frontiers | Research Topics

Developments in sickle cell disease therapy and potentials for gene therapy

Edited by
Robert W. Maitta, Hollie Marie Reeves, Magali J. Fontaine and France Pirenne

Published in
Frontiers in Medicine

ISSN 1664-8714
ISBN 978-2-8325-2864-8
DOI 10.3389/978-2-8325-2864-8

About Frontiers

Frontiers is more than just an open access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers journal series

The Frontiers journal series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the *Frontiers journal series* operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews. Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the *Frontiers journals series*: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area.

Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers editorial office: frontiersin.org/about/contact

Developments in sickle cell disease therapy and potentials for gene therapy

Topic editors

Robert W. Maitta — Case Western Reserve University, United States
Hollie Marie Reeves — University Hospitals Cleveland Medical Center, United States
Magali J. Fontaine — University of Maryland, United States
France Pirenne — Université Paris-Est Créteil Val de Marne, France

Citation

Maitta, R. W., Reeves, H. M., Fontaine, M. J., Pirenne, F., eds. (2023). *Developments in sickle cell disease therapy and potentials for gene therapy*.
Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-2864-8

Table of contents

frontiers | Frontiers in Medicine

TYPE Editorial
PUBLISHED 14 June 2023
DOI 10.3389/fmed.2023.1208192

OPEN ACCESS

EDITED AND REVIEWED BY
Yvette Tanhehco,
Columbia University, United States

*CORRESPONDENCE
Robert W. Maitta
robert.maitta@case.edu

RECEIVED 18 April 2023
ACCEPTED 02 June 2023
PUBLISHED 14 June 2023

CITATION
Maitta RW, Reeves HM and Fontaine MJ (2023) Editorial: Developments in sickle cell disease therapy and potentials for gene therapy. *Front. Med.* 10:1208192. doi: 10.3389/fmed.2023.1208192

Editorial: Developments in sickle cell disease therapy and potentials for gene therapy

Robert W. Maitta[1]*, Hollie M. Reeves[1] and Magali J. Fontaine[2]

[1]Department of Pathology, Case Western Reserve University, Cleveland, OH, United States, [2]Department of Pathology, University of Maryland, Baltimore, MD, United States

KEYWORDS

sickle cell disease, therapy, transplantation, complications, review

Editorial on the Research Topic
Developments in sickle cell disease therapy and potentials for gene therapy

Since first described, sickle cell disease (SCD) has represented a therapeutic challenge for clinicians trying to help patients enduring the ramifications of this dreadful condition. This life-long disease from the first crisis begins with processes that will change the physiological milieu of patients. These processes in the setting of chronic anemia can translate into potentially worsening neurological symptomatology and organ damage that characterize those patients having frequent and recurrent vaso-occlusive crises. Thus, it is readily apparent that reducing the negative effects caused by levels of hemoglobin S does represent the best approach to ameliorate the long-term outlook of patients. The main therapeutic is to provide chronic transfusion support of patients with SCD. However, many patients are unable to tolerate the known adverse events of concomitant chelation therapy needed to reduce the iron load caused by a long transfusion issue, we introduce a variety of SCD-related topics describing concepts and advances in the treatment of this disease.

Most SCD patients manifest symptoms and complications of the disease from childhood. With this in mind, this issue includes a large study from French Guiana describing the incidence and type of complications of pediatric patients over time, and determining the incidence of complications such as acute chest syndrome and ischemic stroke (Gargot et al.). Importantly, even though these data show that the risk of ischemic stroke was low at 3.1%, this risk than doubled by the time patients reached teenage years. These data emphasizes that therapeutic approaches for SCD patients should focus on targeted interventions early in childhood to decrease complications. For diagnosis and testing, this issue also revisits both erythrocyte and reticulocyte counts of SCD patients as predictors of vasoocclusive crises (Feugray et al.). Authors of this study recommend the use of reticulocyte parameters obtained with a complete blood count. Specifically, a higher reticulocyte count in conjunction with higher medium reticulocyte fluorescence have the highest sensitivity and specificity (81% and 88% respectively) for predicting a looming crisis.

Additionally, the current issue includes a multicenter study from Italy looking at over 1,500 SCD patients treated with hydroxycarbamide and different transfusion regimens (Graziadei et al.). This study indicates that a proportion of SCD patients continued to experience symptoms requiring additional intense chronic transfusion support with no reduction in acute SCD-related complications despite receiving hydroxycarbamide. Furthermore, this study exemplifies one of the known complications of transfusion therapy in SCD and that is alloimmunization, which occurred in 8.5% of patients despite matching for Rh and Kell antigens. The authors argue that differences between red blood cell donors and patients explain the high alloimmunization rates seen in SCD patients (1).

Among the known complications of SCD, avascular necrosis of the hip and shoulder are often requiring surgical interventions in this patient population, as symptomatology of the affected joint(s) worsen and crises accumulate. In this Research Topic the use of intense hyperbaric oxygen therapy will be introduced and discussed for SCD patients with avascular necrosis of hip and/or shoulder, that can lead to resolution of the necrosis (Alshurafa, Elhissi et al.). The risk of iron overload, a major complication of chronic transfusions in SCD is being mitigated by performing exchanges instead of regular red cell transfusion. But despite good patient compliance, chronic red cell exchanges did not extend the life expectancy of patients with marked iron overload (Zhou et al.). Moreover, an article in this Research Topic will review the role that systemic hypertension plays in SCD patients in Cameroon by outlining a deep analysis of blood pressure variables that affect renal function over time (Nguweneza et al.).

Lastly, the current issue includes a comprehensive review of most SCD disease-modifying therapies including hydroxyurea, L-glutamine, voxelotor, and crizanlizumab known to reduce pain crises (Tanhehco et al.). Matched-related and haploidentical hematopoitic stem cell transplant (HSCT) with modified conditioning regimens will be compared to and contrasted to new gene therapies entering clinical trials in the same review. Understanding that adverse events to biologicals can also occur, an article proposes how to triage patients presenting with reactions to crizanlizumab while still being beneficial to the patient's treatment outcome [Alshurafa and Yassin (a)]. One of the studies in this Research Topic reviews L-glutamine's role in reducing oxidative stress in SCD over an extended follow-up period (Elenga et al.) and confirms the medication's safety while it reduces hospitalizations, need for transfusions and organ damage. Similarly, this issue also describes the high effectiveness of voxelotor in SCD patients with significant kidney disease [Alshurafa and Yassin (b)].

As mentioned above, allogeneic HSCT is a potentially curative, therapy is limited since finding suitable HLA compatible donors is the first challenge that needs to be overcome. This type of transplantation and graft manipulation including T cell depletion, presents with high infection risks secondary to transplantation conditioning regimens, as well as risks of graft vs. host disease (Bhalla et al.). Despite the potential for allogeneic HSC transplantation, use of new gene-editing tools such as Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and gene transfer technologies to correct the genetic cause of the disease are gaining momentum. CRISPR-associated protein currently represents an opportunity to treat or even cure patients with SCD by targeting either B-cell lymphoma/leukemia 11A gene (BCL11A) and the promoter regions of gamma globin genes (HBG1/2), both of which have been identified to significantly increase HbF protein expression. This issue presents a meta-analysis of the most recent work showing that of these two genes, HBG1/2 has the greater effect on HbF induction (Quagliano et al.). This Research Topic also contains a novel study using the Townes SCD mouse model that shows the extent by which the microRNA29B can induce HbF production *in vivo* (Gu et al.). This report demonstrates that this induction occurs by silencing the *MYB* gene product. Along these lines another article in this issue outlines challenges facing scientists trying to find new HbF-inducing agents and will discuss recently completed or ongoing clinical trials testing some of these agents (Pavan et al.). Additionally, a review in this issue will describe the effects of homology-directed repair of the *HBB* gene, and disruption of *cis*regulatory elements of BCL11A or leukemia/lymphoma related factor binding sites in the γ-globin gene promoters that result in enhancement of HbF expression (Zarghamian et al.). Finally, an article in this Research Topic will describe how inhibition of BACH1 transcription factor using a novel small molecular inhibitor can increase the concentration of HbF and even enhance the effectiveness of hydroxyurea in the setting of drug resistance using *in vitro* and *in vivo* models (Belcher et al.).

In conclusion, approaches to treat and cure SCD are rapidly developing. This is a period of great excitement and hope. Contributors to this Research Topic and the editors of this special topic encourage readers to appreciate this collection as an attempt to provide up to date information while introducing a variety of research areas in the field. Clinical advances including current clinical trials will be covered with focus on all recent developments in the field.

Author contributions

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

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.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated

organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

1. Zheng Y, Maitta RW. Alloimmunisation rates of sickle cell disease patients in the United States differ from those in other geographical regions. *Transfus Med*. (2016) 26:225–30. doi: 10.1111/tme.12314

frontiers in Medicine

ORIGINAL RESEARCH
published: 16 March 2022
doi: 10.3389/fmed.2022.832154

Transfusional Approach in Multi-Ethnic Sickle Cell Patients: Real-World Practice Data From a Multicenter Survey in Italy

Giovanna Graziadei[1†], Lucia De Franceschi[2†], Laura Sainati[3], Donatella Venturelli[4], Nicoletta Masera[5], Piero Bonomo[6], Aurora Vassanelli[7], Maddalena Casale[8], Gianluca Lodi[9], Vincenzo Voi[10], Paolo Rigano[11], Valeria Maria Pinto[12], Alessandra Quota[13], Lucia D. Notarangelo[14], Giovanna Russo[15], Massimo Allò[16], Rosamaria Rosso[17], Domenico D'Ascola[18], Elena Facchini[19], Silvia Macchi[20], Francesco Arcioni[21], Federico Bonetti[22], Enza Rossi[23], Antonella Sau[24], Saveria Campisi[25], Gloria Colarusso[26], Fiorina Giona[27], Roberto Lisi[28], Paola Giordano[29], Gianluca Boscarol[30], Aldo Filosa[31], Sarah Marktel[32], Paola Maroni[33], Mauro Murgia[34], Raffaella Origa[35], Filomena Longo[12], Marta Bortolotti[36], Raffaella Colombatti[3], Rosario Di Maggio[13], Raffaella Mariani[37], Alberto Piperno[37], Paola Corti[5], Carmelo Fidone[6], Giovanni Palazzi[4], Luca Badalamenti[38], Barbara Gianesin[39], Frédéric B. Piel[40] and Gian Luca Forni[14] on behalf of the Italian Society of Thalassemia and Hemoglobinopathies (SITE) the Italian Society of Transfusion Medicine and Immunohematology (SIMTI) and the Italian Association of Hematology and Pediatric Oncology (AIEOP)*

[1] Rare Diseases Center, General Medicine Unit, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy, [2] Department of Medicine, University of Verona and AOUI Verona, Verona, Italy, [3] Dipartimento della Salute della Donna e del Bambino Azienda Ospedaliera, Azienda Ospedaliera Universitaria, Padova, Italy, [4] Servizio Immunotrasfusionale, Azienda Ospedaliero Universitaria Modena, Modena, Italy, [5] Pediatric Clinic Hemato-Oncology Department, University of Milano-Bicocca, MBBM Foundation, San Gerardo Hospital, Monza, Italy, [6] Servizio Immunotrasfusionale, Azienda Ospedaliera Maria Paternò Arezzo, Ragusa, Italy, [7] UOC Medicina Trasfusionale, Azienda Ospedaliera Universitaria, Verona, Italy, [8] Department of Women, Child and General and Specialized Surgery, University "Luigi Vanvitelli", Naples, Italy, [9] Medicina Trasfusionale, Azienda Ospedaliera Universitaria Sant'Anna, Ferrara, Italy, [10] Centro per le Emoglobinopatie – Dipartimento di Scienze Cliniche e Biologiche, Università di Torino, Ospedale San Luigi Gonzaga, Torino, Italy, [11] Campus of Haematology Franco e Piera Cutino, A.O.O.R. "Villa Sofia-Cervello" di Palermo, Palermo, Italy, [12] Hematology, Thalassemia and Congenital Anemia Center, Ospedale Galliera, Genova, Italy, [13] Unità Operativa Semplice Dipartimentale Talassemia P.O. Vittorio Emanuele, Gela, Italy, [14] Italian Association of Pediatric Hematology Oncology (AIEOP) Coagulation Disorders Working Group, Brescia, Italy, [15] Pediatric Hematology/Oncology Unit, Università di Catania, Catania, Italy, [16] Servizio Microcitemia, Presidio Ospedaliero SL 5, Crotone, Italy, [17] UOSD di Talassemia ed Emoglobinopatie, Azienda Ospedaliero-Universitaria Policlinico San Marco, Catania, Italy, [18] Centro Microcitemie, Azienda Ospedaliera "Bianchi-Melacrino-Morelli", Reggio Calabria, Italy, [19] SSD Oncoematologia Pediatrica - Policlinico di S.Orsola, Bologna, Italy, [20] Servizio Trasfusionale, Ospedale Santa Maria delle Croci, Ravenna, Italy, [21] Ospedale Santa Maria della Misericordia, Perugia, Italy, [22] Policlinico San Matteo di Pavia, Pavia, Italy, [23] Unità Operativa Ematologia, Centro di Microcitemia, Azienda Ospedaliera di Cosenza, Presidio Ospedaliero "Annunziata" Cosenza, Cosenza, Italy, [24] Ospedale Spirito Santo, Pescara, Italy, [25] Department of Talassemia, Siracusa Hospital, Siracusa, Italy, [26] SOC Pediatria Nuovo Ospedale Prato Santo Stefano, Prato, Italy, [27] Hematology, Department of Translational and Precision Medicine, Sapienza University, Rome, Italy, [28] Unità Operativa Dipartimentale Talassemia, Presidio Garibaldi-Centro ARNAS Garibaldi, Catania, Italy, [29] UOC Pediatria Universitaria, Università di Bari, Bari, Italy, [30] Ospedale di Bolzano, Bolzan, Italy, [31] UOSD Malattie rare del globulo rosso, AORN A. Cardarelli, Naples, Italy, [32] Hematology and Bone Marrow Transplant Unit, IRCCS San Raffaele Scientific Institute, Milan, Italy, [33] Servizio di Immunoematologia e Medicina Trasfusionale, ASST Sette Laghi, Varese, Italy, [34] Centro Provinciale per le Microcitemia, Ospedale San Martino di Oristano, Oristano, Italy, [35] Ospedale Pediatrico Microcitemico, Università di Cagliari, Cagliari, Italy, [36] Department of Oncology and Oncohematology, University of Milan, Milan, Italy, [37] Rare Disease Centre - Hereditary anemias - ASST-Monza, S. Gerardo Hospital - University of Milano-Bicocca, Monza, Italy, [38] Biomedicina, Neuroscienze e Diagnostica avanzata, University of Palermo, Palermo, Italy, [39] ForAnemia Foundation, Genoa, Italy, [40] Department of Epidemiology and Biostatistics, Imperial College London, London, United Kingdom

OPEN ACCESS

Edited by:
Robert W. Maitta, Case Western Reserve University, United States

Reviewed by:
Magali J. Fontaine, University of Maryland, United States
Sirisha Kundrapu, The MetroHealth System, United States

***Correspondence:**
Gian Luca Forni
gianluca.forni@galliera.it

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Hematology, a section of the journal Frontiers in Medicine

Received: *09 December 2021*
Accepted: *14 February 2022*
Published: *16 March 2022*

Citation:
Graziadei G, De Franceschi L, Sainati L, Venturelli D, Masera N, Bonomo P, Vassanelli A, Casale M, Lodi G, Voi V, Rigano P, Pinto VM, Quota A, Notarangelo LD, Russo G, Allò M, Rosso R, D'Ascola D, Facchini E, Macchi S, Arcioni F, Bonetti F, Rossi E, Sau A, Campisi S, Colarusso G, Giona F, Lisi R, Giordano P, Boscarol G, Filosa A, Marktel S, Maroni P, Murgia M, Origa R, Longo F, Bortolotti M, Colombatti R, Di Maggio R, Mariani R, Piperno A, Corti P, Fidone C, Palazzi G, Badalamenti L, Gianesin B, Piel FB and Forni GL (2022) Transfusional Approach in Multi-Ethnic Sickle Cell Patients: Real-World Practice Data From a Multicenter Survey in Italy. Front. Med. 9:832154. doi: 10.3389/fmed.2022.832154

Sickle cell disease (SCD) is a worldwide distributed hereditary red cell disorder characterized by recurrent acute vaso-occlusive crises (VOCs and anemia). Gold standard treatments are hydroxycarbamide (HC) and/or different red blood cell (RBC) transfusion regimens to limit disease progression. Here, we report a retrospective study

on 1,579 SCD patients (median age 23 years; 802 males/777 females), referring to 34 comprehensive Italian centers for hemoglobinopathies. Although we observed a similar proportion of Caucasian (47.9%) and African (48.7%) patients, Italian SCD patients clustered into two distinct overall groups: children of African descent and adults of Caucasian descent. We found a subset of SCD patients requiring more intensive therapy with a combination of HC plus chronic transfusion regimen, due to partial failure of HC treatment alone in preventing or reducing sickle cell-related acute manifestations. Notably, we observed a higher use of acute transfusion approaches for SCD patients of African descent when compared to Caucasian subjects. This might be related to (i) age of starting HC treatment; (ii) patients' low social status; (iii) patients' limited access to family practitioners; or (iv) discrimination. In our cohort, alloimmunization was documented in 135 patients (8.5%) and was more common in Caucasians (10.3%) than in Africans (6.6%). Alloimmunization was similar in male and female and more frequent in adults than in children. Our study reinforces the importance of donor-recipient exact matching for ABO, Rhesus, and Kell antigen systems for RBC compatibility as a winning strategy to avoid or limit alloimmunization events that negatively impact the clinical management of SCD-related severe complications.

Clinical Trial Registration: ClinicalTrials.gov, identifier: NCT03397017.

Keywords: hydroxycarbamide, multi-ethnicity, sickle cell disease, transfusion therapy, alloimmunization

INTRODUCTION

Sickle cell disease (SCD) is one of the most common inherited red cell disorders worldwide, recognized by the World Health Organization (WHO) and the United Nations as a "global health problem" (1, 2). About 300,000 infants are born each year with SCD; ∼90,000 in Nigeria, 44,000 in India, 40,000 in the Democratic Republic of Congo, 12,000 in the United States (US), 2,000 in Europe, and 300 in the United Kingdom (UK) (3). Although SCD is part of the rare disease group, more than 10,000 SCD patients live in the UK and more than 25,000 in France, making SCD much more common than cystic fibrosis or hemophilia A (4, 5). The median life expectancy is now more than 60 years in Europe (6), but about 80% of patients with SCD in African regions die during childhood (7).

Treatment options for SCD are still limited, particularly in low-income settings. Hydroxycarbamide (HC) is the gold standard treatment for both children and adults with SCD (8). Hydroxycarbamide reduces painful vaso-occlusive events (VOCs) and acute chest syndrome as well as the need for red blood cell (RBC) transfusion. Until now, RBC transfusion has largely been used for clinical management of both acute and chronic sickle cell-related complications (9, 10). Despite the known indications for RBC transfusion and HC therapy (9, 11–16), treatment of patients with SCD in clinical practice is still not homogeneous.

Over the course of a lifetime, patients with SCD might be exposed to different RBC transfusion regimens, ranging from simple transfusion on-demand or manual/automatized RBC exchange to manage acute VOCs or to limit disease progression and organ damage (3, 17). Alloimmunization to RBC blood group antigens carries an increased risk of hemolytic transfusion reactions (DHTR), potentially delaying the identification of compatible RBC units (18, 19). In Europe, Italy presents a distinctive epidemiologic European niche for SCD, characterized by endemic SCD population mainly in Southern Italian regions and multi-ethnic SCD population localized in Central and Northern Italian regions (20–23). This affects the distribution of SCD genotypes, mainly represented by $S\beta^0/S\beta^+$ or SS genotype in patients of Caucasian descent, while SS or SC genotypes characterize patients of African descent. To better understand the scenario of transfusion regimens and alloimmunization of SCD patients in Italy, we carried out a national survey involving the National Comprehensive Reference Centers for SCD and the Italian Society of Thalassemia and Hemoglobinopathies (SITE), in collaboration with the Italian Society of Transfusion Medicine and Immunohematology (SIMTI) and the Italian Association of Hematology and Pediatric Oncology (AIEOP).

MATERIALS AND METHODS

Study Design

This is a retrospective multicenter national study (ClinicalTrials.gov identifier NCT03397017). Data from the clinical records of eligible patients were collected from 2015 to 2018 through a standard web-based application (www.SITE-italia.org) encrypted by the Central Server. The recording of personal, therapy, and complications data of patients with sickle cell anemia included in the National Transfusion Treatment Survey was undertaken by the responsible investigator or sub-investigators selected by each center, following registration on the site. The operator

can subsequently access the patient's clinical data and perform updates to follow clinical evolution over time. The study did not involve any additional tests compared to routine patient management.

The study aimed to identify the lifelong therapeutic approaches, focusing on transfusion regimens, in clinical management of a large multi-ethnic Italian cohort of patients with SCD. The study was coordinated by SITE, in collaboration with SIMTI and AIEOP. A total of 34 Italian reference centers from 15 Italian regions were involved. Data were collected through the National Comprehensive Reference Centers for SCD. The study was approved by the Ethics Committee of Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico of Milan, Italy, the coordinating center of the study.

The inclusion and exclusion criteria were, respectively, the presence or absence of a biochemical and/or genetic diagnosis of SCD and the capability or not to give informed consent. Written informed consent was obtained from all included patients after the study aims and protocol had been explained. When necessary, a Cultural Linguistic Mediator could be engaged to assist in the consent process. Besides demographic data, every center collected data concerning the type and sequence of therapy, acute transfusion therapy (ATR), chronic transfusion therapy (CTR, i.e., regular transfusion regimen for more than 1 year), or HC, the main indications for ACR and CTR, and details on alloimmunization (number and systems of antibodies) over the previous 4 years of treatment.

Statistical Analysis

Descriptive statistics of continuous variables were expressed using medians and interquartile range (IQR: 25th-75th percentile); dichotomous variables were summarized as counts and percentages. The Shapiro-Wilk test was used to test the normality of distribution. As no normal distribution was found, the differences between groups were analyzed with the Wilcoxon signed-rank test. Two- or multiple-proportion comparisons between variables were performed with the Z-test. All tests were two-sided, and p-values < 0.05 were considered as statistically significant. Data management and graphs were created with Excel (Microsoft, Seattle, WA, USA), and statistical analysis was performed using R version 3.5.1 for Windows (R Core Team, Vienna, Austria).

RESULTS

SCD Italian Patients Cluster Into Two Overall Distinct Groups: Children of African Descent and Adults of Caucasian Descent

Our study shows that Italian SCD patients overall cluster into two distinct groups: children of African and adults of Caucasian descent (**Figure 1A**). We collected data from 1,579 patients, of whom 802 were male (51%) and 777 were female (49%) with a median age of 23 (IQR 10–41) years (**Supplementary Figure S1**). No significant difference in age was found between males and females ($p = 0.18$); the median age was 21.5 (IQR 10–40) years in males and 25.3 (IQR 10–42) years in females. Ethnicity was equally distributed between Caucasians (47.9%) and Africans (48.7%), with a minority of African American (2.8%) and Asian (0.4%) SCD patients. Ethnicity was not defined for 0.2% of patients (**Table 1**). In African patients, the most common genotype was homozygosity for HbS (SS; 71.3%). Compound heterozygosity with β-thalassemia mutations was 2.3% for S/β0-thalassemia, 3.9% for S/β+-thalassemia, and 20.8% for SC disease. Otherwise, the most prevalent genotype in Caucasians was S/β-thalassemia (74.5%; in particular, S/β^0-thalassemia 34.8% and S/β^+-thalassemia 39.7%). SS genotype was present in 21.4% and SC in 0.5% of SCD patients of Caucasian descent. The median age of African SCD patients was 12 (IQR 7–22) years compared with 38 (IQR 25–49) years in Caucasian patients ($p < 0.001$).

Combined Transfusion Approaches With Hydroxycarbamide Were Used to Limit HC-Resistant Sickle Cell-Related Clinical Manifestations

Full data on therapeutic management of SCD patients were collected for 1,364 out of the 1,579 patients (**Table 2**; **Figure 1B**; **Supplementary Figure S1**). The following therapeutic regimens were identified: (i) HC: chronic hydroxycarbamide therapy for more than 1 year; (ii) CTR: chronic transfusion regimen; (iii) ATR: acute transfusion approach; and (iv) no treatment (**Figure 2A**). When we considered therapeutic strategies in function of age, we found that blood transfusion approaches were used throughout patient's journey (**Figure 2B**). A total of 292 patients (21.4%) were on CTR, 196 (14.4%) on ATR, and 497 on HC (36.4%), of whom 100 needed transfusions for acute VOCs. Three hundred and seventy-nine (27.8%) patients never received long-term therapy. In both ATR and CTR clinical setting, donor-recipient exact matching for serological ABO, Rhesus and Kell antigen for RBC compatibility was carried out according to international and Italian guidelines, released in 2014 (9, 13, 24–27).

The main indications for CTR were recurrent severe VOCs (371 events; 37.1%), chronic anemia (306 events; 30.6%), primary stroke prevention (78 events, 7.8%) and secondary stroke prevention (55 events, 5.5%), acute chest syndrome (ACS; 107 events; 10.7%), splenic sequestration (31 events, 3.1%), HC-resistant pain (39 events; 3.9%) and leg ulcers (12 events; 1.2%) (**Figure 2B**). The median value of hemoglobin (Hb) before CTR was 9.2 g/dL (IQR: 8.5–10, min-max: 5.2–14.9 g/dL, data available for 85% of patients) and the median value of HbS was 50% of total Hb (IQR: 39–55, min-max: 8.5–89%, data available for 67% of patients).

The main indications for ATR were acute anemia (384 events; 35%), VOCs (352 events; 32.1%), ACS (170 events; 15.5%), surgery (82 events; 7.5%), pregnancy (64 events; 5.8%), splenic sequestration (26 events; 2.4%), stroke (9 events; 0.8%), multi-organ failure (6 events; 0.5%) and priapism (5 events; 0.5%) (**Figure 2B**). Before ATR, the median value of Hb level was 8.0 g/dL (IQR: 6.8–9.0 g/dL, min-max 2.7–15.2 g/dL, data available for 72% of patients), with 67% of HbS (IQR: 56–78%, min-max: 30–93, data available for 43% of patients). Patients who

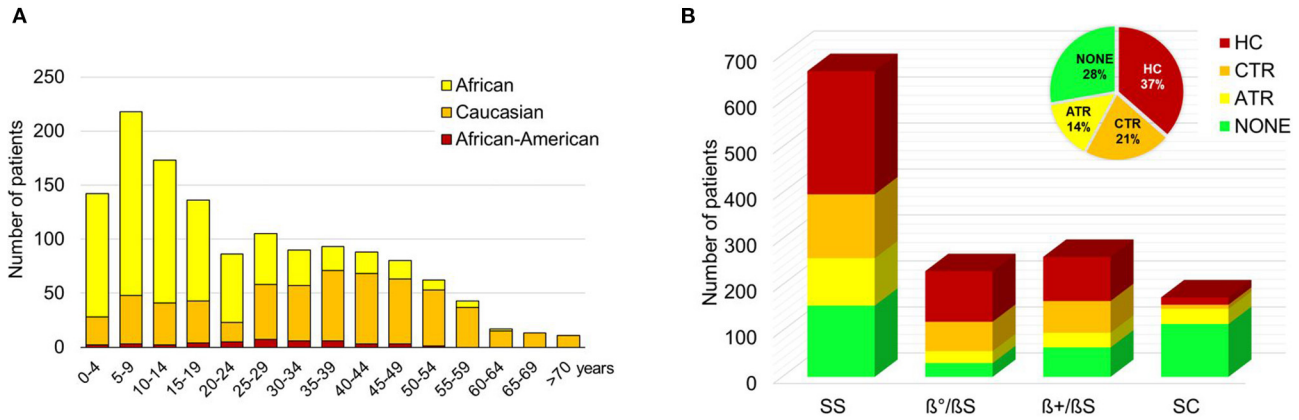

FIGURE 1 | (A) Distribution of sickle cell disease (SCD) patients by age and ethnicity. Data are shown as a column chart that reports, for each age group, the number of Africans and Caucasians patients of the cohort. **(B)** Distribution of SCD patients by genotype and therapy. Data are shown as a column chart that reports, for each genotype, the number of patients that received HC/ATR/CTR/NONE as their therapy regimen. ATR, acute transfusion; CTR, chronic transfusion; HC, hydroxycarbamide.

TABLE 1 | Demographic characteristics of patients with sickle cell disease (SCD).

Ethnicity	Genotype	Counts (%)	Males (%)	Median age (IQR), yr
African ($n = 769$)	HbSS	548 (71.3)	280 (51.2)	11 (6.9–19.8)
	HbS/β°-thalassemia	18 (2.3)	12 (66.7)	14.5 (9.5–19.8)
	HbS/β$^+$-thalassemia	30 (3.9)	16 (53.3)	26.5 (15.6–37.5)
	HbSC	160 (20.8)	78 (48.8)	16.9 (7.8–27.0)
	Not defined	13 (1.7)	3 (23.1)	40 (12–46)
Caucasian ($n = 756$)	HbSS	162 (21.4)	76 (46.9)	35.4 (18–48)
	HbS/β°-thalassemia	263 (34.8)	138 (52.5)	40 (27–50)
	HbS/β$^+$-thalassemia	300 (39.7)	161 (53.7)	39 (25–51)
	HbSC	4 (0.5)	3 (75.0)	31.5 (20–40.8)
	Not defined	27 (3.6)	14 (51.9)	31.9 (22–45)
African-American ($n = 45$)	HbSS	24 (53.3)	9 (37.5)	29 (22.6–36.2)
	HbS/β°-thalassemia	4 (8.9)	2 (50.0)	12.5 (6.8–23.2)
	HbS/β$^+$-thalassemia	4 (8.9)	1 (25.0)	25 (9.5–41.5)
	HbSC	13 (28.9)	4 (30.8)	27.5 (21–35)

Asian patients comprised 0.4%, and people of another ethnicity made up the other 0.2%.
Hb, hemoglobin; IQR, interquartile range; yr, years.

were not treated had a median Hb value of 10.7 g/dL (IQR: 9.3–12 g/dL). When we stratified SCD population according to age: children (<18 years) and adults (>18 years), the proportion of different indications for ATR was similar within the two groups except for pregnancy and priapism, which were present in adults (**Supplementary Figure S2A**). Considering CTR, we found CTR to be more frequently introduced in adults than in children with SCD (**Supplementary Figure S2A**). CTR protocols were more frequently applied in adults than in children to prevent ACS and to treat chronic pain resistant to HC therapy (**Supplementary Figure S2A**). We found a higher proportion of Caucasian adults (older than 20 years) under CTR (135/181, 75%) when compared to African adults (41/94, 44%). In children and adolescents (younger than 20 years), the proportion of Caucasians treated with CTR (46/181, 25%) was lower when compared with Africans (53/94, 56%). The ratio between patients in ATR and CTR decreases with during aging ($p < 0.05$, **Figure 2A**).

HC was generally started after 5 years of age in SCD patients of both Caucasian (6%) and African (94%) descent. Two hundred and fourteen of 246 Caucasian patients were adults (17% SS and 79% Sβ genotypes). As expected, SCD patients with SC genotype showed less intensive medical treatment than SCD patients with either SS or Sβ$^{0/+}$ genotypes (**Figure 1B**).

For 250 out of 1,364 patients (18.3%), it was possible to follow the temporal sequence of change in therapy (**Table 2**). Fourteen patients (1.0% of the 1,364) switched from HC to CTR, and 236 patients (17.3%) switched from CTR to HC. A subset of 60 patients (4.3% of the 1,364 with treatment data available) received a simultaneous blood transfusion and HC for a median period of

TABLE 2 | Treatments of SCD patients (n = 1,364) and distribution according to ethnicity and genotype.

Therapy[a]	Pts (%)	M vs. F	African vs. Caucasian vs. African-American	SS, No. (%)	S/β°, No. (%)	S/β+, No. (%)	SC, No. (%)	*p*-value
CTR	292 (21.4)	152/140	94/181/14	139 (49.5)	64 (22.8)	69 (24.6)	9 (3.2)	<0.001
ATR	196 (14.4)	92/104	116/69/10	103 (53.1)	26 (13.4)	32 (16.5)	33 (17)	<0.001
HC	497 (36.4)	273/224	238/246/11	268 (54.7)	110 (22.4)	96 (19.6)	16 (3.3)	<0.001
None	379 (27.8)	196/183	280/91/7	155 (42.6)	30 (8.2)	64 (17.6)	115 (31.6)	<0.001
[b]ATR/CTR → HC	236 (17.3)	129/107	120/109/7	138	53	37	3	–
[b]HC → CTR	14 (1.0)	8/6	6/7/1	9	2	2	1	–

[a] *The sum of partial counts may not correspond to the total in the case of different and/or not defined ethnicity/genotype.*
[b] *Subset of 250 patients for whom it was possible to follow the timing of therapy.*
A total of 100 patients under HC were transfused for the clinical management of acute vaso-occlusive events (VOCs).
ATR, acute transfusion regimen; CTR, chronic transfusion regimen; F, female; HC, Hydroxycarbamide; M, male; Pts, patients.

