set of pulses

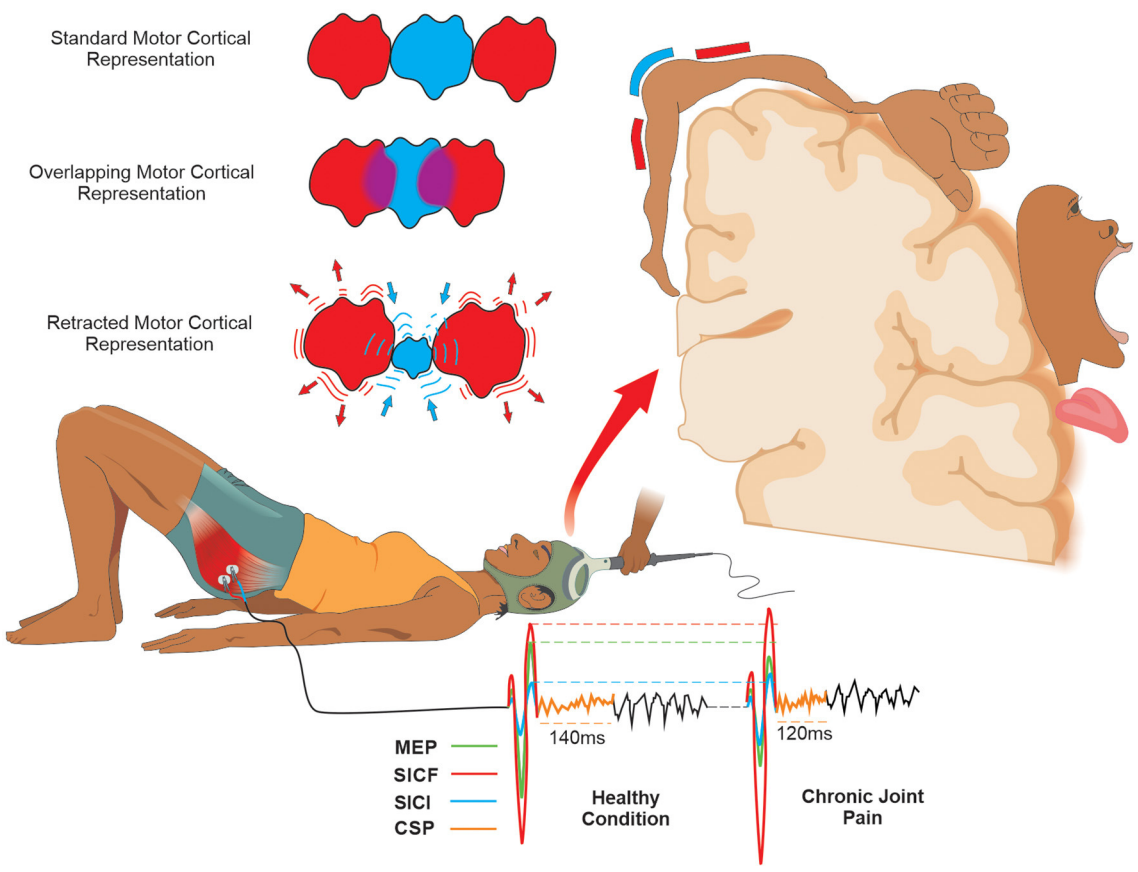

FIGURE 3
Schematic drawing of the measures of motor cortical excitability with tms and mapping of motor cortical representation in joint pain condition: Using TMS, Motor Cortical Excitability can be expressed by corticospinal (MEP) and intracortical measures (ICF, IIC, and ǂCSP). In the CJP, the CSP, SICI, and MEP are decreased, and the SICF is increased compared to the healthy condition. The Standard Motor Cortical Representation in the presence of pain can classically be changed into two possible basic forms. (1) An Overlapping of the Motor Cortical Representation adjacent to that affected by pain; (2) A Retraction of the Motor Cortical Representation affected by pain and an increase in the adjacent representation. ǂCSP is obtained only when the assessment is made during a muscle contraction. Abbreviations: CSP, cortical silent period; SICF, short-interval intracortical facilitation; SICI, short-interval intracortical inhibition; MEP, motor evoked potential; TMS, transcranial magnetic stimulation.

should be applied at various scalp sites using a figure-of-eight coil and a spatial coordinate system referenced to the vertex (83), and the amplitude of MEPs evoked in contralateral muscles is measured (82). However, although the assessment of cortical mapping through TMS can be useful in clinical settings, there are no studies evaluating its diagnostic reliability (Figure 3).