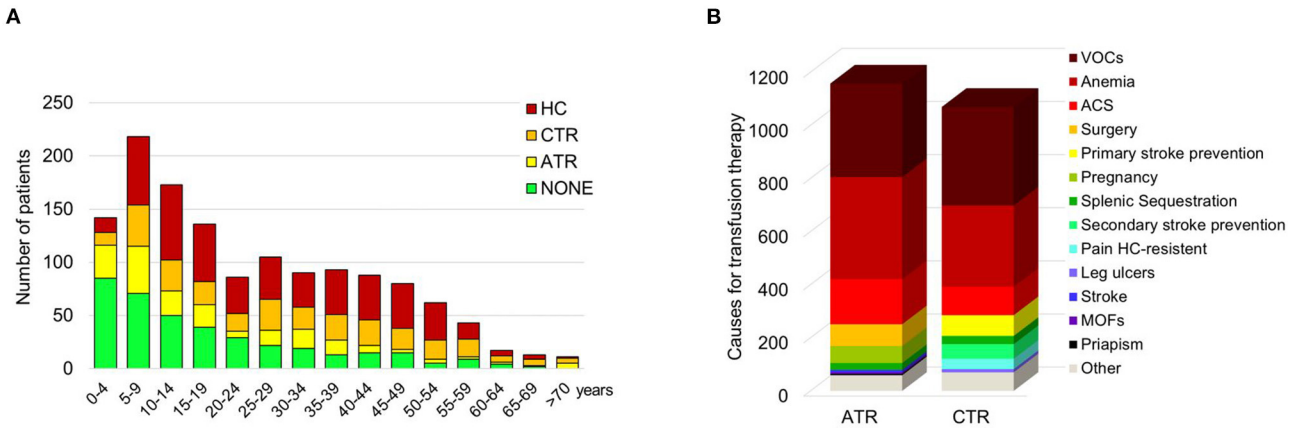

FIGURE 2 | (A) Distribution of sickle cell disease (SCD) patients by age and therapy. Data are shown as a column chart that reports, for each age group, the number of patients that received HC/ATR/CTR/NONE as their therapy regimen. **(B)** Distribution of the indications for transfusion therapy. Data are shown as counts of the indications for ATR and CTR. ACS, acute chest syndrome; ATR, acute transfusion; CTR, chronic transfusion; HC, hydroxycarbamide; MOFs, multi-organ failures; VOCs, vaso-occlusive events.

4.6 years (IQR 2.2–11.1 years). This was due to the severity of sickle cell-related clinical presentation, which was only partially controlled by HC therapy. The main indications for CTR in these 60 patients were, respectively, VOCs (number of events: 33), anemia (n = 25), ACS (n = 13). HC-resistant pain (n = 6), splenic sequestration (n = 4), primary (n = 2) or secondary (n = 3) stroke prevention. Combined therapy (CTR+HC) was started at a median age of 20.5 (IQR 10.7–30.8) years. In 14/250 patients, HC was started at a median age of 30.7 (IQR 28.1–41.4) years, and CTR needed to be added to HC after a mean period of 3.8 (IQR 2.4–5.3) years at a median age of 34.4 (IQR 30.3–48.3) years. The proportion of SCD patients without any treatment decreased with aging (**Figure 2A**). Two out of 1,364 patients with SCD displayed hyperhemolysis reaction after RBC transfusion (1 African SS child: 5 years of age; 1 African SS adult: 23 years of age).

Collectively, our data indicate that acute and chronic transfusion approaches were chosen to limit disease progression in patients of both Caucasian and African descent. RBCs from Caucasian donors were used. The donor-recipient exact matching for ABO, Rhesus and Kell antigen for RBC compatibility strategy limits alloimmunization, which might further complicate clinical management of patients with SCD (9, 18, 19, 28).

Low Immunization Events Characterize the Italian Multi-Ethnic Cohort of SCD Patients

In our study, alloimmunization was documented in 135 (8.5%) out of 1,579 patients with SCD. Sixty-one of these 135 patients (45.2%) were already alloimmunized at their arrival at the comprehensive SCD center. Four of them (6.6%) developed alloimmunization against new RBC antigen (**Supplementary Figure S3A**) 36 of these 135 patients (27%) developed alloantibodies after they arrived at the Italian referral center (**Supplementary Figure S3A**). Data on alloantibody status in relation to the timing of arrival at the SCD center were not available for the other 38 alloimmunized SCD patients.

Among the alloimmunized SCD patients, 33 (24.4%) were younger than 18 years, 53 (39.3%) were aged 18–40 years, and 49 (36.3%) were older than 40 years (**Supplementary Figure S3B**).

Fifty-one alloimmunized patients were African [median age 20 years (IQR 11–28 years)], corresponding to 6.6% of the African group; 78 patients were Caucasian [median age 41 years (IQR 31–51 years)], representing 10.3% of the Caucasian group; five patients were African American [median age 35 years (IQR 30–37 years)] and for one patient (age 29 years) ethnicity was not reported. Thus, alloimmunization was more frequent in adults than in children with SCD ($p < 0.001$). The frequency of alloimmunization was similar in female and male patients with SCD.

RBC-specific antibodies were detected in 124 patients, of whom 70 (41 males, 29 females) had a single antibody and 43 (16 males and 27 females) had multiple antibodies (59.5%, 28.6, 9.5, and 2.4%, respectively, with two, three, four, and six antibodies). Specific antibodies were not identified in 11 patients (3 males and 8 females) (**Supplementary Figure S3C**). A higher proportion of Caucasian patients had multiple antibodies compared with patients of other ethnicities. In the Caucasian group, a single antibody was prevalently found in males (24/40, 60%), while there were no significant differences between genders in the African group. When genotypes were considered, we observed alloimmunization to be more frequently associated with SS genotype in both African and African American patients, whereas SS and $S\beta^{0/+}$ genotypes were equally associated with alloimmunization in SCD patients of Caucasian descent (**Supplementary Figure S3B**).

As shown in **Figure 3**, alloimmunization predominantly involves RhCDE and Kell systems, with Rhesus (45%) the most represented antibody system, followed by Kell (15%), MNS (12%), Duffy (6.2%), Lewis (4.8%), Kidd (4.3%) and Lutheran (2.7%). In addition, antibodies against minor antigens (0.8%) and antibodies not better identified (8.8%) were also detected. Of interest, anti-e antibodies were found in two e-negative African SCD patients. When we considered alloimmunization profile and patients' age, a higher rate (26 vs. 6%) of alloantibodies with non-identified specificity in children's group was observed (**Figure 3**, right pie-charts).

Eleven patients (8.1%) with alloimmunization developed a delayed hemolytic transfusion reaction (DHTR) (8 females and 3 males) with a median age of 39 years (IQR: 18.5–49). Six of these patients had SS genotype (4 Africans, 2 Caucasians), and five were Caucasian with Sβ genotype. In 4 out of the 11 patients, a single antibody (D, C, Cw, Jsb) was detected, and in another 4 of the 11 (all females, 3 Caucasians, and 1 African) multiple antibodies (2–4 different specificities) were detected. In 2 patients, antibodies were not clearly identified. Taken together, these data indicate that low alloimmunization events recorded in both African and Caucasian patients might most likely be related to the donor-recipient exact matching for ABO, Rhesus and Kell antigen for RBC compatibility.

DISCUSSION

Our study highlights the complexity of the clinical management of SCD manifestations throughout the patients' journey. The peculiar multi-ethnic profile of the Italian SCD population, with a roughly equal proportion of Caucasian and African patients, is an added value to the analysis of real-life data. In our series, Caucasian SCD patients were generally older than African patients. This is most likely related to the efficacy of the prenatal program for hemoglobinopathies that started in Italy in the 1980s (29, 30). In contrast, African SCD patients were mainly clustered in pediatric and young adult populations, reflecting the voluntary migration fluxes of the last two decades (22, 23, 31). Indeed, the presence of elderly Caucasian SCD patients represents a unique SCD cohort, providing important information on the combinatory effects of aging and SCD on disease natural history (32, 33).

In our study, the transfusion approach was similar in SS and S/β patients in terms of ATR and CTR regimens, and the percentage of patients with S/β+- and S/β° who were treated with ATR and CTR was similar. Both CTR and ATR were used more frequently in SS patients compared with either S/β+ or S/β°-patients (34). The main reasons for ATR were similar to those described by Rees et al. (35).

Notably, we found that SS African patients were treated with ATR more than Caucasian patients with S/β genotypes. This might be related to (i) patients' low social status; (ii) patients' limited access to family practitioners, generally related to troubles in speaking Italian. All these factors might contribute to the higher access to an emergency department for African SCD patients during acute events when compared to Caucasian SCD subjects (10).

Simple transfusions were the main transfusion strategy used during CTR, and single top-up transfusions were the main one in the acute setting. Manual and automatic exchange transfusions were used more in CTR than in ATR. This might be related to: (i) difficulties in vein access during VOCs; (ii) absence of expertise; (iii) overnight acute events (36, 37).

Of note, we found a subset of patients still symptomatic for acute SCD-related clinical manifestations under treatment with HC, highlighting the biocomplexity of SCD that lies beneath red cell sickling and dehydration (38–44). Indeed, HC partially acts on sickle cell-related inflammatory vasculopathy and chronic inflammation (4, 45–47). Thus, SCD patients considered partial responders to HC require a more intensive treatment, which has until now been represented by the combination of HC and chronic transfusion programs. In our cohort, combination therapy of CTR and HC was administered in a similar percentage of African and Caucasian SS patients. The percentage of SS patients who required long-term combination treatment was higher than the proportion with S/β°-S/β+- genotypes. Considering chronic therapies (CTR and HC), we confirmed that SC patients needed less intensive treatment than other SCD genotypes (14). Thus, our data highlight the presence of a subset of SCD patients with severe clinical manifestations resistant to HC treatment. These might now be eligible for new US Food and Drug Administration and European Medicines Agency therapeutic options such as crizanlizumab, a humanized antibody against P-selectin, or voxelotor, a novel oral anti-sickling agent, alone or in combination with HC (41, 42, 48–52).

In our cohort, alloimmunization was identified in SCD patients aged between 18 and 40 years; those aged <18 years had a

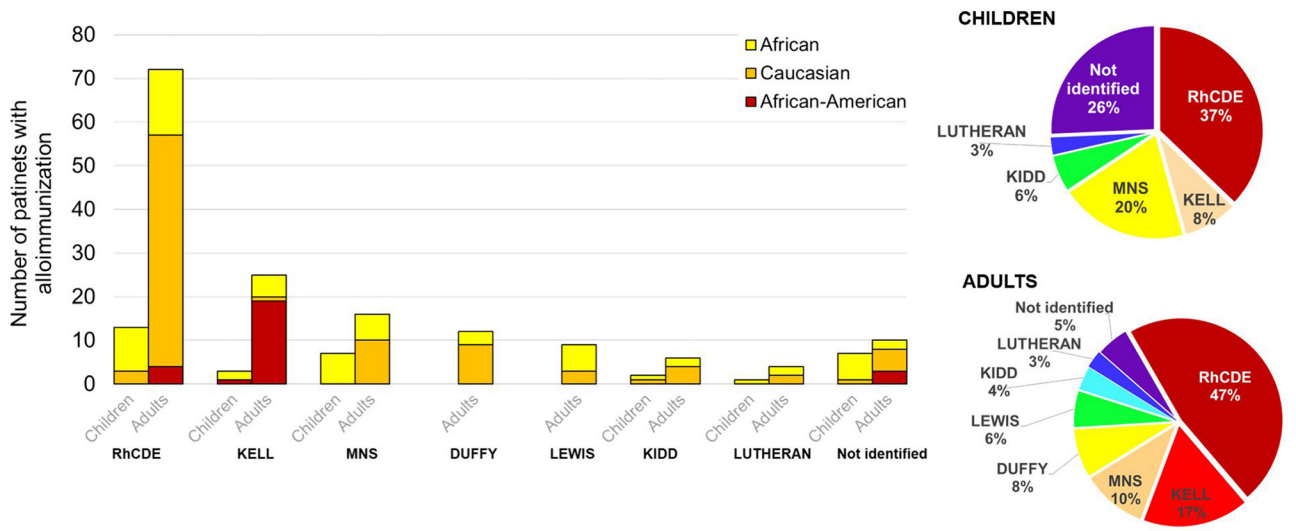

FIGURE 3 | Number of children/adults in SCD patients by antibody system. The pie charts report the overall distribution of the specific antibodies for children and adults. Data are shown as column chart that reports, for each system and differentiating for children/adults, the number of African, Caucasian and African-American patients with the specific antibody.

lower alloimmunization events than those aged ≥18 years, most likely because they had a shorter transfusion history. A higher proportion of Caucasian patients (10.3%) than African patients (6.6%) had alloimmunization, and among these, Caucasian patients again were older than African patients (median 41 vs. 20 years).

Although the alloimmunization rate was not available due to the study's retrospective design, the low alloimmunization events in both African and Caucasian patients, might reflect the satisfactory level of donor-recipient exact matching for ABO Rhesus and Kell antigen for RBC compatibility, consistent with the Italian guidelines for children and adults released on 2014 (11, 15). Antigen mismatch between donor and recipient is the basis for antibody formation, as the recipient recognizes those antigens as non-self and, thereby, an immune response might be elicited (53, 54). Transfused SCD patients have the highest rates of RBC alloimmunization over their lifetimes (up to 40–50%), compared to fewer than 5% of other transfused individuals with either thalassemia major or myelodysplasia (55, 56). Detection of alloantibodies may make locating compatible RBC units for future transfusion difficult, delayed, and sometimes impossible (54). Potential reasons for alloimmunization include (i) number of donor exposures; (ii) antigenic mismatches between donor RBCs and recipient RBCs related to ethnicity (mainly between Caucasian donors and African descent recipients); (iii) the inflammatory state of recipients (57). SCD is characterized by a chronic inflammatory state, worsening during acute VOCs (57, 58). Thus, SCD patients transfused in their baseline state of health are thought to be less likely to become alloimmunized than patients transfused in a state of inflammation, such as during acute VOCs (58). Studies in SCD patients have shown that having an acute chest syndrome at the time of transfusion is a significant risk factor for becoming alloimmunized (53, 58). In our cohort, the antibodies identified were mainly of RhCDE and Kell systems, as reported in studies of transfused SCD patients from the United States and Brazil (9). Different antibodies systems (i.e., MNS and Kidd) and different frequencies of alloimmunization have been recently described in SCD patients in French Guiana when compared to our cohort or those from the United States or Brazil (18). These might reflect the heterogeneity of donor and recipient populations in different geographical settings, which increases the complexity of data analysis and comparison.

Our study presents some limitations: (i) the retrospective design of the study, which did not allow us to evaluate the alloimmunization rate; (ii) lacking data on patients' transfusion history before their arrival to the comprehensive centers for hemoglobinopathies; (iii) no record on patients' mortality.

In conclusion, our study highlights that transfusion regimens are still crucial as intensive treatments for both acute and chronic SCD-related complications. In our cohort, HC was offered to symptomatic SCD children in agreement with national recommendations (10, 15). This clearly favors a more extensive use of transfusion approaches in the pediatric setting in our cohort in case of acute complications. Since alloimmunizations remains a barrier for safe and effective transfusion of patients with SCD, a careful evaluation of RBC transfusion guidelines transfusion becomes a key factor, along with the implementation of transfusion protocols (such as phenotypically-matched RBCs: RhCDE and Kell), genotyping of patients and donors to identify RBC antigenic variants and maintenance of records on patient transfusion history (59, 60). In addition, dissemination of the knowledge on management of DHTR in patients with SCD, might positively impact patients' outcome (61). These measures are expected to reduce the occurrence of alloimmunization as well as the incidence of delayed hemolytic transfusion reactions, optimizing the management of SCD subjects of different descent throughout the patients' journey.

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

The studies involving human participants were reviewed and approved by Ethics Committee of Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico of Milan, Italy. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

GG, LDF, and GLF contributed to the conceptualization and design of the study, acquisition and curation of the data, contributed to data analysis and interpretation, writing, critical appraisal and comments, and reviewing and editing. LS, DV, NM, PB, AV, MC, GL, and VV contributed to the conceptualization and design of the study, acquisition and curation of the data, and critical appraisal and comments. PR, VP, AQ, LN, GR, MA, RR, DD'A, EF, SMar, FA, FB, ER, AS, SC, GC, FG, RL, PG, GB, AF, SMac, PM, MM, RO, FL, MB, RC, RD, AP, PC, CF, GP, and LB contributed to acquisition and curation of the data. BG and FP contributed to data analysis and interpretation. All authors have read and agreed to the published version of the manuscript.

FUNDING

Financial support for Medical Editorial Assistance was provided by Novartis Farma SpA. Novartis Farma SpA was not involved in the study design, collection, analysis, interpretation of data, and the writing of this article or the decision to submit it for publication.

ACKNOWLEDGMENTS

The authors thank Ray Hill, an Independent Medical Writer who provided medical writing assistance with this manuscript on behalf of Springer Healthcare, which was funded by Novartis Farma SpA, Italia in accordance with Good Publication Practice (GPP3) guidelines (http://www.ismpp.org/gpp3). Authors had full control of the content and made the final decision for all aspects of this article.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.832154/full#supplementary-material

Supplementary Figure S1 | Flow-chart of study population. Pts, patients; SCD, sickle cell disease.

Supplementary Figure S2 | Distribution of the indications for transfusion therapy regimen for children and adults, **(A)** Indications for acute transfusion regimen (ATR). **(B)** Indications for chronic transfusion regimen (CTR). Data are shown as counts of the indications for ATR and CTR. ACS, acute chest syndrome; ATR, acute transfusion; CTR, chronic transfusion; HC, hydroxycarbamide; MOFs, multi-organ failures; VOCs, vaso-occlusive events.

Supplementary Figure S3 | (A) Distribution of new antibodies developed in after the arrival at the center for sickle cell disease (SCD) patients with pre-existing (up) and without (low) pre-existing antibodies. **(B)** Number of SCD children/adults patients with alloimmunization by ethnicity. **(C)** Number of SCD children/adults patients with alloimmunization presenting single, multiple or unidentified antibody/ies. Data are shown as pie and column charts with the number of patients with alloimmunization.

REFERENCES

1. World Health Organization. *Sickle-Cell Anaemia: Report by the Secretariat*. Geneva: World Health Organization (2006). Available online at: https://apps.who.int/gb/archive/pdf_files/WHA59/A59_9-en.pdf (accessed June 23, 2021).
2. United Nations Secretary-General. *Secretary-General's Message on Sickle-Cell Anaemia*. (2009). Available online at: https://www.un.org/sg/en/content/sg/statement/2009-06-19/secretary-generals-message-sickle-cell-anaemia (accessed June 18, 2021).
3. Piel FB, Hay SI, Gupta S, Weatherall DJ, Williams TN. Global burden of sickle cell anaemia in children under five, 2010-2050: modelling based on demographics, excess mortality, and interventions. *PLoS Med.* (2013) 10:e1001484. doi: 10.1371/journal.pmed.1001484
4. Brousse V, Makani J, Rees DC. Management of sickle cell disease in the community. *BMJ.* (2014) 348:g1765. doi: 10.1136/bmj.g1765
5. Leleu H, Arlet JB, Habibi A, Etienne-Julan M, Khellaf M, Adjibi Y, et al. Epidemiology and disease burden of sickle cell disease in France: a descriptive study based on a French nationwide claim database. *PLoS ONE.* (2021) 16:e0253986. doi: 10.1371/journal.pone.0253986
6. Gardner K, Bell C, Bartram JL, Allman M, Awogbade M, Rees DC, et al. Outcome of adults with sickle cell disease admitted to critical care - experience of a single institution in the UK. *Br J Haematol.* (2010) 150:610–3. doi: 10.1111/j.1365-2141.2010.08271.x
7. Rees DC. To begin at the beginning: sickle cell disease in Africa. *Lancet Haematol.* (2014) 1:e50–51. doi: 10.1016/S2352-3026(14)00006-4
8. Colombatti R, Palazzi G, Masera N, Notarangelo LD, Bonetti E, Samperi P, et al. Hydroxyurea prescription, availability and use for children with sickle cell disease in Italy: Results of a National Multicenter survey. *Pediatr Blood Cancer*. (2018) 65:26774. doi: 10.1002/pbc.26774
9. Chou ST, Alsawas M, Fasano RM, Field JJ, Hendrickson JE, Howard J, et al. American Society of Hematology 2020 guidelines for sickle cell disease: transfusion support. *Blood Adv.* (2020) 4:327–55. doi: 10.1182/bloodadvances.2019001143
10. Forni GL, Finco G, Graziadei G, Balocco M, Rigano P, Perrotta S, et al. Development of interactive algorithm for clinical management of acute events related to sickle cell disease in emergency department. *Orphanet J Rare Dis.* (2014) 9:91. doi: 10.1186/1750-1172-9-91
11. Società Italiana Talassemie ed Emoglobinopatie – Società and Italiana di Medicina Trasfusionale. *Raccomandazioni per le strategie trasfusionali nelle Emoglobinopatie n.3 [Italian]*. Bonomo P, Carta MP, Forni GL, Prati D, Rigano P, Vassanelli A, editors. Bologna: SITE (2014). Available online at: www.SITE.italia.org (accessed June 16, 2021).
12. Lottenberg R, Hassell KL. An evidence-based approach to the treatment of adults with sickle cell disease. *Hematology Am Soc Hematol Educ Program.* (2005) 58–65. doi: 10.1182/asheducation-2005.1.58
13. Davis BA, Allard S, Qureshi A, Porter JB, Pancham S, Win N, et al. Guidelines on red cell transfusion in sickle cell disease. Part I: principles and laboratory aspects. *Br J Haematol.* (2017) 176:179–91. doi: 10.1111/bjh.14346
14. Davis BA, Allard S, Qureshi A, Porter JB, Pancham S, Win N, et al. Guidelines on red cell transfusion in sickle cell disease Part II: indications for transfusion. *Br J Haematol.* (2017) 176:192–209. doi: 10.1111/bjh.14383

15. Casale M, Casciana ML, Ciliberti A, Colombatti R, Del Vecchio GC, Fasoli S, et al. *Raccomandazioni per la gestione della Malattia Drepanocitica in Età Pediatrica in Italia [Italian].* (2018). Available online at: www.aieop.org (accessed June 16, 2021).
16. Russo G, De Franceschi L, Colombatti R, Rigano P, Perrotta S, Voi V, et al. Current challenges in the management of patients with sickle cell disease - a report of the Italian experience. *Orphanet J Rare Dis.* (2019) 14:120. doi: 10.1186/s13023-019-1099-0
17. Carrara P, Balocco M, Pinto V, Olcese F, Soldà A, Strada P, Forni GL. Manual erythroexchange for chronic transfusion therapy in patients with sickle cell syndromes unresponsive to hydroxyurea: A long-term follow-up. *Am J Hematol.* (2010) 85:974. doi: 10.1002/ajh.21895
18. Conrath S, Vantilcke V, Parisot M, Maire F, Selles P, Elenga N. Increased prevalence of alloimmunization in sickle cell disease? Should we restore blood donation in French Guiana? *Front Med.* (2021) 8:681549. doi: 10.3389/fmed.2021.681549
19. Linder GE, Chou ST. Red cell transfusion and alloimmunization in sickle cell disease. *Haematologica.* (2021) 106:1805–15. doi: 10.3324/haematol.2020.270546
20. Russo-Mancuso G, Sciotto A, Munda SE, Romano V, Schilirò G. Alloimmunization and autoimmunity in Caucasian patients with sickle cell disease. *Int J Pediatr Hematol Oncol.* (1998) 5:443–7.
21. Russo-Mancuso G, La Spina M, Schiliro G. The changing profile of sickle cell disease in Italy. *Eur J Epidemiol.* (2003) 18:923–4. doi: 10.1023/A:1025635118305
22. Rigano P, De Franceschi L, Sainati L, Piga A, Piel FB, Cappellini MD, et al. Real-life experience with hydroxyurea in sickle cell disease: a multicenter study in a cohort of patients with heterogeneous descent. *Blood Cells Mol Dis.* (2018) 69:82–9. doi: 10.1016/j.bcmd.2017.08.017
23. De Franceschi L, Lux C, Piel FB, Gianesin B, Bonetti F, Casale M, et al. Access to emergency departments for acute events and identification of sickle cell disease in refugees. *Blood.* (2019) 133:2100–3. doi: 10.1182/blood-2018-09-876508
24. Alli NA, Patel M, Alli HD, Bassa F, Coetzee MJ, Davidson A, et al. Recommendations for the management of sickle cell disease in South Africa. *S Afr Med J.* (2014) 104:743–51. doi: 10.7196/SAMJ.8470
25. Società Italiana Talassemie ed Emoglobinopatie. *Raccomandazioni per la gestione del paziente adulto affetto da anemia falciforme [Italian].* In: De Franceschi L, Graziadei G, Rigano P, Cianciulli P, Forni GL, editors. Bologna: SITE (2014). Available online at: www.SITE.italia.org (accessed June 16, 2021).
26. Yawn BP, Buchanan GR, Afenyi-Annan AN, Ballas SK, Hassell KL, James AH, et al. Management of sickle cell disease: summary of the 2014 evidence-based report by expert panel members. *JAMA.* (2014) 312:1033–48. doi: 10.1001/jama.2014.10517
27. Qureshi A, Kaya B, Pancham S, Keenan R, Anderson J, Akanni M, et al. Guidelines for the use of hydroxycarbamide in children and adults with sickle cell disease: a British Society for Haematology Guideline. *Br J Haematol.* (2018) 181:460–75. doi: 10.1111/bjh.15235
28. Sins JW, Biemond BJ, Van Den Bersselaar SM, Heijboer H, Rijneveld AW, Cnossen MH, et al. Early occurrence of red blood cell alloimmunization in patients with sickle cell disease. *Am J Hematol.* (2016) 91:763–9. doi: 10.1002/ajh.24397
29. Giambona A, Damiani G, Vinciguerra M, Jakil C, Cannata M, Cassara F, et al. Incidence of haemoglobinopathies in Sicily: the impact of screening and prenatal diagnosis. *Int J Clin Pract.* (2015) 69:1129–38. doi: 10.1111/ijcp.12628
30. Società Italiana Talassemie ed Emoglobinopatie. *Raccomandazioni per lo screening neonatale nelle sindromi falciformi [Italian].* In: De Franceschi L, Russo G, Sainati L, editors. Bologna: SITE (2017). Available online at: www.SITE.italia.org (accessed June 16, 2021).
31. Piel FB, Tatem AJ, Huang Z, Gupta S, Williams TN, Weatherall DJ. Global migration and the changing distribution of sickle haemoglobin: a quantitative study of temporal trends between 1960 and 2000. *Lancet Glob Health.* (2014) 2:e80–89. doi: 10.1016/S2214-109X(13)70150-5
32. Shet AS, Thein SL. A growing population of older adults with sickle cell disease. *Clin Geriatr Med.* (2019) 35:349–67. doi: 10.1016/j.cger.2019.03.006
33. Tumburu L, Ghosh-Choudhary S, Seifuddin FT, Barbu EA, Yang S, Ahmad MM, et al. Circulating mitochondrial DNA is a proinflammatory DAMP in sickle cell disease. *Blood.* (2021) 137:3116–26. doi: 10.1182/blood.2020009063
34. Ware RE, De Montalembert M, Tshilolo L, Abboud MR. Sickle cell disease. *Lancet.* (2017) 390:311–23. doi: 10.1016/S0140-6736(17)30193-9
35. Rees DC, Robinson S, Howard J. How I manage red cell transfusions in patients with sickle cell disease. *Br J Haematol.* (2018) 180:607–17. doi: 10.1111/bjh.15115
36. Swerdlow PS. Red cell exchange in sickle cell disease. *Hematology Am Soc Hematol Educ Program.* (2006) 48–53. doi: 10.1182/asheducation-2006.1.48
37. Biller E, Zhao Y, Berg M, Boggio L, Capocelli KE, Fang DC, et al. Red blood cell exchange in patients with sickle cell disease-indications and management: a review and consensus report by the therapeutic apheresis subsection of the AABB. *Transfusion.* (2018) 58:1965–72. doi: 10.1111/trf.14806
38. Brugnara C, De Franceschi L. Effect of cell age and phenylhydrazine on the cation transport properties of rabbit erythrocytes. *J Cell Physiol.* (1993) 154:271–80. doi: 10.1002/jcp.1041540209
39. De Franceschi L, Corrocher R. Established and experimental treatments for sickle cell disease. *Haematologica.* (2004) 89:348–56.
40. Matte A, Recchiuti A, Federti E, Koehl B, Mintz T, El Nemer W, et al. Resolution of sickle cell disease-associated inflammation and tissue damage with 17R-resolvin D1. *Blood.* (2019) 133:252–65. doi: 10.1182/blood-2018-07-865378
41. Matte A, Zorzi F, Mazzi F, Federti E, Olivieri O, De Franceschi L. New therapeutic options for the treatment of sickle cell disease. *Mediterr J Hematol Infect Dis.* (2019) 11:e2019002. doi: 10.4084/mjhid.2019.002
42. Matte A, Cappellini MD, Iolascon A, Enrica F, De Franceschi L. Emerging drugs in randomized controlled trials for sickle cell disease: are we on the brink of a new era in research and treatment? *Expert Opin Investig Drugs.* (2020) 29:23–31. doi: 10.1080/13543784.2020.1703947
43. Park SY, Matte A, Jung Y, Ryu J, Anand WB, Han EY, et al. Pathologic angiogenesis in the bone marrow of humanized sickle cell mice is reversed by blood transfusion. *Blood.* (2020) 135:2071–84. doi: 10.1182/blood.2019002227
44. Valenti MT, Mattè A, Federti E, Puder M, Anez-Bustillos L, Deiana M, et al. Dietary ω-3 fatty acid supplementation improves murine sickle cell bone disease and reprograms adipogenesis. *Antioxidants.* (2021) 10:799. doi: 10.3390/antiox10050799
45. Hebbel RP, Vercellotti G, Nath KA. A systems biology consideration of the vasculopathy of sickle cell anemia: the need for multi-modality chemoprophylaxsis. *Cardiovasc Hematol Disord Drug Targets.* (2009) 9:271–92. doi: 10.2174/1871529X10909040271
46. De Franceschi L, Cappellini MD, Olivieri O. Thrombosis and sickle cell disease. *Semin Thromb Hemost.* (2011) 37:226–36. doi: 10.1055/s-0031-1273087
47. Sparkenbaugh E, Pawlinski R. Interplay between coagulation and vascular inflammation in sickle cell disease. *Br J Haematol.* (2013) 162:3–14. doi: 10.1111/bjh.12336
48. Telen MJ. Beyond hydroxyurea: new and old drugs in the pipeline for sickle cell disease. *Blood.* (2016) 127:810–9. doi: 10.1182/blood-2015-09-618553
49. Telen MJ, Malik P, Vercellotti GM. Therapeutic strategies for sickle cell disease: towards a multi-agent approach. *Nat Rev Drug Discov.* (2019) 18:139–58. doi: 10.1038/s41573-018-0003-2
50. Delgado J, Voltz C, Stain M, Lapvetelainen T, Urach S, Lahteenvuo J, et al. The European Medicines Agency review of crizanlizumab for the prevention of recurrent vaso-occlusive crises in patients with sickle cell disease. *Hemasphere.* (2021) 5:e604. doi: 10.1097/HS9.0000000000000604
51. Karki NR, Kutlar A. P-selectin blockade in the treatment of painful vaso-occlusive crises in sickle cell disease: a spotlight on crizanlizumab. *J Pain Res.* (2021) 14:849–56. doi: 10.2147/JPR.S278285
52. Weaver SB, Rungkitwattanakul D, Singh D. Contemporary management and prevention of vaso-occlusive crises (VOCs) in adults with sickle cell disease. *J Pharm Pract.* (2021) 8971900211026644. doi: 10.1177/08971900211026644. [Epub ahead of print].
53. Tormey CA, Hendrickson JE. Transfusion-related red blood cell alloantibodies: induction and consequences. *Blood.* (2019) 133:1821–30. doi: 10.1182/blood-2018-08-833962

54. Hendrickson JE. Red blood cell alloimmunization and sickle cell disease: a narrative review on antibody induction. *Ann Blood.* (2020) 5:33. doi: 10.21037/aob-2020-scd-01
55. Chou ST, Jackson T, Vege S, Smith-Whitley K, Friedman DF, Westhoff CM. High prevalence of red blood cell alloimmunization in sickle cell disease despite transfusion from Rh-matched minority donors. *Blood.* (2013) 122:1062–71. doi: 10.1182/blood-2013-03-490623
56. Karafin MS, Westlake M, Hauser RG, Tormey CA, Norris PJ, Roubinian NH, et al. Risk factors for red blood cell alloimmunization in the Recipient Epidemiology and Donor Evaluation Study (REDS-III) database. *Br J Haematol.* (2018) 181:672–81. doi: 10.1111/bjh.15182
57. Yazdanbakhsh K, Ware RE, Noizat-Pirenne F. Red blood cell alloimmunization in sickle cell disease: pathophysiology, risk factors, and transfusion management. *Blood.* (2012) 120:528–37. doi: 10.1182/blood-2011-11-327361
58. Fasano RM, Booth GS, Miles M, Du L, Koyama T, Meier ER, et al. Red blood cell alloimmunization is influenced by recipient inflammatory state at time of transfusion in patients with sickle cell disease. *Br J Haematol.* (2015) 168:291–300. doi: 10.1111/bjh.13123
59. Nickel RS, Horan JT, Fasano RM, Meyer E, Josephson CD, Winkler AM, et al. Immunophenotypic parameters and RBC alloimmunization in children with sickle cell disease on chronic transfusion. *Am J Hematol.* (2015) 90:1135–41. doi: 10.1002/ajh.24188
60. Lin Y, Saskin A, Wells RA, Lenis M, Mamedov A, Callum J, et al. Prophylactic RhCE and Kell antigen matching: impact on alloimmunization in transfusion-dependent patients with myelodysplastic syndromes. *Vox Sang.* (2017) 112:79–86. doi: 10.1111/vox.12455
61. Hendrickson JE, Fasano RM. Management of hemolytic transfusion reactions. *Hematol Am Soc Hematol Educ Program.* (2021) 704–09. doi: 10.1182/hematology.2021000308

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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Medicine

ORIGINAL RESEARCH
published: 13 April 2022
doi: 10.3389/fmed.2022.858911

Assessment of Reticulocyte and Erythrocyte Parameters From Automated Blood Counts in Vaso-Occlusive Crisis on Sickle Cell Disease

*Guillaume Feugray[1], Fiston Kasonga[2], Maximilien Grall[3], Ygal Benhamou[4], Victor Bobée-Schneider[5], Gérard Buchonnet[5], Sylvie Daliphard[5], Véronique Le Cam Duchez[6], Agnès Lahary[5] and Paul Billoir[6]**

[1] General Biochemistry, Normandie Univ, Rouen University, INSERM U1096, CHU Rouen, Rouen, France, [2] CHU Rouen, Vascular Hemostasis Unit, Rouen, France, [3] Department of Internal Medecine, CHU Rouen, Rouen, France, [4] Department of Internal Medecine, Normandie Univ, Rouen University, INSERM U1096, CHU Rouen, Rouen, France, [5] CHU Rouen, Hematology Laboratory, Rouen, France, [6] Normandie Univ, Rouen University, INSERM U1096, CHU Rouen, Vascular Hemostasis Unit, Rouen, France

OPEN ACCESS

Edited by:
Hollie Marie Reeves,
University Hospitals Cleveland Medical Center, United States

Reviewed by:
Mervat Mattar,
Cairo University, Egypt
Mohsen Saleh Elalfy,
Ain Sham University, Egypt

***Correspondence:**
Paul Billoir
paul.billoir@chu-rouen.fr
orcid.org/0000-0001-5632-7713

Specialty section:
This article was submitted to Hematology, a section of the journal Frontiers in Medicine

Received: 20 January 2022
Accepted: 21 March 2022
Published: 13 April 2022

Citation:
Feugray G, Kasonga F, Grall M, Benhamou Y, Bobée-Schneider V, Buchonnet G, Daliphard S, Le Cam Duchez V, Lahary A and Billoir P (2022) Assessment of Reticulocyte and Erythrocyte Parameters From Automated Blood Counts in Vaso-Occlusive Crisis on Sickle Cell Disease. Front. Med. 9:858911. doi: 10.3389/fmed.2022.858911

Sickle cell disease is a complex genetic disease involving cell adhesion between red blood cells, white blood cells, platelets and endothelial cells, inducing painful vaso-occlusive crisis (VOC). We assessed reticulocyte and erythrocyte parameters in a cohort of confirmed SCD patients, and investigated whether a combination of these routine laboratory biomarkers of haemolysis could be used to predict VOC development. Reticulocyte and erythrocyte parameters were evaluated using the Sysmex XN-9000 analyser. A total of 98 patients with SCD were included, 72 in steady state and 26 in VOC. Among the 72 patients in steady state, 22 developed a VOC in the following year (median: 3 months [2–6]). The following parameters were increased in SCD patients with VOC development compared to SCD patients without VOC development in the following year: reticulocyte count (94.6 10^9/L [67.8–128] vs. 48.4 10^9/L [24.9–87.5]), immature reticulocyte count (259 10^9/L [181–334] vs. 152 10^9/L [129–208]) reticulocyte/immature reticulocyte fraction (IRF) ratio (6.63 10^9/(L*%) [4.67–9.56] vs. 4.94 10^9/(L*%) [3.96–6.61]), and medium fluorescence reticulocytes (MFR) (19.9% [17.4–20.7] vs. 17.1% [15.95–19.75]). The association of a reticulocyte count of >189.4 10^9/L and an MFR of >19.75% showed a sensitivity of 81.8% and a specificity of 88% to predict VOC development in the following year. Based on our findings, a combination of routine laboratory biomarkers, as reticulocyte count, immature reticulocyte count and fluorescent reticulocyte fraction at steady state, could be used to predict VOC development in SCD.

Keywords: sickle cell disease, vaso-occlusive crisis, reticulocyte count, erythrocyte parameters, immature reticulocyte fraction

INTRODUCTION

Sickle cell disease (SCD) is an inherited haemoglobinopathy disorder caused by mutations in *HBB* gene with amino-acid substitution on β globin chain. The consequence is synthesis of altered hemoglobin S (HbS) which polymerises in red blood cell (RBC) at deoxygenated state. SCD is associated with chronic haemolytic anemia, vaso-occlusive crisis (VOC) leading to frequent hospitalization, morbidity and mortality caused by organ failure like stroke, acute chest syndrome (ACS), osteonecrosis, leg ulcers, retinopathy, pulmonary hypertension, priapism and nephropathy (1). Moreover, hypercoagulability state is reported in SCD with increased venous thromboembolism (VTE) or pulmonary embolism (PE) (2).

Automated hematology analysers provide whole blood counts with increasing reliability and accuracy (3). However, in daily laboratory practice, no analyser can determine properly RBC morphological abnormalities. These criteria are not always relevant hence the interest to combine several RBC and reticulocyte parameters in order to improve the specificity and sensitivity of microscope examination of a blood smear (4). The introduction of additional parameters in the past years has opened new opportunities for red cell evaluation. The evaluation of automatic parameters of reticulocyte fraction has been reported to be useful in the screening of haemolytic anemia. In Sysmex analysers, reticulocytes are fractionated in low (LFR), medium (MFR) and high fluorescence reticulocytes (HFR). Reticulocytes can be classified according to RNA content into subtypes reflecting successive stages during maturation: immature with HFR to MFR and mature with LFR (5). Whole reticulocyte fraction provides information on bone marrow recovery. However, few studies have evaluated these parameters in sickle cell disease and VOC development (3, 4, 6).

The aim of the study was to assess reticulocyte and erythrocyte parameters in a cohort of confirmed SCD patients, and to investigate whether a combination of these routine laboratory biomarkers of haemolysis could be used to predict VOC development.

METHODS

Study Design and Patients

All patients in the study were diagnosed and treated for SCD at Rouen University Hospital between September 2018 and June 2021. Patients were included during injury evaluation or VOC hospitalization in our tertiary center. The injury evaluation was a standard of care, every year. Patients with VOC were included <24 h after admission to emergency department. All patients received a systematic annual consultation for injury evaluation and determined VOC development.

Prospective data were collected and completed from medical records. Clinical information included age and sex. Pregnant women and patients <18 years were excluded.

The study was performed in accordance with the Declaration of Helsinki on biomedical research involving human subjects. The institutional review board (Rouen University Hospital) approved the study (Authorization protocol number: E2021-78).

Samples and Analysis

Biological standard follow-up included sampling of dipotassium EDTA tubes (BD Vacutainer EDTA, Plymouth) for whole blood counts and plasma from lithium heparin tubes with gel separator (BD Vacutainer LH, Plymouth) for biochemical parameters.

The severity of haemolysis was estimated using plasma lactate dehydrogenase level (LDH). These tests were performed in samples collected for routine follow-up. Whole blood counts were measured on XN-9000 (Sysmex, Villepinte, France). LDH, creatinine, ferritin and indirect bilirubin levels were determined on cobas® 8000 chemistry analyser (Roche Diagnostics, Mannheim, Germany).

Reticulocyte and Erythrocyte Measurement

RBC count was measured using the impedance variation method after hydrodynamic focusing. SCD samples were analyzed in the RET (reticulocytes) channel and classified as per the cell blood count optic (CBC-O) application developed by Sysmex™ that is embedded in the Extended Information Processing Unit (Extended-IPU) (7). Reticulocyte measurement was based on the principle of fluorescence flow cytometry using the nucleic acid dye oxazine 750 which stains the RNA of the cell (8). According to their stage of maturity, reticulocytes have varying fluorescence intensity, and accordingly, they are divided into three subtypes: mature reticulocytes with limited fluorescence are called low-fluorescence reticulocytes (LFR), intermediately mature reticulocytes with medium fluorescence are called medium-fluorescence reticulocytes (MFR), and very immature reticulocytes with high fluorescence are called high-fluorescence reticulocytes (HFR). The immature reticulocyte fraction (IRF), is the percentage of immature reticulocytes calculated as the sum of MFR and HFR and is a reflection of erythropoietic activity (9). Analysis were performed before any transfusion who potentially change results. Analysis were performed only one time in patients.

Parameters of interest provided by reticulocyte and erythrocyte measurement are (10):

- Hemoglobin (Hb), Mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC).

Abbreviations: SCD, sickle cell disease; HbS, hemoglobin S; RBC, red blood cells; VOC, vaso occlusive crisis; ACS, acute chest syndrome; VTE, venous thromboembolism; PE, pulmonary embolism; LFR, low fluorescence reticulocytes; MFR, medium fluorescence reticulocytes; HFR, high fluorescence reticulocytes; LDH, lactate dehydrogenase; CBC-O, cell blood count optic; Extended-IPU, Extended Information Processing Unit; Hb, Hemoglobin; MCV, Mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; IRF, Immature reticulocyte fraction; Ret-He, Hemoglobin concentration in reticulocytes; %MicroR, percentage of microcytic erythrocytes; %Macro-R, percentage of macrocytic erythrocytes; IQR, interquartile ranges; ROC, Receiver operating characteristic; Hypo-He, hypo-hemoglobin content in erythrocytes; Hyper-He, hyper-hemoglobin content in erythrocytes; Ret-He, Hemoglobin concentration in reticulocytes.

- Immature red cells: Reticulocytes, IRF, LFR, MFR, HFR, hemoglobin concentration in reticulocytes (Ret-He), part of young reticulocytes (reticulocytes/IRF).
- Hypo-He and Hyper-He corresponding to red blood cells with hypo-hemoglobin content (<17 pg) and hyper-hemoglobin content (>49 pg), respectively.
- The percentage of microcytic erythrocytes (%Micro-R) and macrocytic erythrocytes (%Macro-R).

Statistical Analysis

Data are expressed as median and interquartile ranges [IQR]. Statistical analyses were performed with GraphPad Prism for Windows, version 9.2. (GraphPad Software, San Diego, California, United States). Fisher exact test, Kruskall–Wallis ANOVA with Dunn's multiple comparisons post-test, Mann-Whitney test or Chi square test were used. Receiver operating characteristic (ROC) curves were built for significant clinical characteristics. $P < 0.05$ were considered to be statistically significant.

RESULTS

Demographic Characteristics

A total of 98 patients with SCD were included in this study, 72 in steady state and 26 during VOC. Demographic and clinical characteristics are reported in **Table 1**. Forty-three patients had homozygous SCD (S/S) or heterozygous SCD and β^0 thalassaemia (S/β^0), 26 had homozygous SCD with $\alpha^{3.7}$ thalassaemia (S/S$\alpha^{3.7}$), 29 had heterozygous SCD with C hemoglobin (S/C) or β^+ thalassaemia (S/β^+). All genotypes were represented in VOC. In the 72 patients in steady state, 22 developed a VOC during the following year (median: 3 months [2–6]).

We observed more splenectomy in the VOC group ($p = 0.04$) and significant differences between patients included during VOC and patients included in steady state, a decrease of RBC ($p = 0.017$), and an increase of MCV ($p = 0.026$). In whole blood count, we observed during VOC, significant increases of leukocytes ($p < 0.001$), neutrophils ($p = 0.0033$), lymphocytes ($p = 0.014$) and monocytes ($p = 0.0048$). Moreover, in biochemistry parameters, in the VOC group, LDH was significantly increased ($p = 0.046$) and creatinine significantly decreased ($p = 0.0065$).

TABLE 1 | Characteristics of study population.

	SCD at steady state $n = 72$	SCD in VOC $n = 26$	*P*
Clinical characteristics			
Age (years)	35.1 ± 12.5	30.7 ± 9.3	0.067
Male *n* (%)	31 (43)	12 (46)	0.82
Hydroxyurea *n* (%)	39 (54)	19 (73)	0.11
Osteonecrosis *n* (%)	23 (32)	3 (11.5)	0.067
Retinopathy *n* (%)	13 (18)	9 (34.6)	0.10
Vasculopathy *n* (%)	9 (12.5)	5 (19.2)	0.51
ACS *n* (%)	20 (27.7)	7 (26.9)	>0.99
Cholecystectomy *n* (%)	28 (38.9)	7 (26.9)	0.34
Splenectomy *n* (%)	2 (2.8)	4 (15.3)	**0.04**
Hematological parameters			
RBC (10^{12}/L)	3.13 [2.67–3.97]	2.73 [2.39–3.18]	**0.017**
Hemoglobin (g/dL)	9.25 [8–10.5]	8.8 [8–10.1]	0.41
Haematocrit (%)	28 [23–30.7]	25 [23–29]	0.28
MCV (fL)	82 [73.6–91.1]	91.7 [79.8–101.3]	**0.026**
MCHC (g/dL)	34.7 [33.0–35.7]	35.1 [34.3–36.0]	0.08
Platelets (10^9/L)	308 [180–391]	328 [258–451]	0.11
Leukocytes (10^9/L)	7.1 [5.73–9.78]	10.7 [7.7–12.5]	**<0.001**
Neutrophils (10^9/L)	3.94 [2.90–5.64]	5.60 [3.99–7.71]	**0.0033**
Lymphocytes (10^9/L)	2.23 [1.53–2.98]	2.68 [2.16–3.59]	**0.014**
Monocytes (10^9/L)	0.69 [0.48–1.00]	0.89 [0.59–1.31]	**0.0048**
HbF (%)	4.9 [2.6–11.3]	11.1 [2.5–17.6]	0.36
Biochemistry parameters			
Indirect bilirubin (μmol/L)	9 [8-11]	10 [8-11]	0.72
LDH (U/L)	347 [230–448]	406 [345–499]	**0.046**
Ferritin (μg/L)	135 [52–282]	83 [58–108]	0.59
Creatinine (μmol/L)	59 [53-78]	49 [41-61]	**0.0065**

Data are expressed as median and [IQR] except for age (mean ± SD) and clinical characteristics with n is the total number of patients (%), ACS, acute chest syndrome; HbF, hemoglobin F; RBC, red blood cells; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; LDH, lactate deshydrogenase. Bold values represent significant differences between the groups.

Reticulocyte and Erythrocyte Variations During VOC

Reticulocyte and erythrocyte parameters are reported in **Table 2**. Briefly, SCD patients with VOC had significantly higher reticulocyte counts ($p = 0.0073$), higher immature reticulocyte ratio (reticulocytes/RET-IRF, $p = 0.004$) and increased Hyper-He ($p = 0.047$). No other parameters had significant differences.

Reticulocyte and Erythrocyte Variations in Patients Developing VOC

We determined which SCD patients included at steady state were hospitalized for VOC ($n = 22$) in the following year. Results are reported in **Table 3** and in **Supplemental Table 1** between genotype. Genotype was not associated with VOC development (Chi Square, $p = 0.08$). In patients with VOC development, RBC were significantly decreased ($p = 0.03$), leukocytes and neutrophils were increased ($p = 0.0068$ and $p = 0.005$, respectively) (**Figure 1**). Moreover, significant increases were observed for reticulocyte count ($p = 0.0026$), immature reticulocyte count ($p = 0.0048$), reticulocytes/IRF ratio ($p = 0.018$) and MFR ($p = 0.0189$).

VOC Prediction With Reticulocyte and IRF Measurement

Then, we determined with a ROC curve, the risk of developing VOC: a reticulocyte count of >189.4 10^9/L (AUC: 0.70, sensitivity: 77.3%, specificity: 64%), an MFR of >19.75% (AUC: 0.67, sensibility: 50%, specificity: 75.5%), and an immature reticulocyte count of >68.6 10^9/L (AUC: 0.69, sensitivity: 77.3%, specificity: 63.3% respectively) (**Figure 2**).

The association of a reticulocyte count of >189.4 10^9/L and an MFR of >19.75% presented a sensitivity of 81.8% and a specificity

TABLE 2 | Reticulocyte and erythrocyte parameter comparison between steady state vs. VOC.

Parameters	Steady state $n = 72$	VOC $n = 26$	*p* value
Reticulocytes (10^9/L)	184 [138–286]	276 [181–385]	**0.0073**
Reticulocytes (%)	6.06 [3.2–10.0]	9.62 [6.05–13.7]	**0.0038**
RET-IRF (%)	35.1 [28.7–40.6]	35.3 [26.6–42.9]	0.94
RET-IRF value (10^9/L)	65.9 [40.4–113.4]	87 [52.6–147.4]	0.056
RET-He (pg)	31.5 [28.2–34.3]	32.9 [29.9–35.2]	0.31
Reticulocytes/RET-IRF (10^9/(L*%))	5.52 [4.09–7.4]	7.77 [5.04–10.5]	**0.004**
Hypo-He (%)	5.2 [1.8–12.3]	1.8 [1.1–5.5]	0.053
Hyper-He (%)	0.50 [0.30–0.80]	0.80 [0.40–3.2]	**0.047**
Micro-R (%)	13.6 [5.25–26.5]	7.4 [3.0–16.2]	0.052
Macro-R (%)	3.9 [3.2–6.0]	5.1 [3.7–8.8]	0.061
LFR (%)	65.3 [59.6–71.5]	66.1 [59.6–71.0]	0.77
MFR (%)	18.0 [16.1–20.1]	18.1 [16.8–19.9]	0.94
HFR (%)	17.1 [11.8–20.8]	15.5 [11.7–21.2]	0.62

Data are expressed as median ± [IQR], n is the total number of patients. LFR: low fluorescent reticulocyte. MFR, Medium fluorescent reticulocyte; HFR, High fluorescent reticulocyte; VOC, Vaso-occlusive crisis. Bold values represent significant differences between the groups.

of 88% to predict VOC development in the following year in patients with SCD at steady state (**Figure 3**).

DISCUSSION

Our study aimed to demonstrate a correlation between routine laboratory biomarkers of haemolysis as reticulocyte and erythrocyte parameters and the occurrence of VOC in SCD. The association between reticulocyte count and MFR had a good correlation with VOC development.

Sickle cell disease is a lifelong blood disorder affecting ∼100,000 people in the United States (11, 12). SCD is a complex genetic disease involving cell adhesion between red blood cells, white blood cells, platelets and endothelial cells, inducing painful VOC (13). Vaso-occlusive crises are characterized by haemolytic anemia, endothelial damage, and potentially life-threatening complications (14). Acute pain is the primary cause of hospitalization in SCD.

Inflammatory syndrome is described in SCD during ACS and VOC (15). Yildrim et al. demonstrated that whole blood count and C-reactive protein at admission could distinguish ACS and VOC (15). In our study, patients with VOC at admission had higher counts of neutrophils and monocytes.

An important disease mechanism involves the release of hemoglobin into the circulation during intravascular haemolysis (14). The release of hemoglobin into the plasma during haemolysis potently inhibits endothelial nitric oxide signaling, leading to endothelial cell dysfunction and nitric oxide resistance (16). Studies have shown correlations between the rate of haemolysis and levels of platelet activation and procoagulant factors in the blood (17). The concentration of LDH has long been recognized as an accurate measure of intravascular haemolysis in SCD (18, 19). In our study, we observed an increase of LDH during VOC. However, this biochemical parameter did not allow to predict VOC development in the following year.

TABLE 3 | Comparison of biologic parameters in SCD patients developing or not a VOC in the following year.

	SCD without VOC development $n = 50$	SCD with VOC development $n = 22$	*P*
RBC (10^{12}/L)	3.36 [2.86–4.27]	2.95 [2.55–3.77]	**0.030**
Hemoglobin (g/dL)	9.8 [8.15–10.58]	8.4 [7.75-10.25]	0.12
Haematocrit (%)	28 [23.5–31]	24 [22–29.3]	0.087
MCV (fL)	76.3 [72.1-89.2]	83.6 [79.7-95.0]	0.082
MCHC (g/dL)	34.6 [32.9–35.7]	35.0 [33.6–35.6]	0.49
Platelets (10^9/L)	302 [168.5–382]	352.5 [238.5–434]	0.083
Leukocytes (10^9/L)	6.4 [5.15–8.65]	9.35 [7.05–10.2]	**0.0068**
Neutrophils (10^9/L)	3.51 [2.66–4.72]	5.61 [3.94–6.74]	**0.005**
Lymphocytes (10^9/L)	2.16 [1.45–2.87]	2.31 [1.67–3.10]	0.29
Monocytes (10^9/L)	0.61 [0.44–0.92]	0.73 [0.55–1.18]	0.09
Reticulocytes (10^9/L)	152 [129–208]	259 [181–334]	**0.0026**
Reticulocytes (%)	4.80 [3.09–8.0]	9.39 [6.65–11.4]	**0.0027**
RET-IRF (%)	33.8 [28.2–39.6]	38.7 [32.8–40.9]	0.16
RET-IRF value (10^9/L)	48.4 [34.9–87.5]	94.6 [67.8–128]	**0.0048**
RET-He (pg)	30.1 [26.5–32.5]	32.5 [31.2–34.7]	0.066
Reticulocytes/RET-IRF (10^9/(L*%))	4.94 [3.69–6.61]	6.62 [4.67–9.56]	**0.012**
HbF (%)	3.5 [1.6–6.6]	10 [4.7–14.0]	**0.01**
Hypo-He (%)	6.4 [2.0–12.8]	4.0 [1.6–7.35]	0.34
Hyper-He (%)	0.40 [0.30–0.60]	0.60 [0.40–0.90]	0.067
Micro-R (%)	16.3 [5.7–29.8]	11.3 [5.2–17.2]	0.27
Macro-R (%)	3.6 [3.05–5.05]	4.1 [3.65–7.2]	0.10
LFR (%)	66.3 [61–72.2]	61 [59–65.6]	0.058
MFR (%)	17.1 [15.95–19.75]	19.9 [17.4–20.7]	**0.018**
HFR (%)	16 [11.05–20.7]	19 [13.6–20.9]	0.37
LDH (U/L)	323 [221–422]	410 [264–487]	0.11

Data are expressed as median and [IQR], n is the total number of patients. RBC, red blood cells; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; HbF, hemoglobin F; LFR, low fluorescent reticulocytes; MFR, Medium fluorescent reticulocytes; HFR, High fluorescent reticulocytes; VOC, vaso-occlusive crisis. Bold values represent significant differences between the groups.

Several routine automated parameters have been developed to evaluate reticulocyte and RBC counts. In SCD, an increase of reticulocyte count is observed in S/S in comparison of S/C (20). In child, S/S patients had most cases of hospital admissions and VOC (21). The reticulocyte count reflects the erythropoietic activity of bone marrow and is thus useful in both diagnosing anemias and monitoring bone marrow response to therapy (22). A lower concentration of RNA in RBC indicates increased maturity, so that immature reticulocytes (HFR and MFR will be classified as a more mature fraction than LFR (10). Immature reticulocyte fraction provides information on bone marrow suppression and recovery of erythropoiesis

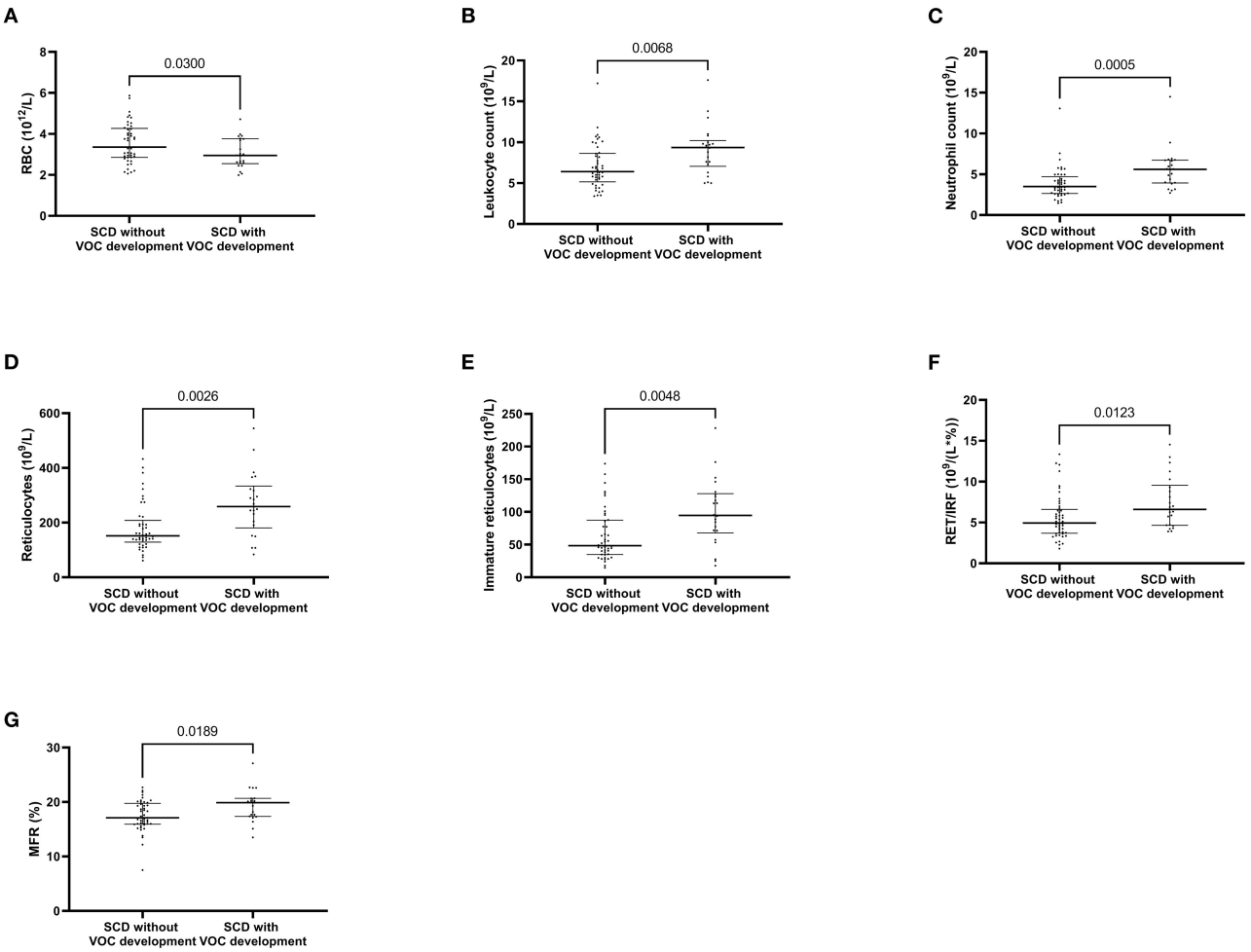

FIGURE 1 | Whole blood count and reticulocyte parameters associated with vaso-occlusive crisis development. Red blood cells **(A)** Leukocyte count **(B)** Neutrophil count **(C)** Reticulocytes **(D)** absolute value of immature reticulocytes **(E)** Part of immature reticulocyte fraction **(F)** medium fluorescent reticulocytes **(G)**. *P* values comparing clinical improvement to clinical worsening are from Mann-Whitney *U*-test.

(23). Erythrocytic parameters has been already used to help the diagnosis of haemoglobinopathy and membranopathy. Automated reticulocyte indices can differentiate hereditary from acquired spherocytosis (4, 6). A previous study, combining reticulocyte count, leukocyte count, and pain in the pelvis and spine, demonstrated a good negative predictive value for ACS risk in SCD (24). Another study demonstrated, with RBC, MCV and MCHC, the possibility of diagnostic orientation in SCD (25). Nivaggioni et al., demonstrated the interest of combining MicroR, MCHC and IRF to differentiate hereditary RBC diseases as spherocytosis and SCD (4). In our study, we observed an association of increased reticulocyte count and increased reticulocyte/IRF during crises. The variation of reticulocyte count was correlated with morbidity in SCD (26).

Laboratory measures can be a powerful supplement to genetic data in predicting morbidity and mortality in SCD (27, 28). Few parameters were associated with VOC prediction. Fetal hemoglobin increase, several β-globin haplotypes and coincidental α-thalassaemia are protective factors of VOC development (26). In children with SCD, ACS was associated with serum level of interleukin 8 and C-reactive protein (29).

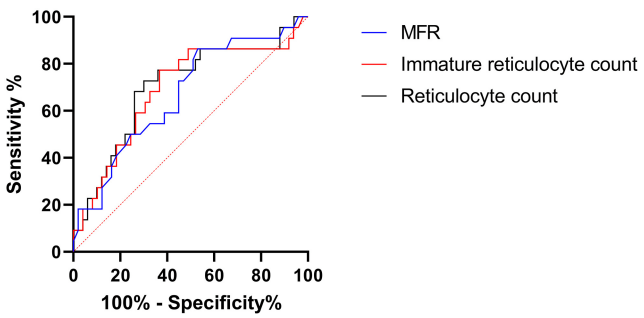

FIGURE 2 | Roc curve of vaso-occlusive crisis prediction by reticulocyte parameters. MFR, medium fluorescent reticulocytes. Immature reticulocyte count: absolute value of immature reticulocytes.

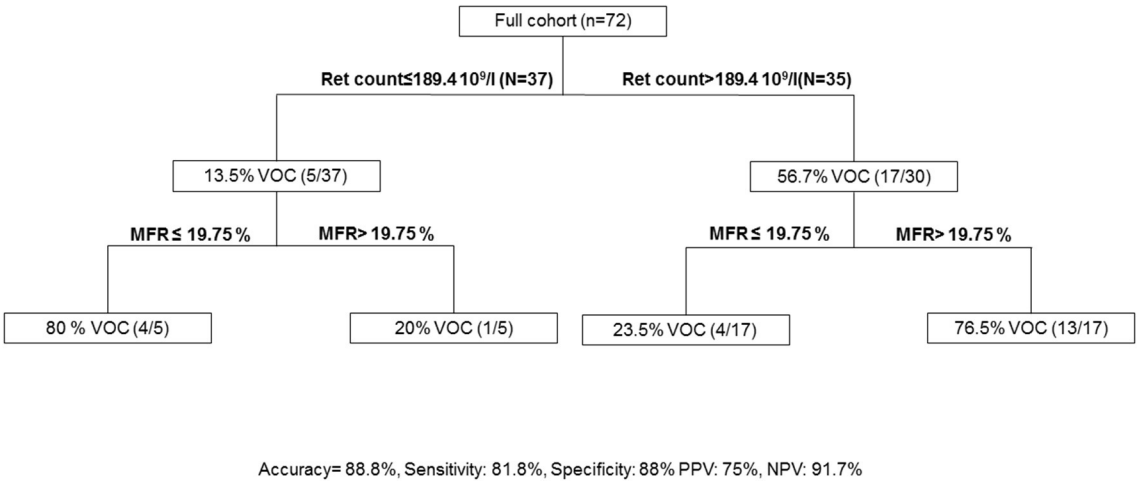

FIGURE 3 | Algorithm to predict vaso-occlusive crisis. MFR, medium fluorescent reticulocytes; Ret count; reticulocyte count; PPV, Positive Predictive Value; NPV, Negative Predictive Value; VOC, Vaso-occlusive crisis.

In adult patients with SCD, a decrease of interleukin 10 in steady state was associated with VOC (AUC ROC curve: 0.688). To our knowledge, our study is the first to evaluate clinical complications in SCD using the reticulocyte parameters. Interestingly, we have shown that a simple score combining reticulocyte count and MFR can be used to predict up to 88.8% of VOC development in the following year. Of note, we observed 17/22 patients with a reticulocyte counts of $>189.4\ 10^9$/L and 13/22 patients with an MFR of >19.75% suggesting the ability of these two parameters to predict clinical outcome.

The ability to predict the phenotype of an individual with sickle cell disease could guide therapeutic decision making. Standard treatment of SCD includes hydroxyurea. In patients with untreated severe SCD, markers of haemolysis were lower in the mild genotype groups regardless of treatment, but they were not lower in severe genotype patients receiving hydroxyurea or chronic transfusions (30). New therapeutic targets are being developed to limit VOC. Voxelotor is an HbS polymerization inhibitor that reversibly binds to hemoglobin to stabilize the oxygenated hemoglobin state (31). Patients treated with voxelotor showed small increases in Hb levels (1 g/dL) and decreased indicators of haemolysis (32). Moreover, a reduced incidence of crises over time was observed. Another treatment is P-selectin target with crizanlizumab (33). P-selectin is expressed on the surface of the endothelium and mediates abnormal rolling and static adhesion of sickle erythrocytes to the vessel surface *in vitro* (34). P-selectin inhibitor crizanlizumab was associated with a significantly lower frequency of sickle cell–related pain crises and appeared to be associated with a low rate of adverse effects (33). The interest of predicting VOC in SCD is to ensure the rapid start of treatment to limit VOC development. Our study has several limitations, including a small sample size. Another limitation was no inclusion of VOC development at home. However, hospitalized VOC are more severe. However, the aim of the study was to build a simple and easy-to-use score to predict VOC development in steady state SCD. Importantly, our score is ready to use in routine laboratory care. The validation of our predictive score is required to ensure the reproducibility of the developed model.

CONCLUSION

Based on our findings, a combination of routine laboratory biomarkers, as reticulocyte count, immature reticulocyte count and fluorescent reticulocyte fraction at steady state, could be used to predict VOC development in 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.

ETHICS STATEMENT

The studies involving human participants were reviewed and the Institutional Review Board (Rouen University Hospital) approved the study (Authorization protocol number: E2021-78). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

GF and FK performed the analysis and wrote the manuscript. MG, YB, and AL included patients and critically revised the manuscript and results. VB-S, GB, and SD critically revised the manuscript and results. VL discussed the obtained results and critically revised the manuscript. PB designed the research, analyzed, interpreted the data, and wrote the

manuscript. All authors read and approved the final version of the manuscript.