In SCD individuals, CJP is possibly associated with maladaptive motor behavior and cortical representation changes due to their chronic and disabling pain (7). The changes in functional connectivity of the structures are involved in the descending inhibitory control of nociceptive information in individuals with SCD (34) and can be associated with intracortical inhibition (84). Thus, although TMS evaluation is not specific to SCD individuals, clinicians and healthcare professionals should be used to investigate these possible cortical alterations both in adult and pediatric SCD individuals with CJP.

Arthrogenic muscle inhibition and CJP

It is common that after joint injuries, there is the presence of weakness in the adjacent involved musculature. The possible cause for this muscle weakness is the presence of a central reflex inhibition that can provoke a failure to fully recruit the motor units and/or a suboptimal firing of the motor units that are recruited (85), preventing the complete activation of the surrounding musculature to the injured joint during a maximal voluntary muscular contraction. This phenomenon has been called Arthrogenic Muscle Inhibition (AMI) (86).

Arthrogenic muscle inhibition can be interpreted as a mechanism of physiological protection to prevent new lesions and potentiation of tissue repair (87). However, AMI may persist for several months or even years after injury (88). This persistence may compromise the rehabilitation process by negatively impacting strengthening protocols, thus, contributing to injury progression and

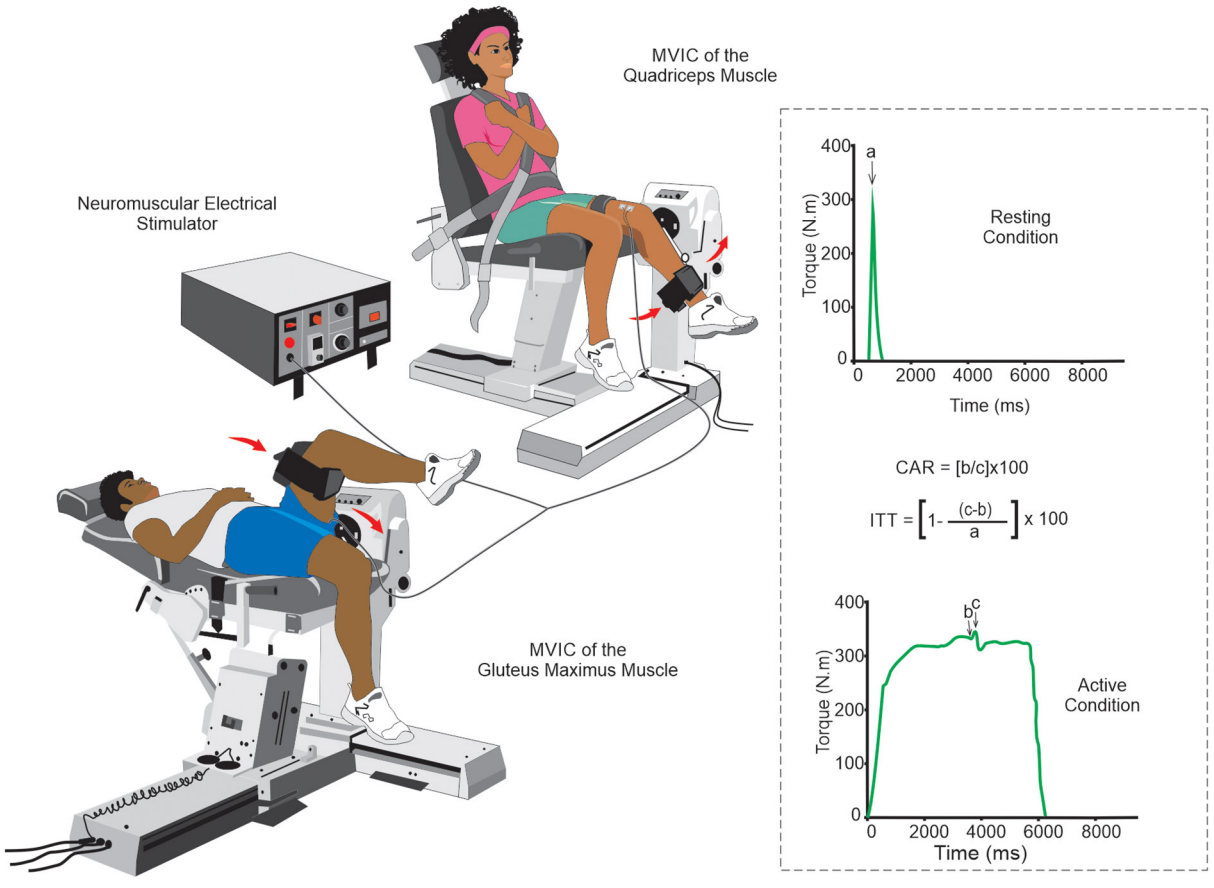

FIGURE 4
Schematic drawing of the arthrogenic muscle inhibition evaluation by central activation rate and interpolated twitch technique: Although the knee joint is the most evaluated with the CAR and the ITT methods, their methodological simplicity allows the assessment of AMI to be also used in other joints (e.g., the hip joint in the figure above). The quantification of the evaluation can be done using specific mathematical formulas for each method. a. stimulus-evoked torque at rest; b. voluntary torque at the time of stimulus delivery; c, peak torque evoked due to the electrical pulse. Abbreviations: AMI, arthrogenic muscle inhibition; CAR, central activation rate; ITT, interpolated twitch technique.

TABLE 1 Summary of the main central nervous system maladaptive changes, assessment methods, clinical interpretation, and diagnostic reliability that can be used in CJP related to sickle cell disease.