ACKNOWLEDGMENTS

The authors are grateful to Dr. Valery Brunel (PharmD., General Biochemistry Laboratory, Rouen University Hospital) for biochemistry dosage and to Nikki Sabourin-Gibbs, Rouen University Hospital, for her help in editing the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.858911/full#supplementary-material

REFERENCES

1. Toledo SL de O, Guedes JVM, Alpoim PN, Rios DRA, Pinheiro M de B. Sickle cell disease: Hemostatic and inflammatory changes, and their interrelation. *Clin Chim Acta Int J Clin Chem.* (2019) 493:129–37. doi: 10.1016/j.cca.2019.02.026
2. Shet AS, Lizarralde-Iragorri MA, Naik RP. The molecular basis for the prothrombotic state in sickle cell disease. *Haematologica.* (2020) 105:2368–79. doi: 10.3324/haematol.2019.239350
3. Fermo E, Vercellati C, Bianchi P. Screening tools for hereditary hemolytic anemia: new concepts and strategies. *Expert Rev Hematol.* (2021) 14:281–92. doi: 10.1080/17474086.2021.1886919
4. Nivaggioni V, Bouriche L, Coito S, Le Floch AS, Ibrahim-Kosta M, Leonnet C, et al. Use of Sysmex XN-10 red blood cell parameters for screening of hereditary red blood cell diseases and iron deficiency anaemia. *Int J Lab Hematol.* (2020) 42:697–704. doi: 10.1111/ijlh.13278
5. Urbina A, Palomino F. In vitro kinetics of reticulocyte subtypes: maturation after red blood cell storage in additive solution-1 (AS-1). *Hematol Transfus Cell Ther.* (2018) 40:143–50. doi: 10.1016/j.htct.2017.12.002
6. Sottiaux JY, Favresse J, Chevalier C, Chatelain B, Jacqmin H, Mullier F. Evaluation of a hereditary spherocytosis screening algorithm by automated blood count using reticulocytes and erythrocytic parameters on the Sysmex XN-series. *Int J Lab Hematol.* (2020) 42:e88–91. doi: 10.1111/ijlh.13125
7. Berda-Haddad Y, Faure C, Boubaya M, Arpin M, Cointe S, Frankel D, et al. Increased mean corpuscular haemoglobin concentration: artefact or pathological condition? *Int J Lab Hematol.* (2017) 39:32–41. doi: 10.1111/ijlh.12565
8. Khodaiji S. "Newer CBC Parameters of Clinical Significance.," In: Saxena R, Pati HP, editors. *Hematopathology: Advances in Understanding*. Singapore: Springer (2019). p. 3–25.
9. Abdul-Hussein HK, Al-Mammori HS, Hassan MK. Evaluation of the expression of red blood cell CD36, interleukin-6 and interleukin-8 in sickle cell anemia pediatric patients. *Cytokine.* (2021) 143:155534. doi: 10.1016/j.cyto.2021.155534
10. Mullier F, Lainey E, Fenneteau O, Da Costa L, Schillinger F, Bailly N, et al. Additional erythrocytic and reticulocytic parameters helpful for diagnosis of hereditary spherocytosis: results of a multicentre study. *Ann Hematol.* (2011) 90:759–68. doi: 10.1007/s00277-010-1138-3
11. Piel FB, Steinberg MH, Rees DC. Sickle Cell Disease. *N Engl J Med.* (2017) 376:1561–73. doi: 10.1056/NEJMra1510865
12. Shafrin J, Thom HHZ, Keeney E, Gaunt DM, Zhao LM, Bhor M, et al. The impact of vaso-occlusive crises and disease severity on quality of life and productivity among patients with sickle cell disease in the US. *Curr Med Res Opin.* (2021) 37:761–8. doi: 10.1080/03007995.2021.1897556
13. Li X, Dao M, Lykotrafitis G, Karniadakis GE. Biomechanics and biorheology of red blood cells in sickle cell anemia. *J Biomech.* (2017) 50:34–41. doi: 10.1016/j.jbiomech.2016.11.022
14. Rees DC, Williams TN, Gladwin MT. Sickle-cell disease. *Lancet Lond Engl.* (2010) 376:2018–31. doi: 10.1016/S0140-6736(10)61029-X
15. Yildirim N, Unal S, Yalcinkaya A, Karahan F, Oztas Y. Evaluation of the relationship between intravascular hemolysis and clinical manifestations in sickle cell disease: decreased hemopexin during vaso-occlusive crises and increased inflammation in acute chest syndrome. *Ann Hematol.* (2021). doi: 10.1007/s00277-021-04667-w. [Epub ahead of print].
16. Hebbel RP, Vercellotti GM. Multiple inducers of endothelial NOS (eNOS) dysfunction in sickle cell disease. *Am J Hematol.* (2021) 96:1505–17. doi: 10.1002/ajh.26308
17. Ataga KI, Moore CG, Hillery CA, Jones S, Whinna HC, Strayhorn D, et al. Coagulation activation and inflammation in sickle cell disease-associated pulmonary hypertension. *Haematologica.* (2008) 93:20–6. doi: 10.3324/haematol.11763
18. Milton JN, Rooks H, Drasar E, McCabe EL, Baldwin CT, Melista E, et al. Genetic determinants of haemolysis in sickle cell anaemia. *Br J Haematol.* (2013) 161:270–8. doi: 10.1111/bjh.12245
19. Kato GJ, McGowan V, Machado RF, Little JA, Taylor J, Morris CR, et al. Lactate dehydrogenase as a biomarker of hemolysis-associated nitric oxide resistance, priapism, leg ulceration, pulmonary hypertension, and death in patients with sickle cell disease. *Blood.* (2006) 107:2279–85. doi: 10.1182/blood-2005-06-2373
20. Aleluia MM, Fonseca TCC, Souza RQ, Neves FI, da Guarda CC, Santiago RP, et al. Comparative study of sickle cell anemia and hemoglobin SC disease: clinical characterization, laboratory biomarkers and genetic profiles. *BMC Hematol.* (2017) 17:15. doi: 10.1186/s12878-017-0087-7
21. da Guarda CC, Yahouédéhou SCMA, Santiago RP, Neres JSDS, Fernandes CF de L, Aleluia MM, et al. Sickle cell disease: A distinction of two most frequent genotypes (HbSS and HbSC). *PloS ONE.* (2020) 15:e0228399. doi: 10.1371/journal.pone.0228399
22. Piva E, Brugnara C, Spolaore F, Plebani M. Clinical utility of reticulocyte parameters. *Clin Lab Med.* (2015) 35:133–63. doi: 10.1016/j.cll.2014.10.004
23. Seliverstov ES. Morphometric properties of immature reticulocytes in health and during acute lymphoblastic and acute myeloid leukemia. *Tissue Cell.* (2021) 71:101578. doi: 10.1016/j.tice.2021.101578
24. Bartolucci P, Habibi A, Khellaf M, Roudot-Thoraval F, Melica G, Lascaux A-S, et al. Score Predicting Acute Chest Syndrome During Vaso-occlusive Crises in Adult Sickle-cell Disease Patients. *EBioMedicine.* (2016) 10:305–11. doi: 10.1016/j.ebiom.2016.06.038
25. Sahli CA, Bibi A, Ouali F, Fredj SH, Dakhlaoui B, Othmani R, et al. Red cell indices: differentiation between β-thalassemia trait and iron deficiency anemia and application to sickle cell disease and sickle cell thalassemia. *Clin Chem Lab Med.* (2013) 51:1595–603. doi: 10.1515/cclm-2012-0842
26. Steinberg MH. Predicting clinical severity in sickle cell anaemia. *Br J Haematol.* (2005) 129:465–81. doi: 10.1111/j.1365-2141.2005.05411.x
27. Habara A, Steinberg MH. Minireview: Genetic basis of heterogeneity and severity in sickle cell disease. *Exp Biol Med Maywood NJ.* (2016) 241:689–96. doi: 10.1177/1535370216636726
28. Gladwin MT, Kato GJ, Gordeuk VR. Identifying adolescent and young adult patients with sickle cell disease at highest risk of death. *Am J Hematol.* (2021) 96:9–11. doi: 10.1002/ajh.26035
29. Adegoke SA, Kuti BP, Omole KO, Smith OS, Oyelami OA, Adeodu OO. Acute chest syndrome in sickle cell anaemia: higher serum levels of interleukin-8 and highly sensitive C-reactive proteins are associated with impaired lung function. *Paediatr Int Child Health.* (2018) 38:244–50. doi: 10.1080/20469047.2018.1519988
30. Njoku F, Zhang X, Shah BN, Machado RF, Han J, Saraf SL, et al. Biomarkers of clinical severity in treated and untreated sickle cell disease: a comparison by genotypes of a single center cohort and African Americans in the NHANES study. *Br J Haematol.* (2021) 194:767–78. doi: 10.1111/bjh.17682
31. Oksenberg D, Dufu K, Patel MP, Chuang C, Li Z, Xu Q, et al. GBT440 increases haemoglobin oxygen affinity, reduces sickling and prolongs RBC

half-life in a murine model of sickle cell disease. *Br J Haematol.* (2016) 175:141–53. doi: 10.1111/bjh.14214
32. Vichinsky E, Hoppe CC, Ataga KI, Ware RE, Nduba V, El-Beshlawy A, et al. A Phase 3 Randomized Trial of Voxelotor in Sickle Cell Disease. *N Engl J Med.* (2019) 381:509–19. doi: 10.1056/NEJMoa1903212
33. Ataga KI, Kutlar A, Kanter J, Liles D, Cancado R, Friedrisch J, et al. Crizanlizumab for the Prevention of Pain Crises in Sickle Cell Disease. *N Engl J Med.* (2017) 376:429–39. doi: 10.1056/NEJMoa1611770
34. Matsui NM, Borsig L, Rosen SD, Yaghmai M, Varki A, Embury SH. P-selectin mediates the adhesion of sickle erythrocytes to the endothelium. *Blood.* (2001) 98:1955–62. doi: 10.1182/blood.V98.6.1955

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.

frontiers | Frontiers in Medicine

ORIGINAL RESEARCH
published: 28 June 2022
doi: 10.3389/fmed.2022.851918

Low Stroke Risk in Children With Sickle Cell Disease in French Guiana: A Retrospective Cohort Study

*Julie Gargot[1], Marie-Claire Parriault[2], Antoine Adenis[2], Jérôme Clouzeau[3], Kim-Anh Dinh Van[4], Balthazar Ntab[5], Antoine Defo[1], Mathieu Nacher[2] and Narcisse Elenga[1]**

[1] Service de Pédiatrie, Centre Hospitalier Andrée Rosemon, Cayenne, French Guiana, [2] CIC INSERM 1424, Centre Hospitalier Andrée Rosemon, Cayenne, French Guiana, [3] Service de Pédiatrie, Centre Hospitalier de l'Ouest-Guyanais «Franck Joly», Saint-Laurent-du-Maroni, French Guiana, [4] Département d'Information Médical, Centre Hospitalier de Kourou, Kourou, French Guiana, [5] Département d'Information Médicale, Centre Hospitalier de l'Ouest-Guyanais «Franck Joly», Saint-Laurent-du-Maroni, French Guiana

One in every 227 babies born in French Guiana has sickle cell disease, which represents the greatest incidence in France. This study aimed to determine the incidence of stroke in children with sickle cell disease and its associated risk factors. This retrospective cohort study included all children with sickle cell disease diagnosed in the neonatal period who were born in French Guiana between 01/01/1992 and 12/31/2002. Of a total of 218 records, 122 patients were included. There were 70 HbSS/Sβ0 (58%), 40 HbSC (33%), and 11 Sβ + thalassemia (9%). The number of emergency admissions was significantly different between genotypes, with a higher number in SS/Sβ0 children ($p = 0.004$). There were significantly more acute chest syndromes ($p = 0.006$) and more elevated Lactate Dehydrogenase in SS/Sβ0 patients ($p = 0.003$). Three of these patients had ischemic strokes at a mean age of 6.9 years, and one had a hemorrhagic stroke at the age of 9,2 years. The incidence rate of ischemic stroke for SS/Sβ0 children was 3.1 (95% CI: 1.0–9.7) per 1,000 patient-years, and the clinically apparent stroke risk by the age of 15 years and 3 months was 6,4%. The incidence of hemorrhagic stroke was 1.1 (95% CI: 0.1–7.4) per 1,000 patients-years. No patient with SC or Sβ + thalassemia genotypes experienced any stroke.

Keywords: sickle cell disease, stroke, incidence, French Guiana, children

OPEN ACCESS

Edited by:
John Joseph Strouse, Duke University, United States

Reviewed by:
Mohsen Saleh Elalfy, Ain Sham University, Egypt
J. Peter Ray Pelletier, UF Health Shands Hospital, United States

****Correspondence:***
Narcisse Elenga
elengafr@yahoo.fr

Specialty section:
This article was submitted to Hematology, a section of the journal Frontiers in Medicine

Received: *10 January 2022*
Accepted: *27 May 2022*
Published: *28 June 2022*

Citation:
Gargot J, Parriault M-C, Adenis A, Clouzeau J, Dinh Van K-A, Ntab B, Defo A, Nacher M and Elenga N (2022) Low Stroke Risk in Children With Sickle Cell Disease in French Guiana: A Retrospective Cohort Study. Front. Med. 9:851918. doi: 10.3389/fmed.2022.851918

INTRODUCTION

Sickle cell disease (SCD) is an autosomal recessive inherited disease characterized by the synthesis of an abnormal hemoglobin sickle hemoglobin S (βs, HbS) resulting in the substitution of a single amino acid (Glu→Val) at the sixth position of β-chain of normal hemoglobin (HbA) molecule (1, 2). This point mutation leads to the polymerization of the HbS molecule under deoxygenated conditions. Polymerization of the HbS leads to the stiffening and weakening of sickled red blood cells. This hereditary disease particularly affects those of African origin because the protection against malaria that heterozygocy conferred to their ancestors selected the sickle cell mutations

genes (3). Homozygous SS (sickle cell anemia or SCA) is generally considered to be the most severe form of SCD. Compound heterozygotes, in whom HbS is combined with a different mutation of the second β-globin gene such as HbC, D, OArab or β-thalassemia (where synthesis of β-globin is reduced), are also considered as symptomatic forms of SCD, with varying phenotypes. SCD is characterized by abnormally shaped adhesive red blood cells that interact with white blood cells and the vascular endothelium, resulting in chronic hemolysis, reperfusion injury, vasculopathy impaired vasomotor tone, and a prothrombotic state. SCD brain vasculopathy causes both overt strokes and "silent" cerebral infarcts, resulting in early death, permanent neurocognitive and physical dysfunction together with life-long suffering and poor quality of life. Pathophysiology of stroke in SCD is complex and includes both diseased large arteries and penetrating arteries (4). SCD is the most common cause of pediatric stroke and children with SCA are at higher risk of stroke, notably in the first decade of life (3). In the absence of early Transcranial Doppler (TCD) screening, strokes occur in 7.4–11% of patients with SCA before age 20 (5–7). As the risk of stroke persists throughout childhood (8), TCD screening has been implemented to detect SCA children at risk, in order to initiate transfusion programs known for significantly reducing stroke incidence in SCA patients with an abnormal TCD (9–12).

French Guiana is an overseas French territory on the north-eastern coast of South America. SCD is a major public health problem in French Guiana (13). The population, most of which is of African ancestry, consists mainly of three groups: Guianese Creoles, Maroons (descendants of runaway slaves), and, more recently, Haitian immigrants (14). The estimated incidence of SCD at birth is one in 227, and the overall frequency of hemoglobin AS carriers is 10% (13, 15). The major SCD groups include the three main genetic forms that combine different structural hemoglobin variants or thalassemia syndromes (hemoglobin S HbS, hemoglobin C HbC, β-thalassemia) (16). There are scarce data on the actual incidence of SCA-related strokes in French Guiana. We aimed to determine the risk for stroke in children with SCD and the risk factors associated with stroke.

MATERIALS AND METHODS

Stroke Definition

Stroke is classically defined as a neurological deficit related to an acute focal injury to the central nervous system (CNS) of vascular origin, including cerebral infarction, intracerebral hemorrhage (ICH), or subarachnoid hemorrhage (SAH) (17). Due to significant advances in the nature, timing, clinical recognition of stroke, its differential diagnoses, and imaging results, authors called for an update of definition of stroke (18).

Definition of Silent Cerebral Infarction

Silent cerebral infarction (SCI) is defined as an area of abnormal hyperintensity on T2-weighted (axial and coronal) MRI images of the brain in a patient without a history of neurological symptoms or signs consistent with the location of the infarct in a given vascular distribution.

Definition of Other Conditions

Acute chest syndrome (ACS) is defined by the presence of fever and/or respiratory symptoms, accompanied by a new radiodensity on the chest X-ray.

Asthma is a chronic inflammatory lung disease, associated with airway hyper responsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness and coughing.

Adenoid hypertrophy is an obstructive disorder related to an increase in the size of the adenoids (a collection of lymphoepithelial tissues located in the upper part of the nasopharynx, medial to the openings of the Eustachian tube).

High blood pressure in children is defined as a mean blood pressure at or above the 95th percentile for their age, sex, and height when measured multiple times over three or more visits.

Monitoring and Management of Sickle Cell Disease in French Guiana

After the announcement of the diagnosis of SCD by the pediatricians, subsequent consultations alternate between visits to the maternal and child protection centers and/or private physicians, who renew explanations, provide vaccinations, carry out surveillance, and in hospital specialized care. We have implemented prophylactic penicillin treatment for all sickle cell patients, comprehensive vaccination and education about regular spleen size measurement for sickle cell families. Hospital visits take place every 3 months in the first 2 years of life, and then at a frequency depending on the severity of the disease. At each visit, the parents' ability to recognize events requiring an urgent consultation (fever higher than 38.5 C°, sudden pallor and/or asthenia, pain not responding to initial analgesic treatment, sudden increase of spleen volume, vomiting) is verified. Similarly, risk factors for vasoocclusive crisis (VOC) are regularly explained. Clinical examination looks for possible hepatosplenomegaly. The splenic overflow is noted in the health booklet, and parents are taught how to measure the spleen, so that they can recognize a sudden increase in its volume. The ears, nose, and throat (ENT) sphere is also examined for upper airway obstruction. The annual check-up varies according to the child's age. A blood count with reticulocyte count, iron status, blood electrolytes and liver function tests are performed annually. From the age of 3, the chest X-ray and abdominal ultrasound are checked annually, and from the age of 6, the pelvic X-ray and cardiac ultrasound are checked. Ophthalmologic surveillance is performed from the age of 6 years in SC children and 10 years in SS children. Cerebral vascularization is systematically checked by TCD from the age of 12–18 months in homozygous children and those who are S-beta0 thalassemic. Before 2009, the frequency of TCD depended on the SCD standard of care of each specialized center. Since 2009, with the establishment of the sickle cell competence centers in French Guiana, TCD was performed annually for each homozygous sickle cell child within the framework of a standardized protocol in all 3 SCD specialized-care centers of French Guiana (Cayenne, Saint-Laurent-du-Maroni and Kourou).

In case of pathological velocities documented by TCD, the child is enrolled in an immediate transfusion exchange program for 2–3 months, followed by brain and cervical magnetic resonance imaging (MRI) with Doppler monitoring. When a stenosis is found, the exchange program is continued in the long term (undetermined duration, HLA typing in siblings and indication of bone marrow transplant in case of HLA compatible donor). When the imaging is normal, the program is continued for 1 year. If after 1 year, there is still no stenosis on imaging patient care depends on TCD velocities:

- TCD velocities are still pathological or borderline: exchanges are continued with annual TCD monitoring until normalization.
- TCD velocities are normalized: the child starts Hydroxyurea (HU) treatment up to the maximum tolerated dose with at least 2 months of overlap with the exchange program, with a TCD check every 3 months and MRI every year. Hydroxyurea is continued if examinations (Doppler and MRI) are normal, with quarterly TCD monitoring for 1 year and then annually. In case of pathological velocities, the exchange transfusion program is reinstated in the long term.

In case of pathological extracranial velocities on TCD, the child is enrolled in a transfusion exchange program. When these extracranial velocities are borderline normal, MRI is indicated because of the significant risk of stenosis. If there is a stenosis, a long-term exchange program is recommended. If there is no stenosis, the child is put on HU with quarterly TCD monitoring. The transfusion or to transfusion exchanges were identical in the three centers of French Guiana.

Study Design

This was a multicenter retrospective cohort study, conducted at the Integrated Sickle Cell Center of French Guiana, a reference center based at Cayenne hospital. A single reviewer (JG) extracted all the records of all children with SCD, admitted to the pediatric units of the three hospitals of French Guiana (Cayenne, Saint-Laurent-du-Maroni and Kourou), born between 01/01/1992 and 12/31/2002, aged < 16 years at the time of inclusion. Patients were identified using the International Classification of Diseases (ICD 10) codes for sickle cell disease (D570, D571, D572) in electronic databases of the different pediatric departments of participating hospitals. The routine sickle cell testing such as newborn screening was implemented in 1992 in French Guiana. All children were diagnosed in the perinatal period. The confirmation of SCA was done using Hb electrophoresis at 6 months of age. Clinical, laboratory, and radiologic monitoring data were collected up to the age of 15 years and 3 months (pediatric age), or the date of the latest news. The failure event was clinical stroke. The biological recorded data were those of the last year of follow-up, reflecting baseline conditions (temporally separated from any acute or chronic event). For each patient, basic information and specific clinical complications were collected: age, gender, hemoglobin type, haplotype, alpha-thalassemia, severity and number of prior acute or chronic sickle cell specific complications (acute splenic or hepatic sequestration, acute chest syndrome, sickling related painful vasoocclusive crisis, neurologic events, severe infections, acute anemia, cholelithiasis), use of opioids for painful events, use and number of transfusions. These data were collected from computerized and/or paper patient records and were directly entered into an electronic form (eCRF) built using Clinsight Capture System® software. The inclusion and data collection period extended from 02/01/2019 to 09/30/2019, and the database was frozen on December 31st, 2019. Only the first stroke admission was included in the analysis. Repeated admissions of the same patient with stroke were excluded.

Statistical Analysis

Statistical data analysis was performed using Stata 12.0 (Stata Statistical Software: Release 12. College Station, TX: StataCorp LP). Categorical data were summarized as count (percentage) and quantitative data as median with 25th/75th percentiles (interquartile range, IQR), and compared using the χ^2 test or Fisher's exact test. Single failure survival analysis was performed and Kaplan-Meier curves were plotted. $P < 0.05$ was chosen as the statistical significance threshold. The exact date of entry of each subject was the birth date. The end of the follow-up period was the date of latest news (up to 15 years 3 months) or date of death (if applicable). The stroke date represented the failure event date if it occurred.

The estimated sample size needed to obtain a 20% difference between the incidence observed in the Freepong study and ours, with a 2-sided test, 90% power, and 5% alpha error, was 70 subjects in total.

Ethical Approval

This study was approved by the « Comité d'Evaluation Ethique de l'Inserm (Number IRB00003888) and the database was declared at the Commission Nationale Informatique et Liberté (CNIL, Number 2200250 v 0). Patients were informed of the utilization of their data with an informative poster in the medical units concerned with SCD. All patients and relatives were personally informed and asked to give approval to participate in the stud. All data were collected, after certification of a written patient's non-opposition. All underage participants had written informed consent provided on their behalf by their parent/legal guardian.

RESULTS

A total of 218 records were extracted 122 of which met the inclusion criteria, as shown in **Figure 1**. Our cohort comprised 56 females (46%), and 66 males (54%). There were 70 HbSS/Sβ0 (58%), 40 HbSC (33%), and 11 Sβ + thalassemia (9%). Of the 122 included patients, 60 lived in the urban Municipality of Cayenne, 49 in Western French Guiana and 11 patients lived in the Savanas. Among them, 21 (17.21%) were treated with HU. The number of emergency room admissions was significantly different between genotypes, with a higher number in SS/Sβ0 children, as expected (**Table 1**). Frequency of acute chest syndrome (ACS) and the level of lactate dehydrogenase (LDH) were significantly higher

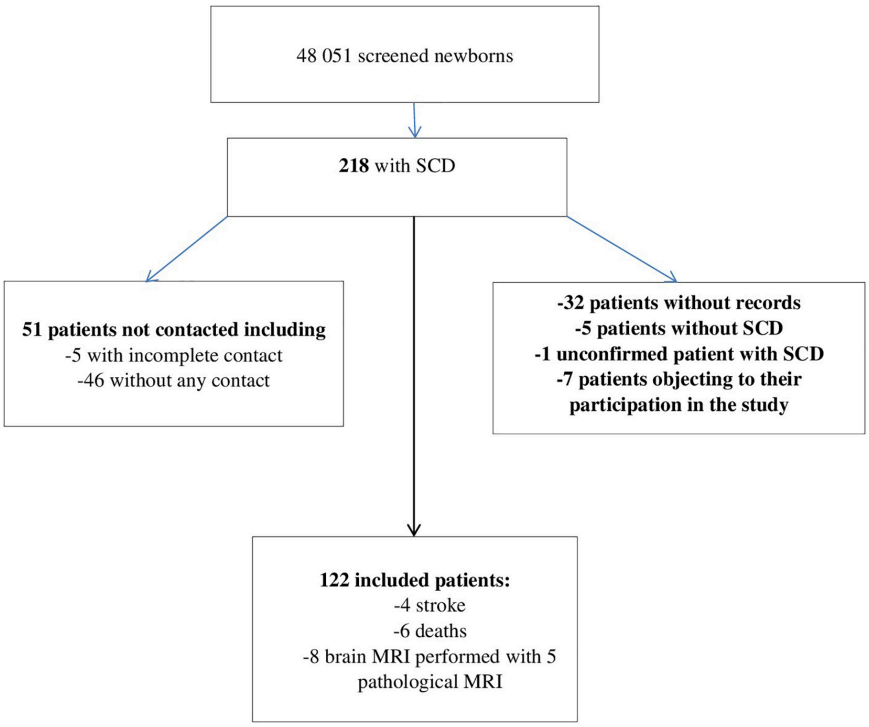

FIGURE 1 | Study flow diagram. SCD, sickle cell disease; Stroke, cerebrovascular accidents; MRI, magnetic resonance imaging.

in patients with genotype SS/Sβ0 (**Tables 2**, **3**). Among the 108 patients who performed a TCD, there were 17% abnormal, 25% conditional, and 58% normal TCD. For all of them, the cerebral MRI angiography was normal. A transfusion exchange program was offered to these 18 patients with pathological TCD. However, only 11 patients were able to benefit from it, for 12 months, after which the transfusion program was stopped. The TCD of the transfused patients was normal by the sixth month after the start of the exchange program. The remaining 7 patients were all treated with HU for the following reasons:

- alloimmunization and difficulty crossmatching units in 4 patients
- refusal of transfusion programs in the other 3 patients

A total of 4 patients had a diagnosis of stroke. Three of these patients experienced a ischemic stroke at a mean age of 6.9 years, and one a hemorrhagic stroke at 9.2 years of age. The cumulative incidence rate of ischemic stroke among SS/Sβ0 children was 3.1 (95% CI: 1.0–9.7) per 1,000 patient-years (**Figure 2**), and the risk of clinical stroke by age 15 years and 3 months was 6,4%. The incidence of hemorrhagic stroke was 1.1 (95% CI: 0.1–7.4) per 1,000 patients-years. All strokes occurred only in children with SS/SB0 genotypes. Risk factors such as lack of alpha-thalassemia, high blood pressure, asthma, and enlarged adenoids were not significantly different between genotypes (**Tables 1**, **2**). 15 patients in this study had moderate splenomegaly. There was no difference in splenomegaly according to genotype. Only 8 patients benefited from a magnetic resonance imaging (MRI) of the brain. Four silent brain infarctions (SBI) were diagnosed (including 2 prior to ischemic stroke), two internal carotid artery stenoses, as well as brain images corresponding to migraine lesions in one patient. There were no cases of transient ischemic attack (TIA) in our study.

Six deaths occurred during the study period, all in patients with SS/Sβ0 SCD except one in a patient with an unknown genotype. The sex ratio was 1. Three patients were followed in Cayenne, and the three others in Saint Laurent du Maroni. Death occurred at a mean age of 4.9 years [range: 9 months–14.75 years]. Two deaths were attributed to severe acute anemia (Hb level 2 and 3 g/dL), two other deaths occurred at home, one in a febrile context, without further information. One death was attributable to an acute chest syndrome complicated by coma, without brain imaging performed. The last death was secondary to post-anesthesia cardiac-respiratory arrest following phlegmon surgery, the injected brain scan was normal. None of our patients benefited from bone marrow transplantation (BMT).

DISCUSSION

The incidence of clinical stroke in children with SS/Sβ0 was low although comparable to previous studies (5, 6, 19). This seems to be explained by a combination of different factors, among which we believe the implementation of systematic screening for cerebral vasculopathy by TCD has played a major role since 2009. The comparison with Ohene-Frempong et al. study (5) (**Table 3**) shows an incidence rate close to ours. In addition,

TABLE 1 | Clinical characteristics of children with sickle cell disease, according to their genotype.

	SS/Sβ 0		SC		Sβ +		NS*	p
	***N* = 70**	**%**	***N* = 40**	**%**	***N* = 11**	**%**	***N* = 1**	**(α = 5%)**
Gender								0.269
Male	42	60	20	50	4	36		
Female	28	40	20	50	7	64	1	
Hospital follow-up								0.366
Cayenne	44	63	20	50	8	73		
Kourou	6	8	2	5	0	0		
Saint Laurent du Maroni	20	29	18	45	3	27	1	
Haplotype	*n* = 28		*n* = 10		*n* = 6			0
Benin/Benin	12	43	0	0	1	17		
Benin/Bantu	5	18	0	0	0	0		
Benin/atypical	4	14	0	0	0	0		
Bantu/Atypical	3	11	0	0	0	0		
Benin/Beta C allele	0	0	7	70	0	0		
Others	4	14	3	30	5	83		
Number of hospitalizations	*n* = 59		*n* = 33		*n* = 11			0
None	1	2	11	33	2	18		
1–4	15	25	17	52	4	36	1	
5–9	13	22	3	9	1	9		
10 or more	30	51	2	6	4	36		
Number of emergency room visits	*n* = 48		*n* = 27		*n* = 9			0.004
None	1	2	3	11	0	0		
1–4	5	10	12	44	1	11	1	
5–9	11	23	4	15	3	33		
10 or more	31	65	8	30	5	56		
Alpha thalassemia	*n* = 34		*n* = 11		*n* = 6			0.634
2/4 alleles	2	6	2	18	0	0		
3/4 alleles	11	32	2	18	2	33		
None	21	62	7	64	4	67		

**NS, not specified.*

TABLE 2 | Distribution of clinical history by genotype.

	Genotypes										*p*
	SS/Sβ 0			SC			Sβ +			NS (*N* = 1)	(α = 5%)
	N	**Presence**	**%**	***N***	**Presence**	**%**	***N***	**Presence**	**%**	**Presence**	
ACS (*n* = 122)	70	20	29	40	2	5	11	2	18	0	0.006
AHT (*n* = 90)	54	1	2	26	0	0	10	1	10		0.292
Infant asthma (*n* = 15)	12	3	25	2	0	0				0	1
Asthma (*n* = 99)	57	9	16	31	6	19	11	0	0		0.33
Hypertrophy of adenoids (*n* = 25)	18	7	39	2	0	0	4	1	25	1	0.798
Enlarged tonsils (*n* = 77)	52	7	13	18	1	6	6	0	0	0	0.835

NS, not specified; ACS, acute chest syndrome; AHT, arterial hypertension.

our prevalence, although lower, is close to that of Ohene-Frempong et al. study (5). According to the literature, stroke rates vary with sickle cell genotype. Children with HbSS or HbSβ^0 thalassemia have a higher risk of stroke compared with SC and HbSβ + thalassemia. In our study, no patient with SC or Sβ + thalassemia genotypes experienced stroke. However, the proportion of SC and HbSβ + patients was high compared with the Frempong cohort. The protective effect of alpha-thalassemia against the development of stroke is well established in the literature. The high rates of subjects with an alpha-thalassemic

TABLE 3 | Comparative incidence of first CVA in children with SCA (SS and β0 thalassemia).

Study by	Ohene-Frempong et al.	Our study		Lagunju et al.
Number of patients	*n* = 4,082	*n* = 122		*n* = 104
Genotyes	67.8% SS	58% SS&S-β0 thal		100% Hb SS
	22.1% SC	33% SC		
	5.2% S-β + thal	9% β + thal		
	4.9% S-β0 thal			
Prevalence of stroke (%)	6.44	6.4		2.3
Incidence rate (per 100 patient-years)	0.69	0.31	(95% CI: 0.1–0.97)	0.27

CVA, cerebrovascular accident; SCA, Sickle cell anemia; Thal, thalassemia.

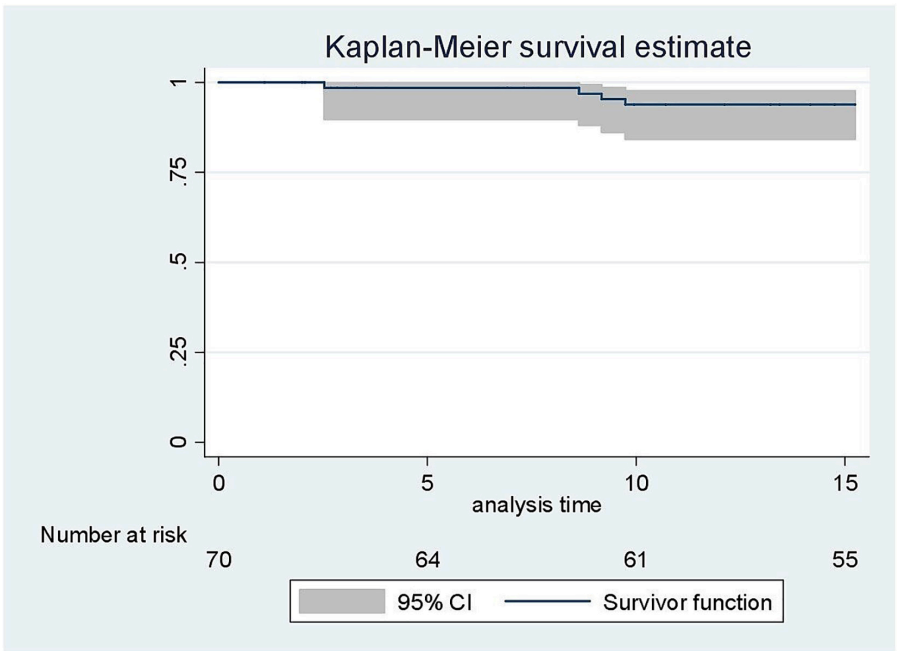

FIGURE 2 | Kaplan-Meier curve on the estimate of the occurrence of clinical stroke before 15 years 3 months.

trait (37.2%) could also explain this low stroke incidence rate. The high Hb F in SSS/Sβ0 could be explained by the young age of our study population. Other factors described in the literature could not be tested because of the retrospective nature of our study. The impact of early TCD screening and intensive therapy on cerebral vasculopathy is now globally recognized (20–22). Thus, our protocol follows the international recommendations to perform an annual TCD from the age of 18 months until the age of 16–18 years (20–22). A limited number of patients under study benefited from a brain MRI. This examination, introduced in our protocol in 2009, has become more accessible since 2015, with the acquisition by each of the three hospitals of an MRI scanner and the presence of another MRI scanner within a private radiology department in Cayenne. For this reason, very few MRIs have been performed. Thus, in a follow-up cohort of children screened for SCA since birth, the systematic detection of pathological TCD and the implementation of an early transfusion program made it possible to reduce the cumulative risk of stroke before age 18 years to 1.9 vs. 11% in the natural history of the disease (10). This is what we have been trying to do since the introduction of TCD. We recognize that stopping the transfusion program might result in a risk of observing another pathological TCD, or even stroke (23–25). However, the transfusion protocol is cumbersome and sometimes inaccessible for some patients. This is why we have implemented this lighter protocol, which allows for the use of HU after 1 year of exchange transfusion (26).

A study in Nigeria showed that HU significantly reduced TCD velocities in children with SCD and high TCD velocities, with a drastic reduction in primary stroke incidence (27). HU is recognized as a potential alternative for primary stroke prevention in low- and middle-income countries (27–29). None of our patients benefited from BMT. However, the place of BMT as a primary prevention strategy was evaluated by the DREPAGREFFE study, which compares the outcome of cerebral vasculopathy following geno-identical allogeneic transplantation vs. chronic transfusion program (30). In French Guiana, the health system is that of France with free access to care and treatment. The neonatal screening for SCD has been generalized

since 1992 (13). And the routine TCD screening with indefinite chronic red blood cell transfusion for children with abnormal TCD as standard of care has been implemented since 2009. Our practices regarding the indication and prescription of brain MRI have therefore improved with the increase in the availability of MRI in French Guiana. Currently, in the 3 hospitals, the protocol for the management of cerebral vasculopathy with annual TCD and angio-MRI in case of pathological TCD (and HLA typing in siblings and indication of bone marrow transplant in case of HLA compatible donor) is scrupulously applied. This has led to an improvement in the management of this serious complication of SCD. The hypothesis that the number of strokes could have been influenced by the number of lost to follow-up is unlikely. Indeed, the occurrence of a clinical stroke implies hospitalization in one of the three hospitals of French Guiana, unless an immediate death occurs at home. In our study, over 10 years, there were 6 deaths in children. In the identified causes of death linked to SCD in France at any age, stroke was the most important cause identified: 34 cases out of 412 deaths (31). The small number of deaths that occurred in our study could be related to the low incidence of stroke. The frequency of silent strokes cannot be discussed in view of the low proportion of children screened with brain MRIs. Screening for silent strokes in children should be improved. Although there are no clear guidelines, sickle cell patients and their families may benefit from training to recognize the signs of TIA, which are sometimes inconspicuous, and to get them to consult urgently even if the symptoms disappeared. A therapeutic education program for sickle cell patients with a dedicated nurse was started in January 2020 at the Integrated Sickle Cell Center at Cayenne hospital.

Our study has some biases and limitations. Our major limitation is the low number of children who had a stroke in the cohort, which makes the identification of risk factors associated with stroke difficult. However, we have not yet collected data on the incidence of stroke in the post-TCD era. In this retrospective study, with many patients lost to follow-up and missing data-the older medical records were sometimes not exhaustive. The beginning of cerebrovascular screening overlapped with a period of our study, and this may have reduced the number of strokes found. However, since the risk of stroke occurred mainly before 9 years, the impact of this bias is difficult to estimate. A recent study carried out in the United States following the STOP and STOP 2 trials showed that the appearance of a first ischemic stroke was due to a failure in the monitoring and treatment of patients in 63% of cases (32).

Despite these limitations, this multicenter study made it possible to estimate the incidence and risk factors of clinical stroke in children with SCD in French Guiana. These results will be taken into account to improve the screening and the primary and secondary prevention of cerebral vasculopathy in children with SCD in French Guiana.

CONCLUSION

The risk of clinical stroke in children with SS/Sβ0 SCD before the age of 15 years and 3 months was low. This was probably explained by the implementation of routine TCD screening with indefinite CRCT for children with abnormal TCD. Additional opportunities for ischemic stroke prevention through the recent implementation of therapeutic patient education program may further reduce the incidence of this complication and will 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 Comité d'Evaluation Ethique de l'Inserm (Number IRB00003888). 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.

ACKNOWLEDGMENTS

We would like to thank the members of the CIC INSERM 1424 team for their logistical support.

REFERENCES

1. Azar S, Wong TE. Sickle cell disease: a brief update. *Med Clin North Am.* (2017) 101:375–93. doi: 10.1016/j.mcna.2016.09.009
2. McGann PT, Nero AC, Ware RE. Ware. Current management of sickle cell anemia. *Cold Spring Harb Perspect Med.* (2013) 3:a011817.
3. Makani J, Williams TN, Marsh K. Sickle cell disease in Africa: burden and research priorities. *Ann Trop Med Parasitol.* (2007) 101:3–14. doi: 10.1179/136485907X154638
4. Guilliams KP, Fields ME, Dowling MM. Advances in understanding ischemic stroke physiology and the impact of vasculopathy in children with sickle cell disease. *Stroke.* (2019) 50:266–73. doi: 10.1161/STROKEAHA.118.020482
5. Ohene-Frempong K, Weiner SJ, Sleeper LA, Miller ST, Embury S, Moohr JW, et al. Cerebrovascular accidents in sickle cell disease: rates and risk factors. *Blood.* (1998) 91:288–94.
6. Connes P, Verlhac S, Bernaudin F. Advances in understanding the pathogenesis of cerebrovascular vasculopathy in sickle cell anaemia. *Br J Haematol.* (2013) 161:484–98. doi: 10.1111/bjh.12300
7. Belisário AR, Silva CM, Velloso-Rodrigues C, Viana MB. Genetic, laboratory and clinical risk factors in the development of overt ischemic stroke in children with sickle cell disease. *Hematol Transfus Cell Ther.* (2018) 40:166–81. doi: 10.1016/j.bjhh.2017.08.008
8. Quinn CT, McKinstry RC, Dowling MM, Ball WS, Kraut MA, Casella JF, et al. Acute silent cerebral ischemic events in children with sickle

cell anemia. *JAMA Neurol.* (2013) 70:58–65. doi: 10.1001/jamaneurol.2013.576
9. Adams RJ, McKie VC, Hsu L, Files B, Vichinsky E, Pegelow C, et al. Prevention of a first stroke by transfusions in children with sickle cell anemia and abnormal results on transcranial Doppler ultrasonography. *N Engl J Med.* (1998) 339:5–11. doi: 10.1056/NEJM199807023390102
10. Bernaudin F, Verlhac S, Arnaud C, Kamdem A, Chevret S, Hau I, et al. Impact of early transcranial Doppler screening and intensive therapy on cerebral vasculopathy outcome in a newborn sickle cell anemia cohort. *Blood.* (2011) 117:1130–40. doi: 10.1182/blood-2010-06-293514
11. Enninful-Eghan H, Moore RH, Ichord R, Smith-Whitley K, Kwiatkowski JL. Transcranial Doppler ultrasonography and prophylactic transfusion program is effective in preventing overt stroke in children with sickle cell disease. *J Pediatr.* (2010) 157:479–84. doi: 10.1016/j.jpeds.2010.03.007
12. Brewin J, Kaya B, Chakravorty S. How I manage sickle cell patients with high transcranial doppler results. *Br J Haematol.* (2017) 179:377–88. doi: 10.1111/bjh.14850
13. Etienne-Julan M, Elana G, Loko G, Elenga N, Vaz T, Muszlak M. La drépanocytose dans les départements français d'outre-mer (Antilles, Guyane, la Réunion, Mayotte): données descriptives et organisation de la prise en charge. *Bull Épidémiol Hebd.* (2012) 27–28:322–5.
14. Simonnet C, Elanga N, Joly P, Vaz T, Nacher M. Genetic modulators of sickle cell disease in French Guiana: markers of the slave trade. *Am J Hum Biol.* (2016) 28:811–6. doi: 10.1002/ajhb.22871
15. Knight-Madden J, Lee K, Elana G, Elenga N, Marcheco-Teruel B, Keshi N, et al. Newborn screening for sickle cell disease in the caribbean: an update of the present situation and of the disease prevalence. *Int J Neonatal Screen.* (2019) 5:5. doi: 10.3390/ijns5010005
16. Elenga N, Cuadro E, Martin É, Cohen-Addad N, Basset T. Associated factors of acute chest syndrome in children with sickle cell disease in French Guiana. *Int J Pediatrics.* (2014) 2014:213681. doi: 10.1155/2014/213681
17. Wijdicks EF, Sheth KN, Carter BS, Greer DM, Kasner SE, Kimberly WT, et al. Recommendations for the management of cerebral and cerebellar infarction with swelling: a statement for healthcare professionals from the American Heart Association/American Stroke Association. *Stroke.* (2014) 45:1222–38. doi: 10.1161/01.str.0000441965.15164.d6
18. Sacco RL, Kasner SE, Broderick JP, Caplan LR, Connors JJ, Culebras A, et al. An updated definition of stroke for the 21st century: a statement for healthcare professionals from the American Heart Association/American Stroke Association. *Stroke.* (2013) 44:2064–89.
19. Hirtz D, Kirkham FJ. Sickle cell disease and stroke. *Pediatr Neurol.* (2019) 95:34–41.
20. Adams RJ, McKie VC, Brambilla D, Carl E, Gallagher D, Nichols FT, et al. Stroke prevention trial in sickle cell anemia. *Control Clin Trials.* (1998) 19:110–29.
21. Adams RJ, Brambilla DJ, Granger S, Gallagher D, Vichinsky E, Abboud MR, et al. Stroke and conversion to high risk in children screened with transcranial Doppler ultrasound during the STOP study. *Blood.* (2004) 103:3689–94. doi: 10.1182/blood-2003-08-2733
22. Lee MT, Piomelli S, Granger S, Miller ST, Harkness S, Brambilla DJ, et al. Stroke prevention trial in sickle cell anemia (STOP): extended follow-up and final results. *Blood.* (2006) 108:847–52. doi: 10.1182/blood-2005-10-009506
23. Adams RJ, Brambilla D. Optimizing primary stroke prevention in sickle cell anemia (STOP 2) trial investigators. discontinuing prophylactic transfusions used to prevent stroke in sickle cell disease. *N Engl J Med.* (2005) 353:2769–78. doi: 10.1056/NEJMoa050460
24. Abboud MR, Yim E, Musallam KM, Adams RJ. STOP II study investigators. discontinuing prophylactic transfusions increases the risk of silent brain infarction in children with sickle cell disease: data from STOP II. *Blood.* (2011) 118:894–8. doi: 10.1182/blood-2010-12-326298
25. DeBaun MR, Gordon M, McKinstry RC, Noetzel MJ, White DA, Sarnaik SA, et al. Controlled trial of transfusions for silent cerebral infarcts in sickle cell anemia. *N Engl J Med.* (2014) 371:699–710. doi: 10.1056/NEJMoa1401731
26. Ware RE, Davis BR, Schultz WH, Brown RC, Aygun B, Sarnaik S, et al. Hydroxycarbamide versus chronic transfusion for maintenance of transcranial doppler flow velocities in children with sickle cell anaemia-TCD With Transfusions Changing to Hydroxyurea (TWiTCH): a multicentre, open-label, phase 3, non-inferiority trial. *Lancet.* (2016) 387:661–70. doi: 10.1016/S0140-6736(15)01041-7
27. Lagunju I, Brown BJ, Oyinlade AO, Asinobi A, Ibeh J, Esione A, et al. Annual stroke incidence in Nigerian children with sickle cell disease and elevated TCD velocities treated with hydroxyurea. *Pediatr Blood Cancer.* (2019) 66:e27252. doi: 10.1002/pbc.27252
28. Adegoke SA, Macedo-Campos RS, Braga JAP, Figueiredo MS, Silva GS. Changes in transcranial Doppler flow velocities in children with sickle cell disease: the impact of hydroxyurea therapy. *J Stroke Cerebrovasc Dis.* (2018) 27:425–31. doi: 10.1016/j.jstrokecerebrovasdis.2017.09.020
29. Hankins JS, McCarville MB, Rankine-Mullings A, Reid ME, Lobo CL, Moura PG, et al. Prevention of conversion to abnormal transcranial Doppler with hydroxyurea in sickle cell anemia: a Phase III international randomized clinical trial. *Am J Hematol.* (2015) 90:1099–105. doi: 10.1002/ajh.24198
30. Chevret S, Verlhac S, Ducros-Miralles E, Dalle JH, de Latour RP, de Montalembert M, et al. Design of the DREPAGREFFE trial: a prospective controlled multicenter study evaluating the benefit of genoidentical hematopoietic stem cell transplantation over chronic transfusion in sickle cell anemia children detected to be at risk of stroke by transcranial Doppler (NCT 01340404). *Contemp Clin Trials.* (2017) 62:91–104. doi: 10.1016/j.cct.2017.08.008
31. Gomes E, Castetbon K, Goulet V. Mortalité liée à la drépanocytose en France: âge de décès et causes associées (1979-2010). *Bull Épidémiol Hebd.* (2015) 8:142–50.
32. Kwiatkowski JL, Voeks JH, Kanter J, Fullerton HJ, Debenham E, Brown L, et al. Ischemic stroke in children and young adults with sickle cell disease in the post-STOP era. *Am J Hematol.* (2019) 94:1335–43. doi: 10.1002/ajh.25635

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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Medicine

TYPE Original Research
PUBLISHED 19 July 2022
DOI 10.3389/fmed.2022.924722

OPEN ACCESS

EDITED BY
Uday Prakash Kulkarni,
Christian Medical College & Hospital, India

REVIEWED BY
Frederick Kaskel,
Children's Hospital at Montefiore, United States
Sushil Selvarajan,
Christian Medical College Hospital, India

*CORRESPONDENCE
Ambroise Wonkam
awonkam1@jhmi.edu

SPECIALTY SECTION
This article was submitted to Hematology, a section of the journal Frontiers in Medicine

RECEIVED 20 April 2022
ACCEPTED 30 June 2022
PUBLISHED 19 July 2022

CITATION
Nguweneza A, Ngo Bitoungui VJ, Mnika K, Mazandu G, Nembaware V, Kengne AP and Wonkam A (2022) Clinical characteristics and risk factors of relative systemic hypertension and hypertension among sickle cell patients in Cameroon. *Front. Med.* 9:924722. doi: 10.3389/fmed.2022.924722

Clinical characteristics and risk factors of relative systemic hypertension and hypertension among sickle cell patients in Cameroon

Arthemon Nguweneza[1], Valentina Josiane Ngo Bitoungui[1], Khuthala Mnika[1], Gaston Mazandu[1], Victoria Nembaware[1], Andre P. Kengne[2] and Ambroise Wonkam[1,3]*

[1]Division of Human Genetics, Department of Pathology, Faculty of Health Sciences, University of Cape Town, Cape Town, South Africa, [2]Non-communicable Diseases Research Unit, South African Medical Research Council, Cape Town, South Africa, [3]McKusick-Nathans Institute and Department of Genetic Medicine, Johns Hopkins University School of Medicine, Baltimore, MD, United States

Increased blood pressure (BP) has been associated with higher risk of stroke and mortality in Sickle Cell Disease (SCD). We investigated risk factors associated with Relative Systemic Hypertension (RSH) or systemic hypertension in SCD patients in Cameroon. Using R, Multivariate multinomial logistic regression modeling was used to examine the effects of the demographic, anthropometric, clinical, and laboratory factors to determine risk factors. A total of 815 individuals with SCD, including 380 (46.6%) males were analyzed. At baseline, the median age [interquartile range] was 18.0 [12.0–25.0] years, ranging from 3 to 66 years. Approximately three-quarters of the patients ($n = 645$; 79.1%) had normal BP, 151 (18.5%) had RSH and 19 (2.3%) had hypertension. Age ($P < 0.001$) and gender ($P = 0.022$) were significantly different across the BP categories. Weight ($P < 0.001$), height ($P < 0.001$), BMI ($P < 0.001$), pulse pressure ($P = 0.020$), history of stroke ($P = 0.012$), hemoglobin level ($P = 0.002$), red blood cell count ($P = 0.031$), creatinine ($P < 0.001$), and (estimated glomerular filtration rate) eGFR ($P = 0.002$) was also significantly different across the three BP categories. After adjustment, the significantly associated factors of RSH in the SCD patients were age [OR = 1.03, (95% CI = 1.01–1.06), $P < 0.010$], male gender [OR = 1.54, (95% CI = 1.04–2.27), $P = 0.029$], BMI [OR = 1.10, (95% CI = 1.04–1.17), $P = 0.001$]. After adjustment, the independent variables significantly associated factors of Hypertension in the SCD patients were age [OR = 1.05, (95% CI = 1.01–1.10), $P = 0.034$], male gender [OR = 3.31, (95% CI = 1.04–10.52), $P = 0.042$], BMI [OR = 1.14, (95% CI = 1.01–1.29), $P = 0.027$]. Creatinine was significantly associated with RSH [OR =1.31 (1.05–1.63), $P = 0.016$]. SCD patients with RSH or hypertension maybe at increased risk of renal dysfunction. We found relatively high prevalence of RSH and hypertension (20.8%) in SCD patients in Cameroon. Tailored Interventions that consider major risk factors (age, gender, and BMI) may lower BP pressure and prevent severe complications.

KEYWORDS

relative hypertension, hypertension, risk factors, sickle cell disease, Cameroon, Africa

Introduction

Sickle cell disease (SCD) patients, generally, have lower systolic, diastolic, and mean blood pressure compared to age and sex-matched controls (1, 2). There are no specific recommendations proposed regarding the defining criteria (and management) of hypertension in SCD patients. The lack of recommendations is a major concern since increased BP has been associated with higher risk of stroke and mortality in SCD patients, even in a range of systolic and diastolic BPs (SBP, DBP) that are considered relatively normal for the general population (i.e., lower than 140 mmHg) (2).

Blood pressure is a potential modulator of clinical severity in SCD patients, recent studies showed that relative systematic hypertension (RSH), defined as BP 120–139/70–89 mmHg, and Systemic Hypertension (BP >140/>90), considerably increased the risk of pulmonary hypertension and renal dysfunction (2). Previous studies have reported demographic, biological, anthropometric, and genetic factors to be associate with blood pressure in SCD patients (3–8). Blood pressure is a heritable trait with estimates of heritability indicating that 30–70% of the trait variance is attributable to genetic variation and a recurrent deleterious and loss of functions mutation with genes associated with lowering BP has been recently associate with long survival in SCD in Africa (9).

Identification of risk factors associated with BP variation in different populations is key to controlling BP, as well as preventing associated causes of mortality in SCD patients. We investigated risk factors associated with RSH or systemic hypertension in SCD patients in Cameroon to gain insight into the pathophysiology of BP variation in this disease in an African setting.

Patients and methods

Ethical approval

A proposal was submitted to the University of Cape Town, Faculty of Health Sciences Human Research Ethics Committee, Cape Town, South Africa (HREC/REF: R015/2018). All patients older than 18 years signed consent forms, while informed consent was given by the parents or guardians for participants younger than 18 years old, in accordance with the Declaration of Helsinki. This study was approved by the National Ethical Committee of the Ministry of Public Health of Cameroon (No 193/CNE/SE/15).

Written and signed informed consent forms were obtained from adult participants and parents/guardians of minor patients. An assent was also obtained from the participants of more than 7 years old.

Participants' recruitment

All SCD patients with complete socio-demographic, clinical, laboratory variables, and complete systolic and diastolic blood pressure measurements were included in the study. The data were obtained from a cross-sectional study conducted in Cameroon from May 2016 to July 2018. The data were collected from nine hospitals from five cities in Cameroon, including Yaoundé, Douala, Bafoussam, Bertoua, and Maroua. Patients who have not experienced a painful crisis a month before, and who had not received a blood transfusion in the past 6 months, were recruited irrespective of age and gender.

Use of variables

Dependent variables

Sickle cell disease patients with a SBP within the range of 120–139 mmHg and/or DBP within the range of 80–89 mmHg is defined as having RSH. Systemic hypertension is further defined as SBPs greater than 140 mmHg or DBPs greater than 90 mmHg. Participants who had incomplete/out of range blood pressure readings were excluded from the analysis.

Independent variables

Information on demographics, including age, residential location, sex, ethnicity, educational level, marital status, and household income status, was collected using a standard questionnaire involving the household and individual levels. Clinical information and laboratory information were also collected. Those who had incomplete/out of range relevant information such as age, gender, BMI, demographic, clinical, laboratory information were also excluded from the dataset.

Statistical analysis

All our analysis was analyzed using R (version 4.0.2). Continuous variables were presented as median and interquartile range (IQR) and categorical variables as percentages (%).

Categorical variables were compared using X^2-test or Fisher exact test if the expected count in a cell was less than five while continuous variables were compared according to BP category with the Kruskal–Wallis test.

Multivariate multinomial logistic regression modeling was used to examine the effects of the demographic, anthropometric, clinical, and laboratory factors to determine the potential independent risk factors for RSH and Systemic hypertension.

A final model was created that included all the predictors and interactions that were significantly associated at the level of $P <$

TABLE 1 Baseline demographic, anthropometric, clinical and laboratory characteristics of Cameroonian SCD patients by BP levels.

Characteristics	All (*n*/*N*, %)	Normal (*n* =, %) or median (IQR)	RSH (*n* = , %) or median (IQR)	Hypertension (*n* = , %) or median (IQR)	*P*-value
Demographics					
Age, years	815/815 (100.0)	17.0 (11.0–24.0)	22.0 (18.0–28.0)	24.0 (18.0–40.5)	<0.001
Aged less than 18	373/815 (45.8)	336/645 (52.0)	33/151 (22.0)	4/19 (21.1)	<0.001
Aged older or equal 18	442/815 (54.2)	309/645 (48.0)	118/151 (78.0)	15/19 (78.9)	
Gender, male	380/815 (46.6)	289/645 (44.7)	77/151 (51.3)	14/19 (73.7)	0.022
Anthropometric and clinical					
Weight	815	46.0 (30.0–55.0)	56.0 (50.0–62.0)	60.0 (50.0–68.0)	<0.001
Height	815	1.58 (1.37–1.67)	1.67 (1.60–1.73)	1.64 (1.58–1.77)	<0.001
Body mass index (BMI)	815	18.0 (16.0–20.0)	20.0 (18.0–22.0)	21.0 (19.5–23.0)	<0.001
Pulse pressure[a]	789	91.0 (81.3–101)	88.0 (80.3–96.8)	84.0 (76.0–92.5)	0.020
History of stroke[a]	28/803 (3.5)	20/637 (3.1)	5/147 (3.4)	3/19 (15.8)	0.012
History of kidney disease[a]	82/807 (10.2)	69/640 (10.8)	10/148 (6.8)	3/19 (15.8)	0.246
History of Pulmonary hypertension[a]	68/807 (8.4)	52/640 (8.1)	13/148 (8.8)	3/19 (15.8)	0.4888
History of transfusion[a]	634/813 (78.0)	504/644 (78.3)	118/150 (78.7)	12/19 (63.2)	0.286
Hydroxuria[a]	72 /807 (8.9)	58/640 (9.1)	14/148 (9.5)	0/19 (0.0)	0.381
Biological data					
Hemoglobin (g/dl)	797/815	7.60 (6.80–8.50)	8.00 (7.10–8.90)	8.10 (7.80–10.8)	0.0020
Hemoglobin F (%)	794/815	6.40 (3.80–11.6)	6.30 (3.40–11.5)	8.35 (4.73–12.3)	0.459
White blood cell count (10^9/L)	797/815	10.3 (7.80–13.0)	9.80 (7.77–12.6)	9.45 (8.30–11.3)	0.637
Mean corpuscular volume (fl)	798/815	88.0 (82.0–95.0)	89.0 (83.0–95.0)	85.0 (77.5–92.0)	0.360
Red blood cell count	794/815	2.70 (2.30–3.13)	2.83 (2.40–3.19)	2.87 (2.63–3.38)	0.031
Creatinine (mg/dl)	770/815	0.45 (0.37–0.60)	0.60 (0.40–0.78)	0.65 (0.50–0.87)	<0.001
(Estimated glomerular filtration rate) eGFR	760/815	175 (151–204)	158 (136–178)	150 (119–182)	0.002

[a]Total number of children may differ because of missing data; IQR, interquartile range.

0.05. The findings presented as crude and adjusted odds ratios with their 95% confidence intervals (CI).

Results

Baseline characteristics

Table 1 Shows the demographic, anthropometric, clinical and laboratory characteristics of the BP categories. Our analysis included 815 individuals with SCD, of whom 645 (79%) had normal BP, 151 (19%) had RSH, 19 (2%) had systemic hypertension. 380 (46.6%) were males. At baseline, the median age [interquartile range] was 18.0 [12.0–25.0] years, ranging from 3 to 66 years. Approximately three-quarters of the patients (645 or 79.1%) were normal BP, 151 (18.5%) had relative hypertension and 19 (2.3%) had hypertension. Age ($P < 0.001$) and gender ($P = 0.022$) were significantly different across the BP categories, with age increasing with BP. Weight ($P < 0.001$), height ($P < 0.001$), BMI ($P < 0.001$), pulse pressure ($P = 0.020$), history of stroke ($P = 0.012$), hemoglobin ($P = 0.002$), red blood cell count ($P = 0.031$), creatinine ($P < 0.001$), and eGFR ($P = 0.002$) were also significantly different across the three BP categories.

Univariate and multivariate analysis

The normal BP group vs. RSH group

Among SCD patients, univariate analyses indicated that these variables were significantly more common risk factors for higher BP values among patients with RSH than those with normal BP: Age ($P < 0.001$), patients >18 years ($P < 0.001$), weight ($P < 0.001$), height ($P < 0.001$), BMI ($P < 0.001$), pulse pressure ($P = 0.046$), creatinine ($P < 0.001$), eGFR ($P < 0.001$) and hemoglobin ($P = 0.020$) (Table 2). Multivariate analyses found that age [OR = 1.02, (95% CI = 1.01–1.05), $P = 0.021$], creatinine [OR = 1.310, 95% CI = 1.05–1.63, $P = 0.016$], BMI [OR = 1.09, (95% CI = 1.03–1.16), $P = 0.002$] were independent risk factors for high BP values in SCD patients with RSH compared with SCD patients with normal BP values (Table 3).

TABLE 2 Univariate multinomial logistic regression analyses of factors associated with RSV and hypertension among SCD patients in Cameroon (reference: Normal BP).

Factors	RSH vs. normal		Hypertension vs. normal BP	
	cOR (95% CI)	*P*-value	cOR (95% CI)	*P*-value
Demographics				
Age*	1.05 (1.03–1.06)	<0.001	1.08 (1.04–1.12)	<0.001
Aged less than 18 (ref.)				
Aged older or equal 18*	3.89 (2.56–5.89)	<0.001	4.07 (1.34–12.41)	0.013
Gender, female (ref.)				
Gender, male*	1.28 (0.89–1.83)	0.170	3.45 (1.22–9.69)	0.019
Anthropometric and clinical				
Weight* (kg)	1.05 (1.04–1.07)	<0.001	1.07 (1.03–1.11)	<0.001
Height (m)*	76.1 (21.77–265.89)	<0.001	15.9 (0.96–264.38)	0.054
Body mass index*	1.16 (1.10–1.23)	<0.001	1.22 (1.11–1.35)	<0.001
Pulse pressure*	0.98 (0.97–1.00)	0.046	0.95 (0.92–1.00)	0.019
History of stroke* vs. No (ref)	1.03 (0.38–2.77)	0.957	5.59 (1.51–20.66)	0.010
History of kidney disease vs. No (ref)	0.60 (030–1.19)	0.145	1.57 (0.44–5.54)	0.480
History of pulmonary hypertension vs. No (ref)	1.11 (058–2.10)	0.749	2.20 (0.26–7.78)	0.223
History of transfusion vs. No (ref)	1.00 (0.65–1.54)	0.985	0.47 (0.18–1.23)	0.128
Hydroxuria	1.00 (0.53–1.89)	0.992	0.00 (−7.07–1.7)	0.762
Biological data				
Hemoglobin (g/dl)*	1.12 (1.01–1.23)	0.020	1.36 (1.14–1.61)	<0.001
Hemoglobin F (%)	0.99 (0.97–1.03)	0.746	1.04 (0.97–1.11)	0.226
White blood cell count (10^9/L)	0.99 (0.95–1.09)	0.782	0.95 (0.85.1.08)	0.485
Mean corpuscular volume (fl)	1.00 (1.00–1.02)	0.528	1.00 (0.94–1.01)	0.062
Red blood cell count*	1.23 (1.00–1.53)	0.061	1.9 (1.24–2.94)	0.03
Creatinine (mg/dl)	1.24 (1.15–1.34)	<0.001	1.38 (1.17–1.62)	<0.001
eGFR	1.00 (0.98–1.00)	<0.001	0.99 (0.98–1.00)	0.007

cOR, Crude odds ratio; CI, Confidence interval; *denotes significant at the 5% level.

The normal BP group vs. systemic hypertension group

Among SCD patients, univariate analyses indicated that these variables were significantly more common risk factors for higher BP values among SCD patients with hypertension than those SCD patients with normal BP: Age ($P < 0.001$), patients >18 years ($P = 0.013$), male gender ($P = 0.019$), weight ($P < 0.001$), BMI ($P < 0.001$), pulse pressure ($P = 0.019$), hemoglobin ($P < 0.001$), creatinine ($P < 0.001$) and Red blood cell count ($P = 0.03$) (Table 2). Multivariate analyses found that age [OR = 1.05, (95% CI = 1.01–1.10), $P = 0.034$], male gender [OR = 3.31, (95% CI = 1.04–10.52), $P = 0.042$], BMI [OR = 1.14, (95% CI = 1.01–1.29), $P = 0.027$] were independent risk factors for higher BP values in SCD patients with hypertension compared with SCD patients with normal BP values (Table 3).

Additionally, Figure 1 Illustrates the relationship between BP and age, gender, BMI. As age increases the probability of SCD patients having RSH or hypertension increases (Figure 1A). Secondly, Males have a higher probability of having RSH or hypertension than females among SCD patients (Figure 1B). For every increase in BMI units, the probability of having RSH or Hypertension increases among SCD patients (Figure 1C). Lastly, for every increase in creatine units, the probability of having RSH or Hypertension increases among SCD patients (Figure 1D).

Discussion

This study determined the role of demographic, anthropometric, clinical and laboratory factors associated with RSH and hypertension among SCD patients in Cameroon, one of the rare attempts from Africa. The main findings, from this relatively large dataset are as follows. Approximately one quarter of our 815 SCD patients were classified in either RSH or systemic hypertension group. At baseline, we observed statistically significant differences in age, gender, weight, height, BMI, pulse pressure, a history of stroke, hemoglobin, and red blood cell count across our three BP groups (normal BP, RSH, hypertension). We found that age, BMI, creatinine, and

TABLE 3 Multivariate multinomial logistic regression analyses of factors associated with RSV and hypertension among SCD patients in Cameroon (reference: Normal BP).

Factors	RSH vs. normal BP		Hypertension vs. normal BP	
	aOR (95% CI)	*P*-value	aOR (95% CI)	*P*-value
Age, years*	1.02 (1.01–1.05)	0.021	1.05 (1.01–1.10)	0.034
Gender, male vs. female (ref.)*	1.20 (1.10–1.80)	0.372	3.31 (1.04–10.52)	0.042
Body mass index (BMI)*	1.09 (1.03–1.10)	0.002	1.14 (1.01–1.29)	0.027
Pulse pressure	1.00 (0.98–1.01)	0.679	0.98 (0.93–1.01)	0.289
History of stroke vs. No (ref)	0.89 (0.30–2.56	0.830	2.24 (0.42–11.79)	0.339
Creatinine (mg/dl)	1.31 (1.05–1.63)	0.016	1.26 (0.75–2.09)	0.373
Hemoglobin (g/dl)	1.00 (0.70–1.40)	0.234	0.88 (0.35–2.47)	0.572
Red blood cell count	1.02 (0.87–1.13)	0.684	1.15 (0.81–2.13)	0.145

aOR, Adjusted odds ratio; CI, Confidence interval; *denotes significant at the 5% level.

male gender were independently associated with an increased risk of RSH and systemic hypertension after adjusting for other variables.

The nearly 19% prevalence of RSH reported in this study was similar to that of 17% reported in studies from North America by Becker et al. (10) and Bodas et al. (11). In similar setting as our study, RSH was lower to that of 45% reported by Benneh Akwasi Kuma et al. (12) and 44% found by Makubi et al. (13), the participants in these studies were adult patients, whereas our study included both pediatric and adult patients. The 2% prevalence of systemic hypertension reported in this study from Cameroon also agrees with previous reports from both high and low incomes settings, which have reported the prevalence of systemic hypertension in SCD patients to be lower than that of the general population (2–8% vs. 28%, respectively) (13–15). Potential explanations of low prevalence of RSH and systemic hypertension in SCD patients include Sodium and water wasting due to the medullary defect, (16) systemic vasodilatation compensating for microcirculatory flow disturbances, (14) increased production of prostaglandins and nitric oxide, (17) reduced vascular reactivity, (16) and premature deaths that remove those individuals whose BP might reach hypertensive levels in middle adulthood (13).

Unsurprisingly, this study also found that age was significantly associated with BP in SCD patients, SCD patients with RSH and systemic hypertension were older than SCD patients with normal BP values suggesting that advancing age contributed to their higher BP values. This finding corroborates with previous reports in developed countries; (1, 8) and in Africa (18), that reported that BP rapidly increases with advancing age in SCD patients starting in the early twenties. With the improved survival of patients with SCD patients, the incidence of RSH or systemic hypertension is expected to rise, thus screening and awareness are necessary to prevent the expected complications, in all part of the world. Indeed, mortality in adult with SCD in the USA and other high-income countries have not changed over the past four decades, mostly dues to debilitating and severe cardiovascular complications (19). Most of the previous data is from 18-year olds. However, younger patients may already show elevated BP and risk for complications.