CNS maladaptive changes in chronic joint pain	Assessment methods or instruments	Clinical interpretation	Diagnostic reliability
Insufficiency of descending inhibitory control	• Conditioned pain modulation (42)	• piPST1 > piPST2 = Descending inhibitory control is functioning • piPST1 < piPST2 = Descending inhibitory control system is faulty	• PST: Pressure threshold meter (ICC >0.75) • PCST: Hot water in 46.5°C (ICC = 0.79)
Central sensitization	• Central sensitization inventory (60,61)	• Severity ratings: Subclinical (≤29) Mild (30-39) Moderate (40-49) Severe (50-59) Extreme (≥60)	• Cut-off at 40 points: Sensitivity (81%) Specificity (75%) Positive predictive value (2.93) Negative predictive value (0.52)
	• Quantitative sensory test (68-70)	• TPT to cold <17.01°C and heat <43.91°C are indicative of impaired nerve sensitivity • PPT <4.42 g is indicative of the existence of an altered sensory function	• TPT: 32°C baselines with decreased/increased temperature at a rate of 1.5°C/s (ICC >0.55) • PPT: Increasing pressure at a rate of 0.5 kgf/s (ICC: 0.64–0.73)
	• Clinical criteria checklist (71,72)	• Pain disproportionate to injury disproportionate aggravating/easing factors; psychosocial symptoms; diffuse palpation	Sensitivity (91.8%) Specificity (97.7%) Positive predictive value (91.9) Negative predictive value (97.7)
Modifications of motor control and cortical reorganization	• Cortical mapping by transcranial magnetic stimulation (77)	• There is an overlap (blurring) or retraction in the areas of somatotopic representation of the motor cortex	
Arthrogenic muscle inhibition	• Central activation rate (91,96)	• When the central activation rate is below 95%, it is an indication that there are muscle fibers that are not being activated by central neural pathways	• CAR: Within- measurement (ICC = 0.94) Between-measurement (ICC = 0.86)
	• Interpolated Twitch Technique (85,87)	• The higher the proportion, the greater the number of muscle fibers that are not centrally activated	• ITT: Within measurement (ICC = 0.89)

Abbreviations: CAR, Central Activation Rate; ITT, Interpolated Twitch Technique; ICC, Intraclass Correlation Coefficient; PCST, Painful Conditioning Stimulus Test; piPST1, pain intensity of first Painful Stimulus Test; piPST2, pain intensity of second Painful Stimulus Test; PST, Painful Stimulus Test; TPT, Thermal Pain Threshold; PPT, Pressure Pain Threshold.

associated dysfunction (87). Common conditions such as joint pain, ligamentous laxity, and joint effusion are potential factors that facilitate the establishment of AMI (86).

In the AMI, there is an alteration of the firing of the joint receptors that send signals for the medullary inhibitory interneurons, causing inhibition of the activity of the alpha motoneurons and, consequently, the musculature involved in the affected joint (87). Joint pain may contribute to the AMI due to the alteration of the excitability of the flexor reflex pathway (86), which has the characteristic of facilitating the flexor and inhibiting the extensor muscles in the region surrounding the painful joint (89). In addition, joint pain in the knee has been associated with decreased muscle activation of the quadriceps (90, 91).

Although a systematic review has shown that the mechanisms of AMI are mostly studied in knee joint injuries (91), it may also be observed in individuals with pathologies in the hip. In this condition, AMI may be represented by a decrease in Gluteus maximus activation during extension activity in pronation (92). In this sense, as the most affected joint in SCD is the hip due to avascular osteonecrosis (7), the healthcare professional must be aware of the possibility of AMI playing an important role in this condition. However, many SCD individuals are likely quite physically deconditioned due to limited physical activity because of fatigue (93) or concerns about triggering vaso-occlusive crises after physical activity (94). This clinical characteristic in SCD individuals can make AMI assessment challenging because of the potential confounding biases related to physical deconditioning or

structural musculoskeletal alterations, especially in bilateral affections. On the other hand, in unilateral affections, these confounding biases can be minimized by comparison with the unaffected side. To date, no studies have evaluated AMI mechanisms in both adults and pediatric SCD individuals, and in future studies, the impact of physical deconditioning on AMI assessment should be better clarified.

In the clinical setting, AMI can be assessed using two quantitative methods: the Central Activation Rate (CAR) and the Interpolated Twitch Technique (ITT) (85, 91, 95). In both methods, the individual is asked to make a maximal voluntary isometric contraction (MVIC), and the force/torque generated by the muscle is registered. Then, when the force/torque plateau is reached, a maximal or supramaximal electrical stimulus is introduced. However, in the ITT method, this electrical stimulus can also be made initially with the muscle at rest (85, 91, 95). For electrical stimulus, 10 pulses, 100 Hz,200 μs pulse duration, and 400 V appear to be a reliable stimulation parameters for muscle contraction (85). Individuals with CAR >95% have a muscle fully activated by voluntary central stimulation, and those with less than that have some central muscle inactivation (91). In the ITT method, the higher the index score, the greater the number of fibers that are not centrally activated (85). Both methods seem simple, easily performed, and therefore feasible in clinical practice and research (Figure 4).

When comparing CAR and ITT in the capacity to estimate the quadriceps muscle activation, there was a significant variation between methods, with an estimated difference of up to 5.5% (85). In addition, it is suggested that ITT is a more accurate measure since the CAR might overestimate voluntary muscle activation (85). Some articles have assessed and reported good reliability of these methods in knee joints (96, 97), and the CAR method was found reliable within- (ICC = 0.94) and between-measurement sessions (ICC = 0.86) (96) while in the ITT method the reliability within measures was (ICC = 0.89) (97). Unfortunately, no studies evaluated the diagnostic value of these methods in joints frequently affected by SCD individuals, such as the hip, shoulder, and elbow. Although both CAR and ITT are not specific to SCD individuals, these methods should be used in clinical practice to evaluate CJP in SCD individuals.

Final remarks and conclusion

Chronic joint pain in patients with SCD might be related to maladaptive plasticity in the CNS, as it shares mechanisms with many known joint pathologies. Some of these maladaptive changes in the CNS are already known and include mainly poor descending inhibitory control, central sensitization, motor control impairments, reorganization of the motor cortex motor, and inhibition of induced maximal voluntary contraction. These changes may be assessed by a set of tests and/or questionnaires that are already available and could be useful in the clinical assessment and research in SCD. In the clinical setting, every healthcare professional can measure these maladaptive changes through instruments and methods with good diagnostic reliability (Table 1). These maladaptive plasticity changes may contribute to persistent pain in SCD, but there is a substantial lack of evidence regarding this aspect. However, future studies should be performed to elucidate and confirm these possible maladaptive changes in the nervous system in SCD individuals related to CJP to understand and treat the pain in those patients with better results.

Author contributions

All authors contributed to the development of the article in specific activities, such as planning, designing, and drafting/revising the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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