Pegelow et al. (1) demonstrated that BP values were higher in males than in females, which is consistent with our results showing that male gender is independently associated with RSH and systemic hypertension in SCD patients. This gender disparity in BP is likely due to gender-related differences in SCD biology or health-seeking behavior between genders (20, 21). For instance, older males with elevated BP relative to the SCD population are at increased risk of stroke than age-matched females (12). SCD males have higher pulse pressure, a predictor of all-cause mortality, than age-matched SCD female patients (12, 22) which further highlights the risk of adverse outcomes associated with RSH and systemic hypertension in males. Another study suggests that regular medical visits are critical for improving hypertension awareness among young adults and reducing gender disparities in cardiovascular health (21).

Consistent with previous studies, Oguanobi et al. (23) in Nigeria, and Pegelow et al. (1) who reported that BMI correlates positively with SBP and DBP and Homi et al. who reported that low weight is a risk factor for low BP. In this study, we found that BMI correlates positively with BP, and BMI was independently associated with RSH and systemic hypertension among SCD patients. Suggesting that a higher BMI in SCD patients with RSH or systemic hypertension may contribute to their higher BP values compared to the SCD patients with normal BP. SCD patients have lower BMI compare to general population but increased BMI in SCD patients has potential to modulate BP (8). In addition, the prevalence of obesity in patients with SCD seems to be on the increase. Obesity is a risk factor for other diseases, including, but not limited to, type 2 diabetes, hypertension, sleep apnea, cardiovascular disease (24). These diseases, in turn,

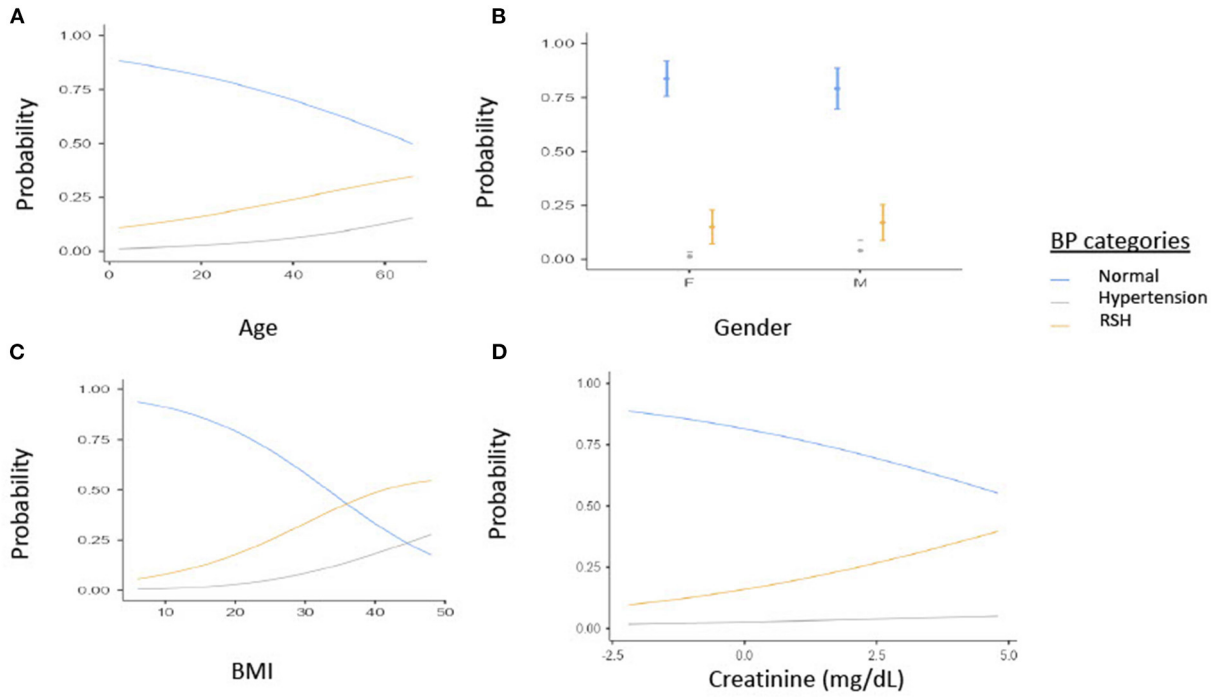

FIGURE 1
Estimated marginal means of **(A)** Age, **(B)** Gender, **(C)** BMI by BP categories, **(D)** creatine (mg/dl). BP, Blood pressure; BMI, Body mass index; RSH, Relative systemic hypertension; F, Females; M, Males.

worsen the clinical picture of SCD and increase the frequency of vaso-occlusive crises (VOCs) (24). Because of the clinical importance as well as public health importance of RSH or systemic hypertension, the ability to identify otherwise normal BMI is of paramount importance, particularly in SCD patients.

Furthermore, measuring BMI alone, in SCD, is sufficient to screen for adiposity and obesity. Previous reports show the body composition of SCD patients with normal mean BMI (22.6 kg/m^2), showed a 32.6% fat composition, indicating high levels of adiposity. Since fat accumulation and adipocyte secretion are responsible for many hormonal changes playing a role in the development of vascular dysfunction and hypertension in the general population, this could be the case in SCD patients too, even if BMI values are normal. Therefore, further studies are needed to better understand the relationship between BMI; hormonal status and BP in SCD.

Previous studies reported that SCD patients with SBP 120–139 mm Hg or DBP 70–89 mm Hg had elevated levels of creatinine compared to SCD patients with SBP <120 mm Hg and DBP <70 mm Hg (2). In this study we found that creatinine was independently associated with RSH. Additionally, SCD patients in RSH and systemic hypertension group had a higher creatinine compared to SCD patients in the normal group. Suggesting that SCD patients with RSH or hypertension are at increased risk of renal dysfunction. Longitudinal studies are needed to better understand temporal relationship between renal dysfunction and RSH.

Previous studies found Increasing hemoglobin, blood viscosity and blood transfusion to be independent risk factors for RSH or hypertension in SCD patients. However, in this study we did not find these factors to be significantly associated with BP among SCD patients (25). These observed differences may be explained by differences in study design, patient's clinical characteristics and thresholds used to define RSH or systemic hypertension.

- Our participants were recruited form referral hospitals. Thus, the findings may not be representative of RSH or systemic hypertension seen in a community. Nevertheless, our analysis is based on the large and well-characterized homozygous study population in a resource-limited country. Therefore, these findings expand the understanding of risk factors for RSH and systemic hypertension in SCD beyond what has been reported from resource-limited settings.
- The exclusion of incomplete records with missing BP might have introduced some bias. Additionally, BP was measured

at single time point which might have increased some patients' likelihood of developing white coat hypertension. Previous studies have highlighted the importance of 24-h ambulatory blood pressure monitoring in diagnosing masked hypertension (26).

- The inability to follow up the cohort as a longitudinal study is a limitation.

In conclusion, this study found evidence of the prevalence of RSH and hypertension in the SCD patients in Cameroon. Age, male gender, BMI was found to be independently associated factors of RSH and hypertension in the SCD patients in Cameroon. Tailored Interventions that consider these risk factors have potential to lower BP pressure in SCD patients and prevent developing severe complications.

Data availability statement

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

Ethics statement

A proposal was submitted to the University of Cape Town, Faculty of Health Sciences Human Research Ethics Committee, Cape Town, South Africa (HREC/REF: R015/2018). All patients older than 18 years signed consent forms, while informed consent was given by the parents or guardians for participants younger than 18 years old, in accordance with the Declaration of Helsinki. This study was approved by the National Ethical Committee of the Ministry of Public Health of Cameroon (No 193/CNE/SE/15). Written and signed informed consent forms were obtained from adult participants and parents/guardians of minor patients. An assent was also obtained from the participants of more than 7 years old. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

AW conceived the study. AN, VN, GM, and AW made substantial contributions to the conception, design of the work, methodology, analysis, data interpretation, and wrote the final manuscript. AN and GM analyzed and interpreted the data. AN issued the first draft of the paper. AN, VN, KM, GM, VN, AK, and AW critically revised successive drafts of the manuscript. VN, GM, AK, and AW supervised the project and compiled the revisions. All authors have read and agreed to the published version of the manuscript.

Funding

This work was supported by the National Heart, Lung, and Blood Institute of the National Institutes of Health (Award Number U24HL135600) and (Award Number 1U24HL135881).

Acknowledgments

Sickle Africa Data Coordinating Center (SADaCC) team, Human genetics Division, Department of Pathology, Health Science Faculty, University of Cape Town.

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.

Publisher's note

References

1. Pegelow CH, Colangelo L, Steinberg M, Wright EC, Smith J, Phillips G, et al. Natural history of blood pressure in sickle cell disease: risks for stroke and death associated with relative hypertension in sickle cell anemia. *Am J Med.* (1997) 102:171–7. doi: 10.1016/S0002-9343(96)00407-X

2. Gordeuk VR, Sachdev V, Taylor J, Gladwin MT, Kato G, Castro OL. Relative systemic hypertension in patients with sickle cell disease is associated with risk of pulmonary hypertension and renal insufficiency. *Am J Hematol.* (2008) 83:15–18. doi: 10.1002/ajh.21016

3. Lamarre Y, Lalanne-Mistrih M, Romana M, Lemonne N, Mougenel D, Waltz X, et al. Male gender, increased blood viscosity, body mass index and triglyceride levels are independently associated with systemic relative hypertension in sickle cell anemia. *PLoS ONE.* (2013) 8:e66004. doi: 10.1371/journal.pone.0066004

4. Bhatnagar P, Barron-Casella EJ, Bean C, Milton JN, Baldwin CT, Steinberg MH, et al. Genome-wide meta-analysis of systolic blood pressure in children with sickle cell disease. *PLoS ONE.* (2013) 8:e74193. doi: 10.1371/journal.pone.0074193

5. Campbell K, Asnani M, Cuningham-Myrie C, Cumming V, Barton EN, Reid M. Determinants of blood pressure in adults with sickle cell disease. *West Indian Med J.* (2007) 56:95. doi: 10.1590/S0043-31442007000300029

6. Novelli EM, Hildesheim M, Rosano C, Vanderpool R, Simon M, Kato GJ, et al. Elevated pulse pressure is associated with hemolysis, proteinuria and chronic kidney disease in sickle cell disease. *PLoS ONE.* (2014) 9:e114309. doi: 10.1371/journal.pone.0114309

7. Lemonne N, Romana M, Lamarre Y, Hardy-Dessources MD, Lionnet F, Waltz X, et al. Association between relative systemic hypertension and otologic disorders in patients with sickle cell-hemoglobin C disorder. *Am J Hematol.* (2014) 89:667–667. doi: 10.1002/ajh.23717

8. Desai PC, Deal AM, Brittain JE, Jones S, Hinderliter A, Ataga KI. Decades after the cooperative study: a re-examination of systemic blood pressure in sickle cell disease. *Am J Hematol.* (2012) 87:65–8. doi: 10.1002/ajh.23278

9. Wonkam A, Chimusa ER, Mnika K, Pule GD, Ngo Bitoungui VJ, Mulder N, et al. Genetic modifiers of long-term survival in sickle cell anemia. *Clin Transl Med.* (2020) 10:e152. doi: 10.1002/ctm2.152

10. Becker AM, Goldberg JH, Henson M, Ahn C, Tong L, Baum M, et al. Blood pressure abnormalities in children with sickle cell anemia. *Pediatr Blood Cancer.* (2014) 61:518–22. doi: 10.1002/pbc.24843

11. Bodas P, Huang A, O'Riordan MA, Sedor JR, Dell KM. The prevalence of hypertension and abnormal kidney function in children with sickle cell disease: a cross-sectional review. *BMC Nephrol.* (2013) 14:237. doi: 10.1186/1471-2369-14-237

12. Benneh-Akwasi Kuma A, Owusu-Ansah AT, Ampomah MA, Sey F, Olayemi E, Nouraie M, et al. Prevalence of relative systemic hypertension in adults with sickle cell disease in Ghana. *PLoS ONE.* (2018) 13:e0190347. doi: 10.1371/journal.pone.0190347

13. Makubi A, Mmbando BP, Novelli EM, Lwakatare J, Soka D, Marik H, et al. Rates and risk factors of hypertension in adolescents and adults with sickle cell anemia in Tanzania: 10 years' experience. *Br J Haematol.* (2017) 177:930–7. doi: 10.1111/bjh.14330

14. Maaten JC, Serné EH, Bakker SJ, van Eps WS, Donker AJ, Gans RO. Effects of insulin on glucose uptake and leg blood flow in patients with sickle cell disease and normal subjects. *Metabolism.* (2001) 50:387–92. doi: 10.1053/meta.2001.21681

15. Bruno D, Wigfall DR, Zimmerman SA, Rosoff PM, Wiener JS. Genitourinary complications of sickle cell disease. *J Urol.* (2001) 166:803–11. doi: 10.1016/S0022-5347(05)65841-7

16. Hatch FE, Crowe LR, Miles DE, Young JP, Portner ME. Altered vascular reactivity in sickle hemoglobinopathy. A possible protective factor from hypertension. *Am J Hypertens.* (1989) 2:2–8. doi: 10.1093/ajh/2.1.2

17. Allon M, Lawson L, Eckman JR, Delaney V, Bourke E. Effects of non-steroidal anti inflammatory drugs on renal function in sickle cell anemia. *Kidney Int.* (1988) 34:500–6. doi: 10.1038/ki.1988.209

18. Akingbola TS, Tayo BO, Salako B, Layden JE, Hsu LL, Cooper RS, et al. Comparison of patients from Nigeria and the USA highlights modifiable risk factors for sickle cell anemia complications. *Hemoglobin.* (2014) 38:236–43. doi: 10.3109/03630269.2014.927363

19. Chaturvedi S, DeBaun MR. Evolution of sickle cell disease from a life-threatening disease of children to a chronic disease of adults: the last 40 years. *Am J Hematol.* (2016) 91:5–14. doi: 10.1002/ajh.24235

20. Ceglie G, Di Mauro M, Tarissi De Jacobis I, de Gennaro F, Quaranta M, Baronci C, et al. Gender-related differences in sickle cell disease in a pediatric cohort: a single-center retrospective study. *Front Mol Biosci.* (2019) 6:1–5. doi: 10.3389/fmolb.2019.00140

21. Everett B, Zajacova A. HHS public access. *Front Mol Biosci.* (2016) 61:1–17. doi: 10.1080/19485565.2014.929488

22. Pikilidou M, Yavropoulou M, Antoniou M, Papakonstantinou E, Pantelidou D, Chalkia P, et al. Arterial stiffness and peripheral and central blood pressure in patients with sickle cell disease. *J Clin Hypertens.* (2015) 17:726–31. doi: 10.1111/jch.12572

23. Oguanobi NI, Onwubere BJC, Ibegbulam OG, Ike SO, Anisiuba BC, Ejim EC, et al. Arterial blood pressure in adult Nigerians with sickle cell anemia. *J Cardiol.* (2010) 56:326–31. doi: 10.1016/j.jjcc.2010.07.001

24. Woods KF, Ramsey LT, Callahan LA, Mensah GA, Litaker MS, Kutlar A, et al. Body composition in women with sickle cell disease. *Ethn Dis.* (2001) 11:30–5. https://pubmed.ncbi.nlm.nih.gov/11289248/

25. Nguweneza A, Oosterwyk C, Banda K, Nembaware V, Mazandu G, Kengne AP, et al. Factors associated with blood pressure variation in sickle cell disease patients: a systematic review and meta-analyses. *Expert Rev Hematol.* (2022) 15:359–68. doi: 10.1080/17474086.2022.2043743

26. Shatat IF, Jakson SM, Blue AE, Johnson MA, Orak JK, Kalpatthi R. Masked hypertension is prevalent in children with sickle cell disease: a Midwest pediatric nephrology consortium study. *Pediatr Nephrol.* (2013) 28:15–120. doi: 10.1007/s00467-012-2275-9

frontiers | Frontiers in Medicine

TYPE Original Research
PUBLISHED 01 August 2022
DOI 10.3389/fmed.2022.931925

OPEN ACCESS

EDITED BY
Hadi Goubran Messiha,
University of Saskatchewan, Canada

REVIEWED BY
Amein AlAli,
Imam Abdulrahman Bin Faisal University, Saudi Arabia
Mohsen Saleh Elalfy,
Ain Sham University, Egypt

*CORRESPONDENCE
Mohamed A. Yassin
yassinmoha@gmail.com

†These authors have contributed equally to this work

SPECIALTY SECTION
This article was submitted to Hematology, a section of the journal Frontiers in Medicine

RECEIVED 29 April 2022
ACCEPTED 11 July 2022
PUBLISHED 01 August 2022

CITATION
Elenga N, Loko G, Etienne-Julan M, Al-Okka R, Adel AM and Yassin MA (2022) Real-World data on efficacy of L-glutamine in preventing sickle cell disease-related complications in pediatric and adult patients. *Front. Med.* 9:931925. doi: 10.3389/fmed.2022.931925

Real-World data on efficacy of L-glutamine in preventing sickle cell disease-related complications in pediatric and adult patients

Narcisse Elenga[1†], Gylna Loko[2], Maryse Etienne-Julan[3], Randa Al-Okka[4], Ahmad M. Adel[4] and Mohamed A. Yassin[5*†]

[1]Centre Hospitalier de Cayenne, Cayenne, France, [2]Centre de reference de la drepanocytose, CHU de la Guadeloupe, Pointe-à Pitre, Guadeloupe, France, [3]Centre de reference de la drepanocytose, CH de Fort de France, Fort de France, France, [4]Department of Pharmacy, NCCCR Pharmacy, Hamad Medical Corporation, Doha, Qatar, [5]Medical Oncology Department—Hematology Section, National Centre for Cancer Care and Research, Hamad Medical Corporation, Doha, Qatar

Background: L-glutamine has been shown to play an important role in the regulation of oxidative stress which is one of the key contributors to the pathophysiology of sickle cell disease (SCD). In a Phase 3 clinical trial, L-glutamine demonstrated a significant reduction in SCD-related complications including vaso-occlusive crises (VOCs), hospitalizations, and acute chest syndrome (ACS) compared to placebo in patients with SCD.

Objective: The primary objective was to confirm the efficacy of L-glutamine (Endari®) therapy in pediatric and adult patients with SCD at follow-up time points of 24, 48 and 72 weeks.

Methods: In the observational study, nineteen patients with SCD were treated orally with L-glutamine twice daily for 72 weeks. Clinical and laboratory parameters were measured at baseline and follow-up time points. Patients with severe VOC and ACS were hospitalized. Blood transfusion was given in case of ACS and uncontrolled pain associated with VOC despite administration of the highest dose of intravenous (IV) narcotic.

Results: Compared to baseline, patients had significantly fewer pain crises (median change from 3.0 to 0.0; $P < 0.00001$), hospitalizations (median change from 3.0 to 0.0; $P < 0.00001$), days of hospitalization (median change from 15.0 to 0.0; $P < 0.00001$), and blood transfusions (median change from 3.0 to 0.0; $P < 0.00001$) at 24, 48, and 72 weeks following L-glutamine therapy. Moreover, there was a drastic decrease in the number of ACS events during this time. A significant increase was observed in mean hemoglobin levels and hematocrit proportions from baseline to 72 weeks ($P < 0.001$). Conversely, compared to baseline, mean reticulocyte counts and lactate dehydrogenase (LDH) levels were considerably lower at follow-up time points ($P = 0.003$ and $P < 0.001$, respectively). No patient reported treatment-related adverse events.

Conclusion: Although the sample size was small, our data clearly demonstrated that L-glutamine therapy was safe and significantly improved clinical outcomes and hemolysis parameters in patients with SCD.

KEYWORDS

L-glutamine, sickle cell disease, clinical outcomes, hemolysis parameters, vaso-occlusive crisis

Introduction

Sickle cell disease (SCD) is a heterogeneous group of life-threatening inherited blood disorders that is prevalent worldwide and affects millions of people (1). An estimate by Piel et al. (2) predicted the overall number of births affected by SCD to be 14.2 million between 2010 and 2050 (2). SCD occurs due to the presence of abnormal hemoglobin S (HbS) which is formed by a point mutation resulting in a single amino acid substitution (glutamic acid to valine) at position 6 in the gene that encodes for the β-globin chain of hemoglobin (3, 4). Red blood cells (RBCs) undergo alteration in structure and function as a result of stress-induced intracellular polymerization of HbS (4). These deformed ("sickle shaped") RBCs become very adhesive and upon interaction with white blood cells (WBCs) and the endothelium cause chronic hemolysis and occasional microvascular occlusion in multiple body organs (4). These processes can result in serious clinical complications that include acute pain (also called as vaso-occlusive crisis or VOC), tissue ischemia, multi-organ damage, stroke, acute chest syndrome (ACS) (5, 6).

The only approved curative treatment for SCD patients is bone marrow transplantation (BMT), also called as hematopoietic stem cell transplantation (HSCT). However, its application is limited by the cost and availability of few matched donors (7). Transfusion with RBC-rich blood is a useful and growing treatment option for controlling and avoiding SCD complications but this approach also has several limitations (4). The currently available United States Food and Drug Administration (US FDA)-approved therapeutic options for SCD patients include hydroxyurea (HU), L-glutamine oral powder (Endari®), intravenous Crizanlizumab (Adakveo), and Voxelotor/GBT440 (Oxbryta). Among these, HU and Voxelotor target hemoglobin S (HbS) polymerization whereas L-glutamine and Crizanlizumab target vaso-occlusion (4).

Oxidative stress is one of the key contributors to the pathophysiology of SCD and related complications (8, 9). It is reported that patients with SCD experience increased oxidative stress due to increased production of reactive oxygen species, mainly during vaso-occlusion and ACS (8). A previous *in vitro* study revealed that adhesion and damage of RBC membrane were affected by depletion of glutamine and patients with SCD have increased uptake of L-glutamine (9). L-glutamine, a precursor of nicotinamide adenine dinucleotide (NAD), may play an important role in the regulation of oxidative stress by normalizing the altered NAD redox system in patients with SCD (10). Indeed, L-glutamine increases the NADH and NAD redox potential to increase the amount of free glutamine in the blood. Sickle-shaped RBCs take up this free glutamine and use it to generate antioxidant molecules. These new antioxidants help to neutralize the oxidative stress in sickle cells (11). In the pivotal Phase 3 clinical trial, L-glutamine demonstrated significant reduction in pain crises, hospitalizations, and ACS events compared to placebo in patients with SCD with or without HU over a 48-week period (12). In September 2021, a re-analysis of the pivotal Phase 3 clinical data using a similar statistical method that was used for all approved medications (HU, Crizanlizumab, and Voxelotor) demonstrated that L-glutamine decreased the number of VOCs by 45% (13).

Endari® is a pharmaceutical-grade L-glutamine approved by the US FDA in July 2017 for reducing acute complications of SCD in patients 5 years of age and older (14). After this announcement, Endari® became the first approved treatment for children with SCD and the first innovative treatment for adults with SCD in almost two decades.

The objective of this study is to confirm the efficacy of L-glutamine therapy in both pediatric and adult patients with SCD at follow-up time points of 24, 48 and 72 weeks. Here, we present the preliminary results from the analysis of data from 19 patients who were initially enrolled of the120 patients who are planned to be recruited in this study.

Materials and methods

Study design

This observational study was conducted from October 2019 through April 2021 and included patients with confirmed SCD diagnosis with standard laboratory investigations obtained prior to initiation of L-glutamine therapy under the Early Access Programs (EAPs) approved by the local ethics committee (MRC-04-20-1240 in Qatar and Commission Nationale Informatique et Libertés approval Number 3Yj157849 3 in French Guiana). Written informed consent was obtained from patients or their

parents or legal guardian. Patients were treated with L-glutamine and examined at follow-up time points of 24, 48 and 72 weeks.

Treatment plan

Pharmaceutical-grade L-glutamine (Endari®; Emmaus Medical, Inc.) was administered orally twice daily for 72 weeks at a dose recommended in the package insert (0.3 g per kg of body weight per dose). Patients with severe VOC and ACS were hospitalized. Blood transfusion was given in case of ACS and uncontrolled pain associated with VOC despite administration of the highest dose of intravenous (IV) narcotic.

Data collection

The following data were collected at:

(1) Baseline—age, gender, SCD genotype (HbSS), weight, age at the time of diagnosis, HU use and laboratory parameters including hemoglobin levels, hematocrit, WBC count, reticulocyte count, lactate dehydrogenase (LDH) levels. (2) The 12 months prior to therapy initiation (considered as baseline values)—clinical parameters including number of hospitalizations, days spent in hospital, VOCs, ACS events, and number of packed red blood cells (PRBCs) transfusions. (3) Follow-up clinic visits at 24, 48 and 72 weeks—both laboratory and clinical parameters. The data values for clinical parameters at 24, 48, and 72 weeks have been annualized. Severe VOC was defined as either ACS or painful crisis requiring IV narcotic analgesics.

Safety outcomes

Any patient and/or healthcare practitioner-reported adverse events (AEs) or serious adverse events (SAEs) experienced during Endari® treatment and follow-up clinic visits were recorded.

Statistical analysis

It is planned to achieve a target sample size of 120 globally. For the clinical observations, non-parametric analysis was performed using the Friedman test with $P < 0.05$ for statistical significance followed by calculation of mean rank for multiple comparisons. The laboratory observations were analyzed with repeated measures ANOVA at $P < 0.05$ followed by *post-hoc* analysis with Bonferroni correction at $P < 0.02$ for significance. Statistical analysis was performed using MedCalc software Version 20.015 (MedCalc Software Ltd, Belgium).

TABLE 1 Baseline characteristics of patients with sickle cell disease (SCD).

	Patients with SCD ($N = 19$)
Age, years	
Median	17
Range <18 years >18 years	8-54 10 (53%) 9 (47%)
Gender, *n* (%)	
Females	9 (47%)
Males	10 (53%)
SCD genotype	HbSS
Race	
Black	15 (79%)
Arab	4 (21%)
Weight, kg	
Median	50
Range	25-75
Age at time of SCD of diagnosis, *n* (%)	
At Birth	12 (63%)
Endari® dose, g (twice daily), *n* (%)	
10 g	8 (42%)
15 g	11 (58%)
HU therapy at baseline, *n* (%)	
Yes	12 (63%)
No	7 (37%)
HU therapy at follow-up time points, *n* (%)	
Yes	9 (47%)
No	10 (53%)

Results

Baseline characteristics

A total of 19 patients (four patients from Qatar and fifteen patients from French Guiana) with confirmed diagnosis of SCD and having HbSS genotype were initially enrolled and retrospectively analyzed for this study. The 4 patients from Qatar had Arab-Indian haplotype and 15 patients from French Guiana had African haplotype of SCD. All 19 patients completed the study. The median age of patients was 17 years (range 8–54 years) with 53% of patients below 18 years and 47% above 18 years, and median weight was 50 kg (range 25-75 kg). More patients were males than females (53% vs. 47%). Higher proportion of patients received 15 g of L-glutamine twice daily (58%) than patients receiving L-glutamine at 10 g twice daily (42%). At baseline, 63% of the patients were taking HU therapy, but during the follow-up clinic visits only 47% had continued HU therapy in addition to L-glutamine (Table 1).

TABLE 2 Clinical observations of patients with sickle cell disease (SCD) at baseline and follow-up time points.

Clinical endpoints	Follow–up time points				P*	P
	Baseline ($N = 19$)	24 weeks ($N = 19$)	48 weeks ($N = 19$)	72 weeks ($N = 19$)		
Annualized no. of VOCs						
Median (Range)	3(1–14)	0(0–6)	0(0–6)	0(0–6)	<0.00001	<0.05†
Total no. of ACS events (n)	11	NA	2	NA	NA	NA
Annualized no. of hospitalizations						
Median (Range)	3(1–8)	0(0–8)	0(0–6)	0(0–6)	<0.00001	<0.05†
Annualized no. of days spent in hospital						
Median (Range)	15(3–30)	0(0–24)	0(0–24)	0(0–16)	<0.00001	<0.05§
Annualized no. of blood transfusions						
Median (Range)	3(0–6)	0(0–4)	0(0–4)	0(0–0)	<0.00001	<0.05§

* Friedman test.
† Multiple comparisons: 72 weeks vs. baseline, 24 and 48 weeks.
§ Multiple comparisons: baseline vs. 24, 48 and 72 weeks.
NA, Not available.

TABLE 3 Laboratory parameters of patients with sickle cell disease (SCD) at baseline and follow–up time points.

Laboratory parameters	Baseline ($N = 19$) Mean ± SE	24 weeks ($N = 19$) Mean ± SE	Follow–up time points: Change in mean from baseline to 24 weeks	48 weeks ($N = 19$) Mean ± SE	Change in mean from baseline to 48 weeks	72 weeks ($N = 19$) Mean ± SE	Change in mean from baseline to 72 weeks	P*	P
Hemoglobin, g/dL	8.2 ± 0.35	8.7 ± 0.27	0.48 ± 0.20	9.2 ± 0.26	0.92 ± 0.19	8.8 ± 0.33	0.57 ± 0.18	< 0.001	0.0008†
Hematocrit, %	24.1 ± 0.92	26.0 ± 0.86	1.86 ± 0.71	27.9 ± 0.83	3.75 ± 0.58	26.6 ± 1.10	2.49 ± 0.76	< 0.001	<0.0001†
WBC count, x10^9/L	11.9 ± 0.97	10.8 ± 1.10	−1.0737 ± 1.24	9.5 ± 0.83	−2.41 ± 0.71	9.6 ± 0.83	−2.32 ± 1.14	0.149	0.0205†
Reticulocyte count, x10^9/L	284.4 ± 21.72	253.0 ± 19.18	−31.37 ± 21.45	241.6 ± 17.44	−42.8 ± 20.61	203.6 ± 19.85	−80.74 ± 22.39	0.003	0.0121‡
LDH, U/L	561.8 ± 50.68	484.9 ± 43.37	−76.90 ± 38.72	331.2 ± 26.85	−230.63 ± 37.26	436.4 ± 48.95	−125.42 ± 61.89	<0.001	<0.0001†

SE, Standard error.
*Repeated measures ANOVA.
Pairwise comparison of mean difference (Bonferroni correction): †baseline vs. 48 weeks; ‡ baseline vs. 72 weeks.

Clinical observations

Compared to baseline, patients had significantly fewer number of annual pain crises (VOCs) at 24, 48, and 72 weeks following L-glutamine therapy (median change from 3.0 to 0.0; $P < 0.00001$). The *post-hoc* analysis revealed a statistically significant difference at 72 weeks compared to baseline, 24 and 48 weeks ($P < 0.05$) (Table 2). The rate of annual hospitalizations was significantly reduced over 72 weeks from baseline (median change from 3.0 to 0.0; $P < 0.00001$). In addition, after treatment with L-glutamine, patients spent fewer days in hospital compared to baseline (median change from 15.0 to 0.0; $P < 0.00001$) (Table 2). The data at 72 weeks was found to be statistically significant in comparison to baseline and other two time points ($P < 0.05$). Compared to baseline, the number of blood transfusions were significantly lower at follow-up time points of 24, 48- and 72-weeks following L-glutamine therapy (median change from 3.0 to 0.0; $P < 0.00001$) (Table 2). In the year prior to therapy initiation, a total of 11 ACS events were reported in nine patients. However, after 48 weeks of L-glutamine therapy, only two such events were observed (Table 2). The general well-being of patients improved considerably with L-glutamine therapy without any treatment-related AEs (data not shown).

Taken together, in this group of SCD patients, there was a significant decline in the median (Table 2) as well

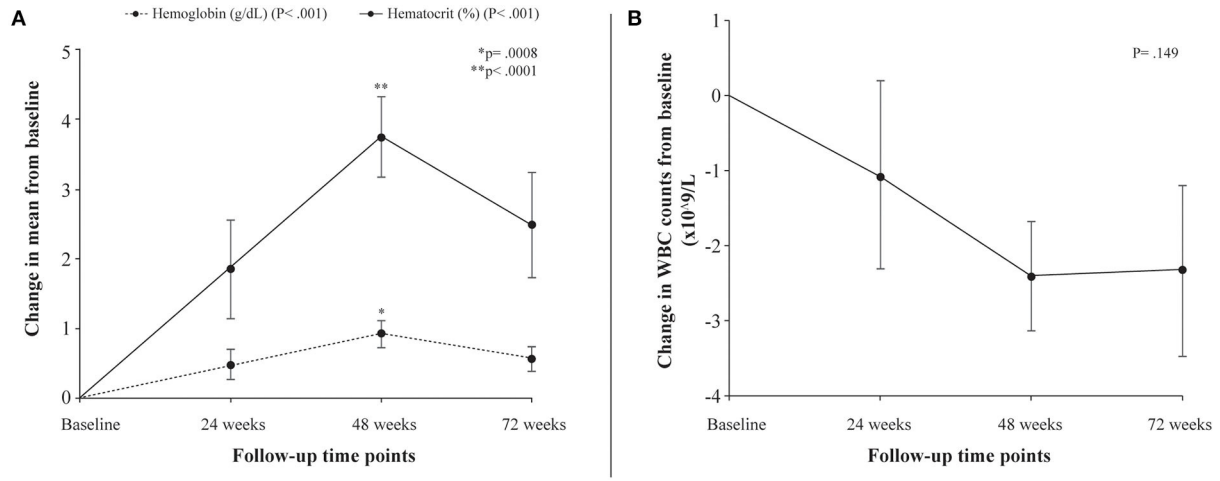

FIGURE 1
Differences in laboratory parameters from baseline to follow-up time points. **(A)** Change in mean levels of hemoglobin (g/dL) and hematocrit (%) from baseline to follow-up time points. **(B)** Change in mean WBC counts from baseline to follow-up time points. P denotes probability values for repeated measures ANOVA; p denotes probability value of pairwise comparison of mean difference (Bonferroni correction) in hemoglobin levels (*) and hematocrit (**) at 48 weeks from baseline.

as average (Supplementary Table 1) number of annual VOCs, hospitalizations, days spent in the hospital, and blood transfusions over 72 weeks from baseline following L-glutamine therapy.

Laboratory observations

Following treatment with L-glutamine, the mean levels of hemoglobin increased significantly from baseline to 72 weeks (8.2 to 8.8 g/dL; $P < 0.001$) with peak mean increase from baseline of 11.2% at 48 weeks. The *post-hoc* analysis showed mean difference in hemoglobin levels from baseline to be statistically significant at 48 weeks ($P = 0.0008$). The mean hematocrit proportions increased markedly from baseline to 72 weeks (24% to 27%; $P < 0.001$) with highest mean improvement from baseline of 15.5% at 48 weeks. The mean difference from baseline was found to be significant at 48 weeks ($P < 0.0001$) (Table 3, Figure 1A). There was a decrease from baseline in the average WBC counts at the follow-up time points but the difference was not statistically significant ($P = 0.149$) (Table 3, Figure 1B). A similar pattern of decline in mean reticulocyte counts from baseline was observed at 24, 48 and 72 weeks (284.4 to 203.6 x10^9/L; $P = 0.003$) with mean difference from baseline reaching statistical significance at 72 weeks ($P = 0.0121$) (Table 3, Figure 2). The mean LDH levels significantly decreased at the follow-up time points compared to baseline (561.8 to 436.4 U/L; $P < 0.001$) and mean difference from baseline was found to be significant at 48 weeks ($P < 0.0001$) (Table 3, Figure 2).

Discussion

This study including 19 patients with SCD and nearly half of the patients receiving HU simultaneously (47%) demonstrated a clinically significant reduction in the median frequency of VOCs at 24, 48, and 72 weeks following L-glutamine therapy compared to the preceding year. Compared to baseline, the number and duration of hospitalizations were significantly reduced at all follow-up time points. These results are consistent with published data which indicates that about 95% of patients with SCD get hospitalized due to severe episodes of pain or VOCs (15) and alleviating VOCs reduces both the frequency and duration of hospitalizations (16). ACS is one of the common reasons for hospitalization and a topmost cause of mortality among patients with SCD (17). In this study, L-glutamine therapy resulted in considerably lesser number of ACS events in patients at the follow-up time points.

Following L-glutamine therapy, significant changes were observed in the hemolysis parameters. Compared to baseline, both hemoglobin and hematocrit increased in patients at 24, 48, and 72 weeks from the start of L-glutamine therapy with peak mean increase from baseline of 11.2% and 15.5% respectively at 48 weeks. The median number of blood transfusions were significantly reduced from 15 in the preceding year to none following treatment with L-glutamine. This would result in fewer transfusion-related adverse events (AEs) and complications (4). Several clinical studies have demonstrated elevated levels of serum LDH in sickle cell patients in steady state due to oxidative stress associated with hemolysis (18, 19). For this reason, LDH is used as a marker of hemolysis in SCD (20). The LDH levels are significantly much higher during painful

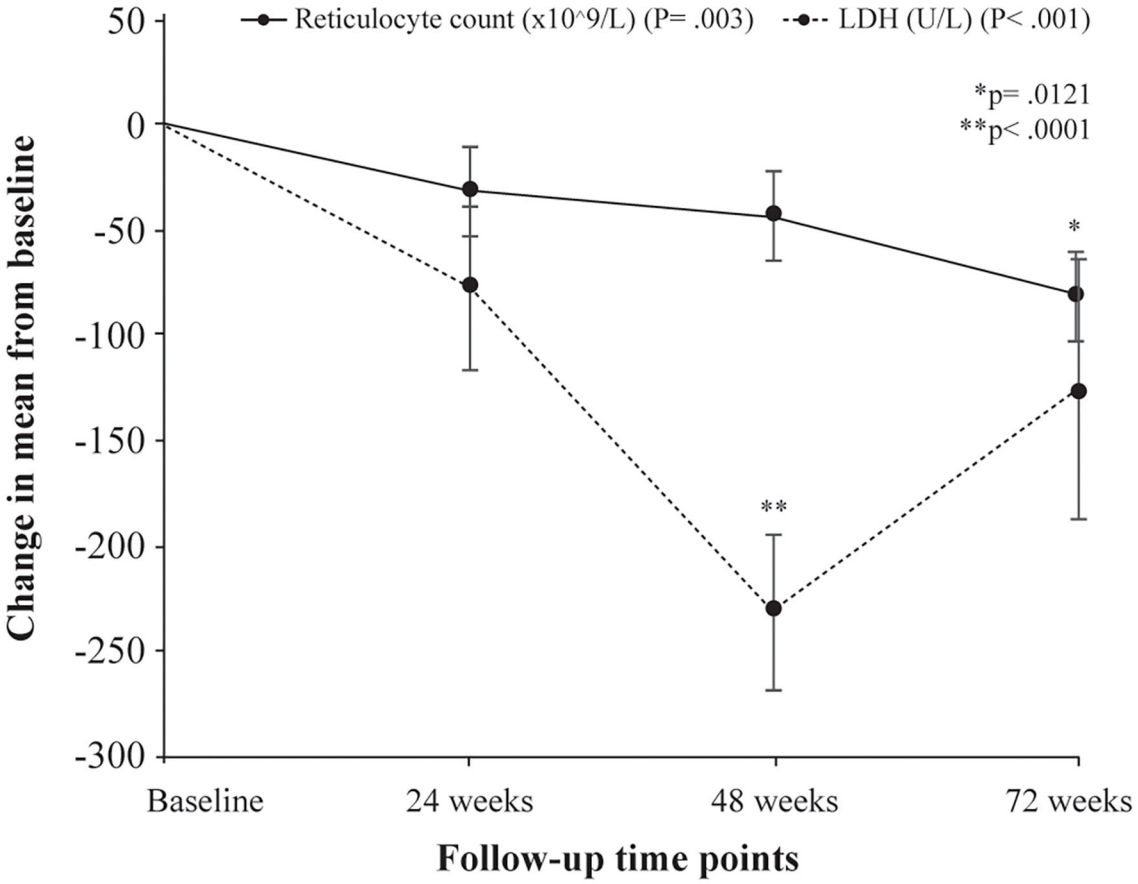

FIGURE 2
Change in mean levels of hemolysis markers (reticulocyte counts and LDH levels) from baseline to follow-up time points. P denotes probability values for repeated measures ANOVA; p denotes probability value of pairwise comparison of mean difference (Bonferroni correction) in reticulocyte counts at 72 weeks from baseline (*) and LDH levels at 48 weeks from baseline (**).

crises (21). In our study, the serum levels of LDH decreased dramatically from baseline at all follow-up time points following L-glutamine therapy (561.8 to 436.4 U/L). This suggested a decrease in hemolysis with an associated reduction in VOC. It is important to note here that improved hemolysis parameters do not always indicate reduced VOCs. As an example, Voxelotor showed improvement in hemolysis with no reduction in VOCs (22), whereas Crizanlizumab showed decreased VOCs without improvement of hemolysis parameters (23).

It is reported that sickle cell patients have higher number of reticulocytes compared to non-sickle cell subjects (24). The increased reticulocytosis is caused by chronic peripheral hemolysis (25). This supports the use of elevated reticulocyte count as a marker of hemolysis in SCD (20). Our results showed significant reduction in reticulocyte counts from baseline through 72 weeks after treatment with L-glutamine. Leukocytes or WBCs contribute to the pathophysiology of SCD (26). In a cohort study, the number of WBCs was higher in SCD patients compared to non-SCD subjects (24). However, reducing the number of leukocytes ameliorates SCD (26). Supporting this, a double-blind randomized clinical study showed the association of decrease in neutrophil count with clinical efficacy of hydroxyurea in SCD patients (27). Consistent with these observations, in the present study, treatment with L-glutamine resulted in mean decrease in WBC counts of up to 20% from baseline to 72 weeks.

There were no L-glutamine-associated AEs reported by patients or healthcare practitioners, thus confirming the reported safety profile (12). Thus, L-glutamine (Endari®) provides beneficial effects in patients unable to receive hydroxyurea or who may have undesirable side effects from hydroxyurea or in addition to hydroxyurea to lower the incidence of pain crises for those who may have partial response to HU (28).

This study provides the description of real-world data on L-glutamine therapy in patients with SCD supporting the previously published results (12). Most importantly, for the first time, the clinical and laboratory findings from this study

demonstrate significant improvement in clinical parameters along with improvement of hemolysis parameters. These promising results may have influenced patients' willingness to continue L-glutamine medication. The small sample size is the main limitation of this study; however, with the anticipated approval of L-glutamine (Endari®) in Qatar, larger studies will be conducted to confirm these encouraging results.

Conclusion

This study demonstrated for the first time that L-glutamine (Endari®) therapy in SCD patients from Qatar and French Guiana resulted in significant improvements in clinical outcomes (number of VOCs, number and duration of hospitalizations, and number of blood transfusions) accompanied by noteworthy increases in hemoglobin levels and reductions in markers of hemolysis (reticulocyte counts and LDH levels). Moreover, there was a drastic decrease in the number of ACS events following L-glutamine therapy. Furthermore, treatment with L-glutamine resulted in clinically significant outcomes from baseline through 72 weeks suggesting sustained long-term efficacy.

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 Local Ethics Committee (Hamad Medical Corporation - PO Box 3050, Doha - Qatar, MRC-04-20-1240 in Qatar and Commission Nationale Informatique et Libertés - 3 Place de Fontenoy - 75007 Paris, France, approval number 3Yj157849 3 in French Guiana). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

NE and MY: conceptualization, investigation, methodology, validation, and writing—review and editing. GL, ME-J, RA-O, and AA: validation and writing—review and editing. All authors contributed to the article and approved the submitted version.

Acknowledgments

The authors would like to acknowledge Turacoz Healthcare Solutions and Emmaus Medical, Inc. for medical writing assistance.

Conflict of interest

NE and MY had received fees for consultancy from Emmaus Medical, Inc.

The remaining 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.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.931925/full#supplementary-material

References

1. Williams TN, Thein SL. Sickle cell anemia and its phenotypes. *Annu Rev Genomics Hum Genet.* (2018) 19:113–47. doi: 10.1146/annurev-genom-083117-021320

2. Piel FB, Hay SI, Gupta S, Weatherall DJ, Williams TN. Global burden of sickle cell anaemia in children under five, 2010-2050: modelling based on demographics, excess mortality, and interventions. *PLoS Med.* (2013) 10:e1001484. doi: 10.1371/journal.pmed.1001484

3. Antwi-Boasiako C, Dankwah GB, Aryee R, Hayfron-Benjamin C, Donkor ES, Campbell AD. Oxidative profile of patients with sickle cell disease. *Med Sci (Basel).* (2019) 7:17. doi: 10.3390/medsci7020017

4. Salinas Cisneros G, Thein SL. Recent advances in the treatment of sickle cell disease. *Front Physiol.* (2020) 11:435. doi: 10.3389/fphys.2020.00435

5. Jang T, Poplawska M, Cimpeanu E, Mo G, Dutta D, Lim SH. Vaso-occlusive crisis in sickle cell disease: a vicious cycle of secondary events. *J Transl Med.* (2021) 19:397. doi: 10.1186/s12967-021-03074-z

6. Ware RE, de Montalembert M, Tshilolo L, Abboud MR. Sickle cell disease. *Lancet.* (2017) 390:311–23. doi: 10.1016/S0140-6736(17)30193-9

7. Gluckman E, Cappelli B, Bernaudin F, Labopin M, Volt F, Carreras J, et al. Sickle cell disease: an international survey of results of HLA-identical sibling hematopoietic stem cell transplantation. *Blood.* (2017) 129:1548–56. doi: 10.1182/blood-2016-10-745711

8. Klings ES, Farber HW. Role of free radicals in the pathogenesis of acute chest syndrome in sickle cell disease. *Respir Res.* (2001) 2:280–5. doi: 10.1186/rr70

9. Morris CR, Suh JH, Hagar W, Larkin S, Bland DA, Steinberg MH, et al. Erythrocyte glutamine depletion, altered redox environment, and pulmonary hypertension in sickle cell disease. *Blood.* (2008) 111:402–10. doi: 10.1182/blood-2007-04-081703

10. Niihara Y, Zerez CR, Akiyama DS, Tanaka KR. Oral L-glutamine therapy for sickle cell anemia: I. Subjective clinical improvement and favorable change in red cell NAD redox potential. *Am J Hematol.* (1998) 58:117–21. doi: 10.1002/(sici)1096-8652(199806)58:2<117::aid-ajh5>3.0.co;2-v

11. Sadaf A, Quinn CT. L-glutamine for sickle cell disease: knight or pawn? *Exp Biol Med (Maywood).* (2020) 245:146–54. doi: 10.1177/1535370219900637

12. Niihara Y, Miller ST, Kanter J, Lanzkron S, Smith WR, Hsu LL, et al. A phase 3 trial of l-glutamine in sickle cell disease. *N Engl J Med.* (2018) 379:226–35. doi: 10.1056/NEJMoa1715971

13. Zaidi AU, Estepp J, Shah N, Alkindi S, Ezzat H, Lam H, et al. A reanalysis of pain crises data from the pivotal l-glutamine in sickle cell disease trial. *Contemp Clin Trials.* (2021) 110:106546. doi: 10.1016/j.cct.2021.106546

14. FDA. *FDA Approves New Treatment for Sickle Cell Disease. FDA News Release 2017.* (2017). Available online at: https://www.fda.gov/news-events/press-announcements/fda-approves-new-treatment-sickle-cell-disease (accessed November 19, 2021).

15. Ballas SK, Lusardi M. Hospital readmission for adult acute sickle cell painful episodes: frequency, etiology, and prognostic significance. *Am J Hematol.* (2005) 79:17–25. doi: 10.1002/ajh.20336

16. Panepinto JA, Brousseau DC, Hillery CA, Scott JP. Variation in hospitalizations and hospital length of stay in children with vaso-occlusive crises in sickle cell disease. *Pediatr Blood Cancer.* (2005) 44:182–6. doi: 10.1002/pbc.20180

17. Vichinsky EP, Neumayr LD, Earles AN, Williams R, Lennette ET, Dean D, et al. Causes and outcomes of the acute chest syndrome in sickle cell disease. National acute chest syndrome study group. *N Engl J Med.* (2000) 342:1855–65. doi: 10.1056/NEJM200006223422502

18. Kato GJ, McGowan V, Machado RF, Little JA. Taylor Jt, Morris CR, et al. Lactate dehydrogenase as a biomarker of hemolysis-associated nitric oxide resistance, priapism, leg ulceration, pulmonary hypertension, and death in patients with sickle cell disease. *Blood.* (2006) 107:2279–85. doi: 10.1182/blood-2005-06-2373

19. Stankovic Stojanovic K, Steichen O, Lefevre G, Bachmeyer C, Avellino V, Grateau G, et al. High lactate dehydrogenase levels at admission for painful vaso-occlusive crisis is associated with severe outcome in adult SCD patients. *Clin Biochem.* (2012) 45:1578–82. doi: 10.1016/j.clinbiochem.2012.07.114

20. Stankovic Stojanovic K, Lionnet F. Lactate dehydrogenase in sickle cell disease. *Clin Chim Acta.* (2016) 458:99–102. doi: 10.1016/j.cca.2016.04.035

21. Ehtesham MA, Lakhkar B, Adkane R. Lactate dehydrogenase as predictor for severity in sickle cell disease. *Int J Cur Res Rev.* (2020) 12:S1–5. doi: 10.31782/IJCRR.2020.SP84

22. Vichinsky E, Hoppe CC, Ataga KI, Ware RE, Nduba V, El-Beshlawy A, et al. A phase 3 randomized trial of voxelotor in sickle cell disease. *N Engl J Med.* (2019) 381:509–19. doi: 10.1056/NEJMoa1903212

23. Ataga KI, Kutlar A, Kanter J, Liles D, Cancado R, Friedrisch J, et al. Crizanlizumab for the prevention of pain crises in sickle cell disease. *N Engl J Med.* (2017) 376:429–39. doi: 10.1056/NEJMoa1611770

24. Mikobi TM, Lukusa Tshilobo P, Aloni MN, Mvumbi Lelo G, Akilimali PZ, Muyembe-Tamfum JJ, et al. Correlation between the lactate dehydrogenase levels with laboratory variables in the clinical severity of sickle cell anemia in congolese patients. *PLoS ONE.* (2015) 10:e0123568. doi: 10.1371/journal.pone.0123568

25. Dhaliwal G, Cornett PA, Tierney LM. Jr. Hemolytic anemia. *Am Fam Physician.* (2004) 69:2599–606.

26. Okpala I. The intriguing contribution of white blood cells to sickle cell disease - a red cell disorder. *Blood Rev.* (2004) 18:65–73. doi: 10.1016/S0268-960X(03)00037-7

27. Charache S, Terrin ML, Moore RD, Dover GJ, Barton FB, Eckert SV, et al. Effect of hydroxyurea on the frequency of painful crises in sickle cell anemia. Investigators of the multicenter study of hydroxyurea in sickle cell anemia. *N Engl J Med.* (1995) 332:1317–22. doi: 10.1056/NEJM199505183322001

28. Cieri-Hutcherson NE, Hutcherson TC, Conway-Habes EE, Burns BN, White NA. Systematic review of l-glutamine for prevention of vaso-occlusive pain crisis in patients with sickle cell disease. *Pharmacotherapy.* (2019) 39:1095–104. doi: 10.1002/phar.2329

frontiers | Frontiers in Medicine

TYPE Opinion
PUBLISHED 08 September 2022
DOI 10.3389/fmed.2022.1002063

OPEN ACCESS

EDITED BY
Robert W. Maitta,
Case Western Reserve University,
United States

REVIEWED BY
Mohamed A. Yassin,
Hamad Medical Corporation, Qatar

*CORRESPONDENCE
Chung Man Chin
chung@unilago.edu.br
Jean Leandro dos Santos
jean.santos@unesp.br

SPECIALTY SECTION
This article was submitted to Hematology,
a section of the journal
Frontiers in Medicine

RECEIVED 24 July 2022
ACCEPTED 22 August 2022
PUBLISHED 08 September 2022

CITATION
Pavan AR, Lopes JR, Lima Imperador CH, Man Chin C and dos Santos JL (2022) Perspectives and challenges to discovering hemoglobin-inducing agents in Sickle Cell Disease. *Front. Med.* 9:1002063. doi: 10.3389/fmed.2022.1002063

Perspectives and challenges to discovering hemoglobin-inducing agents in Sickle Cell Disease

Aline Renata Pavan[1,2], Juliana Romano Lopes[1], Carlos Henrique Lima Imperador[3], Chung Man Chin[1,3]* and Jean Leandro dos Santos[1]*

[1]Department of Drugs and Medicines, School of Pharmaceutical Sciences, São Paulo State University (UNESP), São Paulo, Brazil, [2]Institute of Chemistry, São Paulo State University (UNESP), São Paulo, Brazil, [3]Advanced Research Center in Medicine (CEPAM), School of Medicine, Union of the Colleges of the Great Lakes (UNILAGO), Sao Jose do Rio Preto, SP, Brazil

KEYWORDS

Sickle Cell Disease, fetal hemoglobin, fetal hemoglobin inducers, epigenetic, NO/sCG/cGMP pathway, clinical trials, Cd34 blood cells+

Introduction

Sickle Cell Disease (SCD), a distinct group of β-hemoglobinopathies, includes Sickle Cell Anemia (SCA) and β-thalassemia (1). An estimate of about 300,000 newborns was diagnosed with SCA worldwide, mainly in low-income countries such as sub-Saharan Africa, which contributed to about 75% of this statistic. SCD, a point mutation in the sixth codon of the β-globin gene (GAG to GTG), led to the replacement of glutamic acid by valine in the adult hemoglobin (HbA), thus, forming HbS, which in the deoxygenated state, prone to polymerization, modified the erythrocyte cytoskeleton into the well-known sickle shaped-form. These cells were susceptible to hemolysis after continuous oxy-deoxy cycles, contributing to chronic inflammation and nitric oxide depletion, which would worsen the vascular damage and cause the vaso-occlusion process (1–5).

Inflammation and vaso-occlusion, associated with multisystemic damage, were responsible for the clinical manifestations, including cardiovascular and pulmonary diseases, retinopathy, stroke, pain, acute chest syndrome, nephropathy, and priapism, among others. The diversity of symptoms was associated with the β-globin haplotypes among SCA patients (2). For example, fetal hemoglobin levels (HbF) could range from 0.1 to 30%, and those with SCA phenotypes exhibiting HbF persistence might have minor or lack symptoms (3). The polymorphism in genes associated with the pathophysiology of SCD, those involved in the chronic inflammatory process and vascular endothelial dysfunction, were responsible for the various clinical manifestations. The current research aimed to reduce the disease burden through symptom management to increase the expectancy and quality of life, which is a serious concern, mainly for developing countries, where the child mortality rate could go up to 90% before the age of five (4). For developed countries, such as the US, a reduction of up to 30 years in life expectancy was found by comparing SCD patients with healthy individuals, which seems inconceivable given the scientific progress in recent years (5).

Allogeneic hematopoietic stem cell transplant (allo-HSCT), the curative approach for SCA, could be applied to a small number of patients since about only 20% of them have a healthy HLA-identical sibling donor (6). In addition, the high cost, which was valued in the US estimate at $406,193, chronic graft vs. host disease, and high rates of morbidities made such an approach difficult in the clinical routine (7). Genetically modified autologous stem cells were an alternative for curing that included correcting the mutation associated with the disease through gene editing (i.e., CRISPR-Cas9), restoring HbF production by knockout transcription factors such as BCL11A, or including modified β-globin genes that avoid hemoglobin polymerization (i.e., LentiGlobin BB305) (7, 8). The cost for gene therapy was estimated to be above 1 million USD (6), and access to this strategy was limited, but in the long run, gene therapy could be a safer alternative than allo-HSCT.

Clinical care included focusing on hydration, immunization, blood transfusion, and pain management (9). The most difficult part of SCA care was the limited number of approved drugs to reduce and prevent the symptoms, which included only four drugs, approved by the US FDA: hydroxyurea (HU), L-glutamine, crizanlizumab, and voxelotor (10). Under the preclinical perspective, the drug discovery was focused on preventing HbS polymerization, vascular adhesion, and coagulation; reducing the inflammatory process, oxidative stress, and nitric oxide/sCG/cGMP pathway impacts; and promoting HbF induction (11, 12). HU promoted the HbF induction, a validated approach, for which it acted through pleiotropic effects, including the activation of the enzyme sGC, which increases the level of cGMP; and the downregulation of the silencing transcription factors BCL11A, KLF-1, and MYB (13, 14). The regulation of the expression of the gamma-globin gene was the molecular basis for HbF induction. Medicinal Chemistry approaches were used to design new compounds to inhibit epigenetic enzymes, and transcription factors, act directly on the NO/sCG/cGMP pathway and induce HbF production.

During the preclinical stage, the identification of HbF inducer used phenotypic assays culture cells (i.e., K562; CD34+, HUDEP-2 cells). The preliminary results obtained from screening were validated by a secondary assay using different cell lines since the use of human CD34+ progenitor cells were mandatory to reduce false-positive results. *In vivo* assays using transgenic animals were performed to confirm the efficacy of the HbF-inducing agent. One of the first HbF-inducing agents, except for HU, investigated, was the short-chain fatty acids. The preclinical data suggested its potential as a new drug; however, the irregular and poor pharmacokinetics limited its use in humans. Moreover, the clinical trials of sodium 2,2-dimethylbutyrate revealed a limited effect by increasing at 2% the levels of HbF, and only 2.7% when combined with HU (NCT01322269) (15). Molecular studies for short-chain fatty acids induced HbF through inhibition of histone deacetylase (HDAC) enzymes. HDAC, an epigenetic enzyme constituted of eighteen HDAC isoforms distributed in four classes: I (HDAC-1, 2, 3, and 8), IIA (HDAC-4, 5, 7, and 9), and IIB (HDAC-6 and 10), III (sirtuins 1–7), and IV (HDAC-11). HDAC, acted by removing the acetyl group from ε-N-acetyl-lysine in histone tails, and thus, regulated gene transcription of the γ-globin gene, whose expression provided selective inhibition of HDAC-1 and HDAC-2 (16). Structural requirements to design selective class I, specifically HDAC-1 and HDAC-2, were described elsewhere (17). Compound ACY-957, a 2-aminobenzamide derivative, selectively inhibited HDAC-1 and HDAC-2 with IC50 values of 7 nM and 18 nM, respectively, and showed a favorable pharmacokinetic profile in mammals and rodents in preclinical studies. For example, monkeys treated with ACY-957 (25 and 75 mg/Kg) increased HbF levels, but white blood suppression observed during the treatment disappeared after washout time. Alternative schemes considering non-daily administration were well tolerated (Figure 1).

The inhibition of epigenetic enzymes was pursued, including lysine-demethylase 1 (LSD-1), which had pronounced effects on HbF induction. *In vitro* and *in vivo* studies using transgenic sickle mice validated LSD-1 as a promising target as a HbF-inducing agent (18, 19). A phase I study (US Clinical trial: NCT03132324) was initiated using the LSD-1 inhibitor named INCB059872 but was terminated due to a business decision. Pharmacological interventions were important to regulate γ-globin gene expression, and their interference with several transcription factors, including TR4, BCL11A, KLF-1, MYB, SOX-6, GATA-1, Nrf2, and FOXO3 had been associated with HbF production. However, their involvement was a vital process, and low druggability caused serious concerns about long-term safety in the use of compounds interfering with transcription factors. The drug pomalidomide, which modulates the levels of SOX-6, here, held importance. The additional anti-inflammatory effects, beyond its interference, helped in SCA treatment. Phase I trials using pomalidomide at 4 mg/day for 12 weeks reported a significant increase in the HbF levels (Clinical Trial: NCT01522547) (20).

Guanylate cyclase and the NO/cGMP signaling pathway presented another promising approach to finding out new HbF-inducing agents (Figure 1). The involvement of the soluble guanylate cyclase (sGC) in physiological processes such as vasodilation, platelet, and leukocyte adhesion had beneficial pleiotropic effects beyond HbF induction. Olinciguat, an sGC stimulator able to induce γ-globin mRNA expression up to 2.9-fold at 10 μM, received orphan drug status from the US FDA in 2018 to treat SCA. This drug showed both anti-inflammatory effects and prevented vaso-occlusion events (21, 22). A phase-II trial (Clinical Trial: NCT03285178) was terminated, revealing that the drug was safe and well tolerated by SCA patients. As NO levels were reduced in SCA patients, its reestablishment could improve vascular homeostasis. The beneficial effects of NO-donors to increase HbF levels are accompanied by concerns regarding its adverse

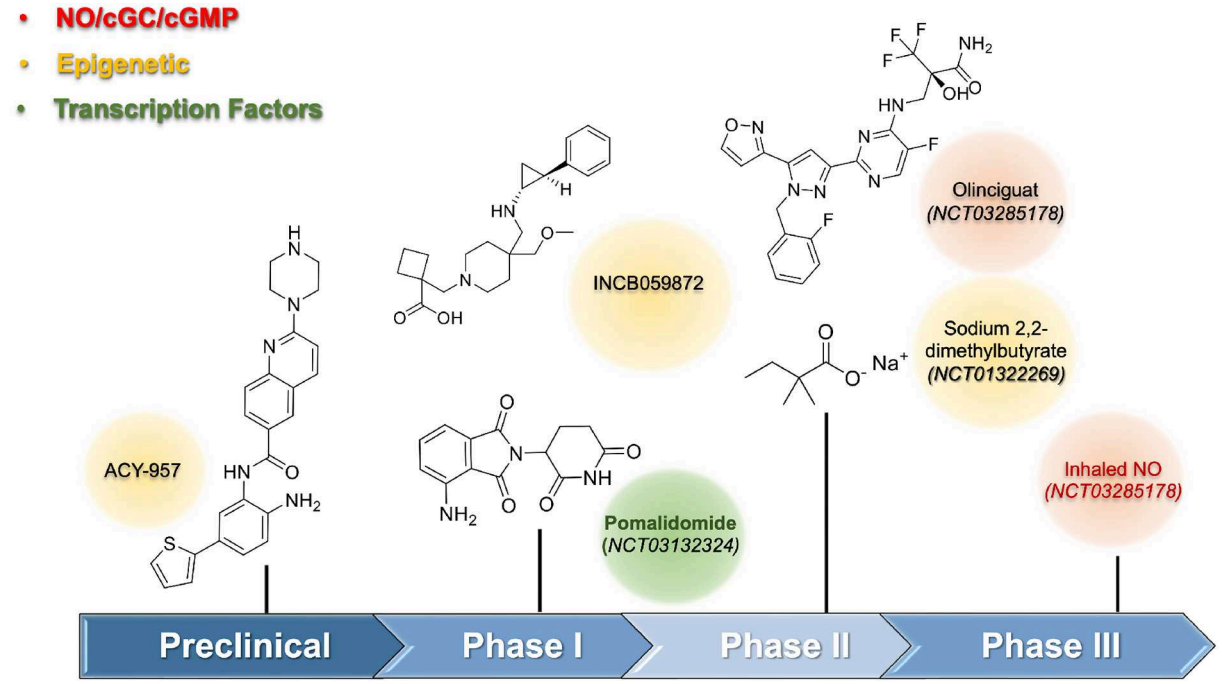

FIGURE 1
Emerging HbF inducers for Sickle Cell Disease.

cardiovascular effects. Thus, the kinetic effects of NO-release as well as the use of appropriate formulations need further investigation. A phase III trial with inhaled NO (Clinical Trial: NCT03285178) did not show a reduction in the time to solve the vaso-occlusive crisis, although other outcomes improved (23).

The current status of some clinical trials found in the US clinical trials database (https://clinicaltrials.gov/) involving HbF-inducing agents was still limited. Some of these studies are investigating the use of HU in pediatric patients (NCT01506544; NCT00305175) or in combination with other drugs (i.e., crizanlizumab - NCT03814746; tadalafil - NCT05142254; and clotrimazole - NCT00004492). It has been estimated that the rate of HU failure is about 30%, however, patients-related issues such as the lack of treatment adherence, adverse-effects, failures to health access and medicines, and a non-optimal dose schemes could be the main reasons to contribute for so high levels of failure, suggesting that rate of non-responsiveness must be lower. The investigations on safe and efficacious new drugs acting as HbF-inducing agents are valuable and could represent an alternative for HU. Despite the expectancy toward the gene editing technologies regarding the cure of genetic diseases, the implementation of this approach into the clinic is expensive and demands medical facilities, highly specialized workers, and involves high risks for patients. Considering the diversity of SCD symptoms, the use of HbF-inducing drugs will represent an alternative for many patients, mainly in low-income countries for the next few years. An efficacious treatment was to take into account interventions due to the multifactorial aspects of SCA and the diversity of phenotypes through various pathways to control the main symptoms in many aspects. There were a lot of perspectives regarding the future of HbF-inducers, and the authors believed that additional efforts to investigate the drug discovery of polypharmacology drugs could provide a promising start. Even after advancements in gene therapy, the use of small molecules would be an important part of the treatment, considering that the diversity of clinical manifestations and the NO/sCG/cGMP pathway to be a promising approach for drug discovery in the future.

Author contributions

Conceptualization and writing—original draft preparation: AP, JL, CL, JS, and CM. Supervision, project administration, and funding acquisition: JS and CM. All authors have read and agreed to the published version of the manuscript.

Funding

AP was supported by the School of Medicine, the Union of the Colleges of the Great Lakes (UNILAGO). SJRP, SP, and the Program for the Scientific Development

(PADC-UNILAGO), Brazil. Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) process numbers 21/100059-9, 20/13279-7, 19/10746-6, and 18/19523-7.

Acknowledgments

We thank the National Council for Scientific and Technological Development (CNPq) for productivity fellowship level 2 [CNPq process numbers: 305174/2020-7 (JS) and 313435/2019-7 (CM), respectively]. We also thank the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) process numbers 21/100059-9, 20/13279-7, 19/10746-6, and 18/19523-7.

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.

Publisher's note

References

1. Eaton WA. Linus pauling and sickle cell disease. *Biophys Chem.* (2002) 100:109–16. doi: 10.1016/S0301-4622(02)00269-7

2. Habara AH, Shaikho EM, Steinberg MH. Fetal hemoglobin in sickle cell anemia: the arab-indian haplotype and new therapeutic agents. *Am J Hematol.* (2017) 92:1233–42. doi: 10.1002/ajh.24872

3. Forget BG. Molecular basis of hereditary persistence of fetal hemoglobin. *Ann N Y Acad Sci.* (1998) 30:38–44. doi: 10.1111/j.1749-6632.1998.tb10460.x

4. Simpson S. Sickle cell disease: a new era. *Lancet Haematol.* (2019) 6:e393–4. doi: 10.1016/S2352-3026(19)30111-5

5. Payne AB, Mehal JM, Chapman C, Haberling DL, Richardson LC, Bean CJ, et al. Trends in sickle cell disease–related mortality in the United States, 1979 to 2017. *Ann Emerg Med.* (2020) 76:S28–36. doi: 10.1016/j.annemergmed.2020.08.009

6. Walters MC, De Castro LM, Sullivan KM, Krishnamurti L, Kamani N, Bredeson C, et al. Indications and results of HLA-identical sibling hematopoietic cell transplantation for sickle cell disease. *Biol Blood Marrow Transplant.* (2016) 22:207–11. doi: 10.1016/j.bbmt.2015.10.017

7. Leibovitch JN, Tambe AV, Cimpeanu E, Poplawska M, Jafri F, Dutta D, et al. L-glutamine, crizanlizumab, voxelotor, and cell-based therapy for adult sickle cell disease: hype or hope? *Blood Rev.* (2022) 53:100925. doi: 10.1016/j.blre.2021.100925

8. Frangoul H, Altshuler D, Cappellini MD, Chen Y-S, Domm J, Eustace BK, et al. CRISPR-Cas9 gene editing for sickle cell disease and β-thalassemia. *N Engl J Med.* (2021) 384:252–60. doi: 10.1056/NEJMoa2031054

9. Pinto VM, Balocco M, Quintino S, Forni GL. Sickle cell disease: a review for the internist. *Intern Emerg Med.* (2019) 14:1051–64. doi: 10.1007/s11739-019-02160-x

10. Ali MA, Ahmad A, Chaudry H, Aiman W, Aamir S, Anwar MY, et al. Efficacy and safety of recently approved drugs for sickle cell disease: a review of clinical trials. *Exp Hematol.* (2020) 92:11–8. doi: 10.1016/j.exphem.2020.08.008

11. Pavan AR, dos Santos JL. Advances in sickle cell disease treatments. *Curr Med Chem.* (2021) 28:2008–32. doi: 10.2174/0929867327666200610175400

12. Bosquesi PL, Melchior ACB, Pavan AR, Lanaro C, de Souza CM, Rusinova R, et al. Synthesis and evaluation of resveratrol derivatives as fetal hemoglobin inducers. *Bioorg Chem.* (2020) 100:103948. doi: 10.1016/j.bioorg.2020.103948

13. Pule GD, Mowla S, Novitzky N, Wonkam A. Hydroxyurea down-regulates BCL11A, KLF - 1 and MYB through miRNA-mediated actions to induce γ-globin expression: implications for new therapeutic approaches of sickle cell disease. *Clin Transl Med.* (2016) 5:15. doi: 10.1186/s40169-016-0092-7

14. Pule GD, Mowla S, Novitzky N, Wiysonge CS, Wonkam A. A systematic review of known mechanisms of hydroxyurea-induced foetal haemoglobin for treatment of sickle cell disease. *Expert Rev Hematol.* (2015) 8:669–79. doi: 10.1586/17474086.2015.1078235

15. Kutlar A, Reid ME, Inati A, Taher AT, Abboud MR, El-Beshlawy A, et al. A dose-escalation phase IIa study of 2,2-dimethylbutyrate (HQK-1001), an oral fetal globin inducer, in sickle cell disease. *Am J Hematol.* (2013) 88:255–60. doi: 10.1002/ajh.23533

16. Bradner JE, Mak R, Tanguturi SK, Mazitschek R, Haggarty SJ, Ross K, et al. Chemical genetic strategy identifies histone deacetylase 1 (HDAC1) and HDAC2 as therapeutic targets in sickle cell disease. *Proc Natl Acad Sci U S A.* (2010) 107:12617–22. doi: 10.1073/pnas.1006774107

17. Ho TCS, Chan AHY, Ganesan A. Thirty years of HDAC inhibitors: 2020 insight and hindsight. *J Med Chem.* (2020) 63:12460–84. doi: 10.1021/acs.jmedchem.0c00830

18. Rivers A, Vaitkus K, Ibanez V, Ruiz MA, Jagadeeswaran R, Saunthararajah Y, et al. The LSD1 inhibitor RN-1 recapitulates the fetal pattern of hemoglobin synthesis in baboons (P. anubis). *Haematologica.* (2016) 101:688–97. doi: 10.3324/haematol.2015.140749

19. Le CQ, Myers G, Habara A, Jearawiriyapaisarn N, Murphy GJ. Inhibition of LSD1 by small molecule inhibitors stimulates fetal hemoglobin synthesis. *Blood.* (2019) 133:2455–9. doi: 10.1182/blood.2018892737

20. Kutlar A, Swerdlow PS, Meiler SE, Natrajan K, Wells LG, Clair B, et al. Pomalidomide in sickle cell disease: phase i study of a novel anti-switching agent. *Blood.* (2013) 122:777. doi: 10.1182/blood.V122.21.777.777

21. Tchernychev B, Feil S, Germano P, Warren W, Lonie E, Feil R, et al. The clinical-stage sGC stimulator IW-1701 prevents increase of plasma biomarkers of intravascular inflammation and suppresses leukocyte-endothelial interactions in tnfα-treated mice. *Blood.* (2017) 130:447. doi: 10.1182/blood.V130.Suppl_1.447.447

22. Mittleman RS, Wilson P, Sykes K, Mihova M, Chickering JG, Ruff D, et al. Multiple-ascending-dose study of the soluble guanylate cyclase stimulator, iw-1701, in healthy subjects. *Blood.* (2017) 130:3533. doi: 10.1182/blood.V130.Suppl_1.3533.3533

23. Gladwin MT, Kato GJ, Weiner D, Onyekwere OC, Dampier C, Hsu L, et al. Nitric oxide for inhalation in the acute treatment of sickle cell pain crisis: a randomized controlled trial. *JAMA.* (2011) 305:893–902. doi: 10.1001/jama.2011.235

frontiers | Frontiers in Medicine

TYPE Case report
PUBLISHED 15 September 2022
DOI 10.3389/fmed.2022.931924

OPEN ACCESS

EDITED BY
Hollie Marie Reeves,
University Hospitals Cleveland Medical Center, United States

REVIEWED BY
Salam Alkindi,
Sultan Qaboos University, Oman
Nicola Conran,
State University of Campinas, Brazil

*CORRESPONDENCE
Awni Alshurafa
dr.a.shurafa@gmail.com
Mohamed A. Yassin
yassinmoha@gmail.com

SPECIALTY SECTION
This article was submitted to Hematology, a section of the journal Frontiers in Medicine

RECEIVED 24 May 2022
ACCEPTED 15 August 2022
PUBLISHED 15 September 2022

CITATION
Alshurafa A and Yassin MA (2022) Case report: Safety and efficacy of voxelotor in a patient with sickle cell disease and stage IV chronic kidney disease. *Front. Med.* 9:931924. doi: 10.3389/fmed.2022.931924

Case report: Safety and efficacy of voxelotor in a patient with sickle cell disease and stage IV chronic kidney disease

Awni Alshurafa * and Mohamed A. Yassin *

Hamad Medical Corporation, Doha, Qatar

Sickle cell disease (SCD) is a heterogeneous group of inherited disorders characterized by the production of sickle hemoglobin which is less soluble than an adult or fetal hemoglobin. Voxelotor is a hemoglobin S polymerization inhibitor that has been approved for sickle cell disease treatment in the adult and adolescent populations. It acts as a hemoglobin modulator by increasing its affinity to oxygen which prevents red blood cells from sickling. Chronic kidney disease is a common but under-reported complication of SCD and it is a leading cause of morbidity and mortality. The data about the safety and efficacy of voxelotor use in chronic kidney disease is limited. Herein we report a 49-year-old man, with sickle cell disease and stage IV chronic kidney disease, who was managed successfully with voxelotor and resulted in decreasing transfusion requirement and vaso-occlusive painful crisis without affecting kidney function.

KEYWORDS

sickle cell disease, voxelotor, hemolysis, chronic kidney disease, vaso-occlusive crisis (VOC)

Introduction

Sickle cell disease (SCD) is a heterogeneous group of inherited disorders characterized by the production of sickle hemoglobin, which polymerizes when deoxygenated resulting in rigid and very fragile red blood cells. Hemoglobin SS is the most common genotype, however, other genotypes like C, D, and E present with different phenotypes of chronic hemolysis and recurrent vaso-occlusive crisis (1, 2). SCD affects millions of people worldwide. It is estimated that the number of patients with SCD in the United States may approach 100,000 (3, 4). SCD is a multi-organ disorder that is associated with high morbidity and mortality. The major clinical features are chronic hemolysis, infection, vaso-occlusive events that cause pain, tissue ischemia, and sometimes infarction (5, 6).

Voxelotor is a hemoglobin S polymerization inhibitor that has been approved for sickle cell disease treatment by the United States Food and Drug Administration (FDA) November 2019 for the adult and adolescent population (≥12 years) (7). It reversibly binds to hemoglobin and increases its affinity to oxygen, stabilizing sickle hemoglobin and preventing polymerization. It can be considered for patients who did not

tolerate hydroxyurea or can be prescribed as an additional treatment for patients who are refractory on maximum doses of hydroxyurea. The most common adverse effects are headache, abdominal pain, nausea, skin rash, and fever (8).

Chronic kidney disease is a common but under-reported complication of SCD and is a leading cause of morbidity and mortality. The annual incidence of acute renal failure and chronic kidney disease was found to be 2–3 times higher compared to the non-sickle group in a retrospective study (9). Renal impairment is a risk for drug toxicity as well as decreased drug efficacy.

The data about the safety and efficacy of voxelotor in chronic kidney disease is limited. Herein we report a case of a 49-year-old man, a known case of sickle cell disease and stage IV chronic kidney disease who was requiring frequent transfusion and suffering from severe body pain, managed successfully with voxelotor without affecting kidney function.

Case report

A patient is a 49-year-old man with SCD (hemoglobin S/D double heterozygous), diabetes mellitus, and hypertension. His clinical course was complicated by chronic kidney stage IV, due to sickle glomerulopathy (baseline creatinine 250–300 umol/L and estimated GFR around 25 ml/min/1.73 m^2), splenectomy at age 20, osteonecrosis of the left femoral head status post left hip replacement at age of 25. The patient required recurrent hospital visits for vaso-occlusive pain crises and blood transfusions despite being compliant with hydroxyurea 1000 mg twice daily.

The patient was started on voxelotor 1,500 mg daily. After starting voxelotor, transfusion frequency decreased from every 2 weeks to every 4 weeks to maintain hemoglobin above 7 g/dL as can be seen in **Figure 1**. The patient reported that his body pain decreased by more than 70% and he did not report any of the known voxelotor side effects such as headache, nausea, diarrhea, or abdominal pain.

TABLE 1 Laboratory results 3 months before, at starting voxelotor, 3 months, and 6 months after.

Time	3 months before	0 months	3 months after	6 months after
Creatinine (umol/L)	262	255	264	251
eGFR (ml/min/1.73 m^2)	25	26	25	26
Hemoglobin (gm/dl)	6.2	6.8	7.6	7.8
Red blood cells ($\times 10^{12}$/L)	2.2	2.4	2.6	2.9
Hematocrit (%)	19.2	19.8	22.5	23.3
White blood cells ($\times 10^9$/L)	8.2	8.8	9.7	7.9
Platelets ($\times 10^9$/L)	151	166	157	170
LDH (U/L)	527	440	516	365
T. bilirubin (μmol/L)	34	39.5	24	22
D. bilirubin (μmol/L)	19	9		

eGFR, estimated glomerular filtration rate; LDH, lactate dehydrogenase; T. bilirubin, total bilirubin; D. bilirubin, direct bilirubin.

Close follow-up of kidney function over 6 months on voxelotor showed that creatinine and GFR remained stable, as seen in **Table 1**.

Discussion

Polymerization of the deoxygenated sickle hemoglobin is the main driver of the pathophysiology of sickle cell disease. Voxelotor acts as a hemoglobin modulator by increasing its affinity to oxygen which prevents red blood cells from sickling. Voxelotor received FDA approval in late 2019 for sickle cell disease treatment following a multicenter, phase 3, double-blind, randomized, placebo-controlled trial. This trial showed that voxelotor provided a sustained increase in hemoglobin and a decrease in hemolytic markers (10, 11).

Pharmacokinetics and pharmacodynamics studies of voxelotor in healthy people have shown it is a rapidly absorbed

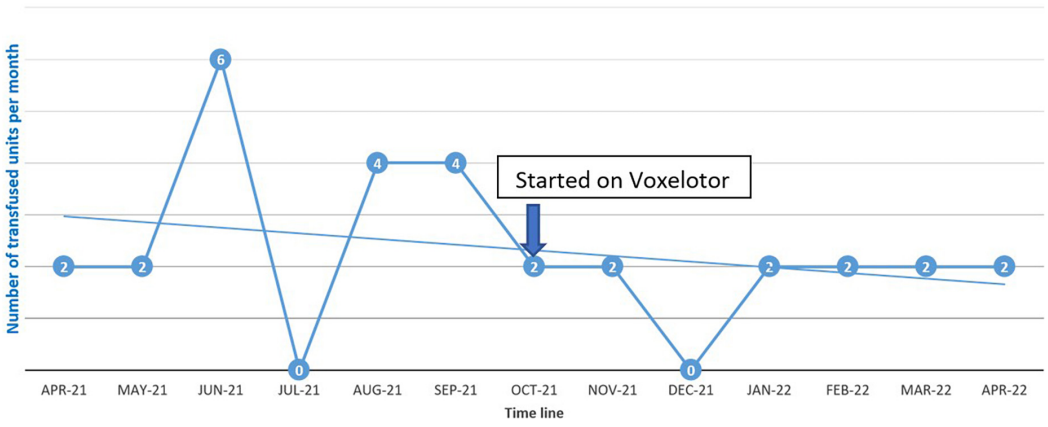

FIGURE 1
Transfusion requirement before and after starting voxelotor.

drug, has an oral bioavailability of 35%, binds to protein at 99.8%, and its post-peak primarily metabolism in the liver. Hepatic metabolism is *via* phase I (oxidation and reduction) and phase II (glucuronidation). Around two-thirds of voxelotor and its metabolites are excreted in feces (62.6%) while around one-third are excreted in the urine (35.4%) after oral administration (12).

The data about the safety and efficacy of voxelotor in chronic kidney disease is limited. Preston et al. reported the only study in this regard (13). Its two open-label, parallel-group, phase I clinical trials, studied the safety and pharmacokinetics of voxelotor in patients with liver or kidney impairment. Considering that impaired kidney function may change voxelotor exposure, a total of eight patients with SCD and chronic kidney disease (estimated GFR < 30 mL/min/1.73 m2) and eight healthy, matched controls were given voxelotor 900 mg daily following an overnight fast. Subjects with normal kidney function were matched to patients with SCD having severe kidney impairment based on age, sex, and body mass index. Mean creatinine was 394 μmol/L and creatinine clearance was 12.5 ml/min in the renal impairment group. End-stage renal disease patients on dialysis were excluded. The result showed no apparent effect of renal function on voxelotor excretion on the basis of similar post-peak mean plasma voxelotor concentration decline between the two groups. There were no serious adverse events (AE), mortality, or medication discontinuation due to AE. Based on these results, Preston et al. (13) concluded that; voxelotor is safe and tolerable in patients with SCD along with severe chronic kidney disease and no dose adjustment is needed.

In our case, the patient had a poor quality of life due to recurrent hospital visits for blood transfusion and a vaso-occlusive painful crisis. After starting a 1500 mg daily dose of voxelotor, his transfusion requirement decreased, and he had a subjective decrease in body pain. In the Preston study, they used the dose of 900 mg daily as it was expected to be within the upper range of the therapeutic dose and was well tolerated in healthy subjects, however, we used the dose of 1,500 mg daily based on the HOPE trial which showed a higher hemoglobin response compared to the dose of 900 mg and pl acebo (7). Indices of kidney function remained stable 6 months after starting treatment which supports the previously reported study. Up to our knowledge, this is the first real-world reported case of voxelotor use in sickle cell disease patients with stage IV chronic kidney disease with the approved dose of voxelotor 1500 mg daily.

Conclusion

In conclusion, considering the limited data, this case report indicates that voxelotor is safe and tolerable, and dose adjustment is not required in patients with SCD along with severe renal impairment; however, further studies are required to confirm this finding.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by the Medical Research Council, Hamad Medical Corporation. The patients/participants provided their written informed consent to participate in this case study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

AA contributed to acquisition of data and drafted the manuscript. Both authors equally contributed to writing and editing.

Funding

Open access funding was provided by the Qatar National Library.

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.

Publisher's note

References

1. Yassin M, Soliman A, De Sanctis V, Nashwan A, Abusamaan S, Moustafa A, et al. Liver Iron Content (LIC) in adults with Sickle Cell Disease (SCD): correlation with serum ferritin and liver enzymes concentrations in Trasfusion Dependent (TD-SCD) and Non-Transfusion Dependent (NT-SCD) patients. *Mediterr J Hematol Infect Dis.* (2017) 9:e2017037. doi: 10.4084/MJHID.2017.037

2. Khamees I, Ata A, Choudry H, Soliman AT, De Sanctis V, Yassin MA. Manifestations of HbSE sickle cell disease: a systematic review. *J Transl Med.* (2021) 19:262.

3. Hassell KL. Population estimates of sickle cell disease in the U.S. *Am J Prev Med.* (2010) 38:S512–21. doi: 10.1016/j.amepre.2009.12.022

4. Wali Y, Kini V, Yassin MA. Distribution of sickle cell disease and assessment of risk factors based on transcranial doppler values in the Gulf region. *Hematol Amst Neth.* (2020) 25:55–62. doi: 10.1080/16078454.2020.1714113

5. Rees DC, Williams TN, Gladwin MT. Sickle-cell disease. *Lancet.* (2010) 376:2018–31. doi: 10.1016/S0140-6736(10)61029-X

6. Yassin MA, Soliman AT, De Sanctis V, Abdula MAJ, Riaz LM, Ghori FF, et al. Statural growth and prevalence of endocrinopathies in relation to liver iron content (LIC) in adult patients with beta thalassemia major (BTM) and sickle cell disease (SCD). *Acta Bio Medica Atenei Parm.* (2018) 89(Suppl 2):33–40. 10.23750/abm.v89i2-S.7085

7. Vichinsky E, Hoppe CC, Ataga KI, Ware RE, Nduba V, El-Beshlawy A, et al. A phase 3 randomized trial of voxelotor in sickle cell disease. *N Engl J Med.* (2019) 381:509–19. doi: 10.1056/NEJMoa1903212

8. Estepp JH. Voxelotor (GBT440), a first-in-class hemoglobin oxygen-affinity modulator, has promising and reassuring preclinical and clinical data. *Am J Hematol.* (2018) 93:326–9. doi: 10.1002/ajh.25042

9. Yeruva SLH, Paul Y, Oneal P, Nouraie M. Renal failure in sickle cell disease: prevalence, predictors of disease, mortality and effect on length of hospital stay. *Hemoglobin.* (2016) 40:295–9. doi: 10.1080/03630269.2016.1224766

10. Blair HA. Voxelotor: first approval. *Drugs.* (2020) 80:209–15. doi: 10.1007/s40265-020-01262-7

11. Ali MA, Ahmad A, Chaudry H, Aiman W, Aamir S, Anwar MY, et al. Efficacy and safety of recently approved drugs for sickle cell disease: a review of clinical trials. *Exp Hematol.* (2020) 92:11–8.e1. doi: 10.1016/j.exphem.2020.08.008

12. Hutchaleelaha A, Patel M, Washington C, Siu V, Allen E, Oksenberg D, et al. Pharmacokinetics and pharmacodynamics of voxelotor (GBT440) in healthy adults and patients with sickle cell disease. *Br J Clin Pharmacol.* (2019) 85:1290–302. doi: 10.1111/bcp.13896

13. Preston RA, Marbury T, Balaranam G, Green M, Dixon S, Lehrer-Graiwer J, et al. Pharmacokinetics of voxelotor in patients with renal and hepatic impairment. *J Clin Pharmacol.* (2021) 61:493–505. doi: 10.1002/jcph.1757

frontiers | Frontiers in Medicine

TYPE Opinion
PUBLISHED 27 September 2022
DOI 10.3389/fmed.2022.892967

OPEN ACCESS

EDITED BY
John Joseph Strouse,
Duke University, United States

REVIEWED BY
Yan Zheng,
St. Jude Children's Research Hospital, United States

*CORRESPONDENCE
Robert W. Maitta
robert.maitta@case.edu

SPECIALTY SECTION
This article was submitted to Hematology, a section of the journal Frontiers in Medicine

RECEIVED 09 March 2022
ACCEPTED 13 September 2022
PUBLISHED 27 September 2022

CITATION
Zhou YY, Reeves HM, Webb L, Santiago Z and Maitta RW (2022) Chronic red cell exchange in sickle cell patients with iron overload may not affect mortality. *Front. Med.* 9:892967. doi: 10.3389/fmed.2022.892967

Chronic red cell exchange in sickle cell patients with iron overload may not affect mortality

Yi Yuan Zhou[1,2], Hollie M. Reeves[1,2], LaRhonda Webb[1], Zamira Santiago[1] and Robert W. Maitta[1,2]*

[1]University Hospitals Cleveland Medical Center, Cleveland, OH, United States, [2]Case Western Reserve University School of Medicine, Cleveland, OH, United States

KEYWORDS

sickle cell, iron overload, survival, red cell exchange, mortality

Introduction

Sickle cell disease (SCD) is an inherited blood disorder that affects ~100,000 Americans (1, 2). In SCD, red blood cells (RBC) containing aberrant sickle hemoglobin (HgbS) become sickle-shaped at low oxygen tension and stick together leading to obstructed blood flow. The resulting decrease in tissue oxygenation causes chronic complications such as vasculo-occlusive pain crises among others that require frequent hospitalizations (3). Chronic RBC transfusions play a prominent role in the treatment of this disease by improving oxygenation through addition of normal RBC and dilution of RBC containing HgbS (4, 5). However, the major disadvantage of management with simple transfusion is the introduction of excess iron into the body which outpaces dedicated mechanisms for iron removal. Thus, over the course of the disease, excess iron is deposited into multiple organs causing organ damage leading to eventual organ failure. By contrast, automated red cell exchange (RCE) is an alternative therapeutic approach that exchanges the patient's sickle RBC with normal RBC reducing the sickle cell RBC more efficiently while being iron neutral (6). Despite the apparent advantage of RCE (7) and inclusion in current management recommendations (8), its benefits for patients with iron overload remains controversial. In this study we examined if RCE improved long-term survival for SCD patients with iron overload treated at our institution.

Methods

The records of patients enrolled in the chronic RCE program at our institution, a tertiary academic medical center, were reviewed to identify patients treated for iron overload over a 2-year period. For each identified patient, multiple parameters were noted and followed including number of RCEs, procedure frequency, program adherence, pre- and post-procedural hematocrit and HgbS, serum ferritin, RBC units used in the exchange, and length of enrollment within the program. Study was approved by our institutional review board.

TABLE 1 Demographics and RCE parameters of study cohort.

Age	Gender	Ht/wt (cm/Kg)	Pre-Hct (%)	Target Hct (%)	Pre-HgbS (%)	Target HgbS (%)	Ferritin (8-150 μg/L)	Units/RCE	Frequency	Duration in program
62	F	162.6/56.2	20 (18–22)	28	15 (14–20)	10 (8–10)	5800	4	4 wks.	14 months
67	F	160/50.3	26 (23–33)	28	2 (1–2)	1	5580	4–5	4 wks.	2 months
30	M	190.5/110.7	23 (19–25)	28	10 (9–13)	5 (3–5)	3435	6–7	4–5 wks.	16 months
40	F	162.6/67.6	30 (26–33)	28	20 (19–22)	10 (9–15)	2941	5	4 wks.	7 months
35	M	162.6/58.1	20 (16–22)	28	5 (5–7)	3 (1–4)	8060	4	4 wks.	19 months
31	M	180.0/71.0	21 (20–22)	28	5 (4–8)	2 (2–4)	4740	4–5	4 wks.	21 months
44.2 56.3:32.0	3:3	169.2/69.0	23.3	28	9.5	5.2	5092.7	5	4	13.2 months

Hct, hematocrit; wt, weight; HgbS, hemoglobin S; pre-HgbS, mean and range is provided; RCE, automated red cell exchange. Target Hct and HgbS represent those expected at completion of RCE. Results represent mean/patient with range provided in parenthesis. Ferritin measurements are provided with normal range on top. For patients who had autopsies findings were consistent with end-stage renal disease, liver hemosiderosis, encephalopathy, myocardial infarction, diffuse intravascular coagulation and acute respiratory distress syndrome.

Results

A total of 6 patients of 11 treated with chronic RCE for iron overload expired during the study period (Table 1). Patients were 3 females and 3 males with mean age of 44.2 years (range 30–67). Females were older at time of death (mean 56.3 years) compared to males (mean 32.0 years). An analysis of patients who expired showed that their pre-RCE HgbS were an average of 9.5% (range 2–20%). Patients received between 4 and 7 RBC units every 4–5 weeks with a target HCT of 28%, average target HgbS of 5.2% (range 1–10%). Overall patients who expired had poor tolerance to iron chelation. Serum ferritin measured toward the end of patients' lives was high (mean 5092.7 μg/L). Functional studies including iron-related imaging were not performed in the patients. The 6 patients who expired participated in the chronic RCE program for a mean duration of 13.2 months (range 2–21 months) and were compliant with scheduled exchanges. Patients died of cardiac complications, multiorgan failure, end stage renal and liver disease despite treatment adherence and maintenance of low HgbS concentration.

Discussion

In our chronic RCE program, over half of patients treated for iron overload expired secondary to long-standing complications. Previous studies found that sudden death due to cardiac failure is the most common adverse event of chronic transfusions and results from chronic deposition of excess iron within the heart (9). Our data concur with these findings as all of the six expired patients died of cardiac complications with underlying multiorgan failure. Additionally, all 6 of the expired patients demonstrated chronic low pre-RCE HgbS concentration suggesting some degree of impaired RBC synthesis. Although conditions of excess iron such as hemochromatosis have been noted as a risk factor for anemia, the exact relationship between the two is still not completely understood (10). Furthermore, even though iron overload most commonly affect liver, heart, and endocrine systems, damage to organs related to hematopoiesis such as the kidneys can also occur (11). Regardless of the etiology for the low pre-RCE HgbS levels, the benefits of RCE in these patients is primarily improving patients' anemia while remaining iron neutral since HgbS at baseline was low. Finally, since RCE does not help actively remove excess iron, our results support reports that indicate that once iron is deposited in tissues the damage is often irreversible (12).

In the literature, data looking at adults treated via automated RCE for iron overload is mostly lacking. We present our experience which suggests a need for much larger studies with greater patients numbers to determine the benefit that RCE has in these patients. It would appear that stronger preventative measures, and earlier intervention against iron overload are more important to improve long-term survival of SCD patients. Although RCE can help slow iron overload-induced organ damage and prevent further iron deposition (8, 13), our data suggests that once sufficient organ damage has occurred, RCE may not have as much potency in improving patients' survival.

Author contributions

YZ and HR gathered data and co-wrote the manuscript. LW and ZS gathered data. RM conceived study, gathered data, and edited manuscript to its final version. All

authors contributed to the article and approved the submitted version.

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.

Publisher's note

References

1. Zheng Y, Maitta RW. Alloimmunisation rates of sickle cell disease patients in the United States differ from those in other geographical regions. *Transfus Med*. (2016) 26:225–30. doi: 10.1111/tme.12314

2. Brousseau DC, Panepinto JA, Nimmer M, Hoffmann RG. The number of people with sickle-cell disease in the United States: national and state estimates. *Am J Hematol*. (2010) 85:77–8. doi: 10.1002/ajh.21570

3. Kauf TL, Coates TD, Huazhi L, Mody-Patel N, Hartzema AG. The cost of health care for children and adults with sickle cell disease. *Am J Hematol*. (2009) 84:323–7. doi: 10.1002/ajh.21408

4. Yawn BP, Buchanan GR, Afenyi-Annan AN, Ballas SK, Hassell KL, James AH, et al. Management of sickle cell disease: summary of the 2014 evidence-based report by expert panel members. *JAMA*. (2014) 312:1033–48. doi: 10.1001/jama.2014.10517

5. Yazdanbakhsh K, Ware RE, Noizat-Pirenne F. Red blood cell alloimmunization in sickle cell disease: pathophysiology, risk factors, and transfusion management. *Blood*. (2012) 120:528–37. doi: 10.1182/blood-2011-11-327361

6. Maitta RW. Current state of apheresis technology and its applications. *Transfus Apher Sci*. (2018) 57:606–13. doi: 10.1016/j.transci.2018.09.009

7. Fasano RM, Leong T, Kaushal M, Sagiv E, Luban NL, Meier ER. Effectiveness of red blood cell exchange, partial manual exchange, and simple transfusion concurrently with iron chelation therapy in reducing iron overload in chronically transfused sickle cell anemia patients. *Transfusion*. (2016) 56:1707–15. doi: 10.1111/trf.13558

8. Chou ST, Alsawas M, Fasano RM, Field JJ, Hendrickson JE, Howard J, et al. American Society of Hematology 2020 guidelines for sickle cell disease: transfusion support. *Blood Adv*. (2020) 4:327–55. doi: 10.1182/bloodadvances.2019001143

9. Kohgo Y, Ikuta K, Ohtake T, Torimoto Y, Kato J. Body iron metabolism and pathophysiology of iron overload. *Int J Hematol*. (2008) 88:7–15. doi: 10.1007/s12185-008-0120-5

10. Khan AA, Hadi Y, Hassan A, Kupec J. Polycythemia and anemia in hereditary hemochromatosis. *Cureus*. (2020) 12:e7607. doi: 10.7759/cureus.7607

11. McLaren GD, Muir WA, Kellermeyer RW. Iron overload disorders: natural history, pathogenesis, diagnosis, and therapy. *Crit Rev Clin Lab Sci*. (1983) 19:205–66. doi: 10.3109/10408368309165764

12. van Tuijn CF, Schimmel M, van Beers EJ, Nur E, Biemond BJ. Prospective evaluation of chronic organ damage in adult sickle cell patients: a seven-year follow-up study. *Am J Hematol*. (2017) 92:E584–E90. doi: 10.1002/ajh.24855

13. Tsitsikas DA, Sirigireddy B, Nzouakou R, Calvey A, Quinn J, Collins J, et al. Safety, tolerability, and outcomes of regular automated red cell exchange transfusion in the management of sickle cell disease. *J Clin Apher*. (2016) 31:545–50. doi: 10.1002/jca.21447

frontiers | Frontiers in Medicine

TYPE Systematic Review
PUBLISHED 29 September 2022
DOI 10.3389/fmed.2022.943631

OPEN ACCESS

EDITED BY
Robert W. Maitta,
Case Western Reserve University,
United States

REVIEWED BY
Ljiljana Vasovic,
Weill Cornell Medical Center,
NewYork-Presbyterian, United States
Mégane Brusson,
INSERM U1163 Institut Imagine, France

*CORRESPONDENCE
Anthony Quagliano
aq181@mynsu.nova.edu
Samiksha Prasad
sprasad@nova.edu

SPECIALTY SECTION
This article was submitted to Hematology, a section of the journal Frontiers in Medicine

RECEIVED 13 May 2022
ACCEPTED 08 September 2022
PUBLISHED 29 September 2022

CITATION
Quagliano A, Acevedo D, Hardigan P and Prasad S (2022) Using Clustered Regularly Interspaced Short Palindromic Repeats gene editing to induce permanent expression of fetal hemoglobin in β-thalassemia and sickle cell disease: A comparative meta-analysis.
Front. Med. 9:943631.
doi: 10.3389/fmed.2022.943631

Using Clustered Regularly Interspaced Short Palindromic Repeats gene editing to induce permanent expression of fetal hemoglobin in β-thalassemia and sickle cell disease: A comparative meta-analysis

Anthony Quagliano*, Daniel Acevedo, Patrik Hardigan and Samiksha Prasad*

Dr. Kiran C. Patel College of Allopathic Medicine, Nova Southeastern University, Fort Lauderdale, FL, United States

β-hemoglobinopathies like sickle cell disease (SCD) and β-thalassemia are characterized by differing mutations in the hemoglobin subunit beta gene (HBB). These disorders vary in phenotypic presentation and severity, with more severe manifestations leading to transfusion dependence along with associated complications such as infection and iron overload. β-hemoglobinopathies symptoms rapidly worsen after birth as the levels of fetal hemoglobin (HbF) begin to decline. To reverse this decline, current treatment plans typically involve the use of pharmacological agents such as hydroxyurea to raise expression levels of HbF. However, these treatments only result in transient effects and must be consistently administered. Gene editing technologies such as CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats- CRISPR associated protein) offer the opportunity to create novel treatments which can raise HbF expression with potential permanent effects. Two gene targets, B-cell lymphoma/leukemia 11A gene (BCL11A) and the promoter regions of gamma globin genes (HBG1/2), have been identified to significantly increase HbF protein expression. In order to differentiate the effectiveness of BCL11A and HBG1/2 editing, a meta-analysis was performed by first identifying 119 studies for inclusion based on the search terms terms "β-Thalassemia," "beta-thal" "sickle cell disease," "SCD," and "CRISPR." Following application of exclusion and inclusion criteria, we performed analysis on 8 peer-reviewed published studies from 2018 to 2021 were included in the study. Forest plots were generated using R (version 4.1.2). Primary comparative analysis shows HBG1/2 had a significantly ($p < 0.01$)greater impact on induction of HbF expression compared to BCL11A.

This analysis leads us to conclude that HBG1/2 merits further investigation as a possible gene editing target for treatment of SCD and β-thalassemia.

KEYWORDS

β-thalassemia, BCL11A, HBG1/2, hemoglobinopathies, fetal hemoglobin (HbF), gene editing (CRISPR/Cas9), sickle cell disease

Introduction/Background

The CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) – Cas (CRISPR-associated protein) systems are part of the adaptive immune system used by archaea and bacteria against foreign genetic elements (e.g., viruses or plasmids). The foreign genetic material is stored in the CRISPR sequence of the host's genome and is referred to as a "spacer." Different spacers are separated by short palindromic repeats. Spacers can be transcribed to form crRNA (CRISPR-RNA). crRNA is then attached to the Cas protein by trcrRNA (trans-activating CRISPR RNA), this complex is used to guide the Cas proteins to the foreign genetic material, which the nucleases (Cas) will then cleave (1–3).

This system can be manipulated by using different gRNAs (guide RNA; dual tracrRNA:crRNA chimera) (4) and different Cas proteins to potentially silence genes, fix mutated genes, or even embed new genes. β-hemoglobinopathies are among the most prevalently inherited disorders. With symptoms typically worsening after birth as fetal hemoglobin (HbF) levels decline, genome editing allows an interesting opportunity to alleviate these symptoms.

Disorders such as Sickle cell disease (SCD) or β-thalassemia are characterized by differing mutations in the hemoglobin subunit beta gene (HBB) on chromosome 11 (5, 6). While these β-hemoglobinopathies have a wide array of phenotypic manifestations, individuals who have more severe manifestations tend to have higher rates of morbidity and mortality (7, 8).

Sickle cell disease is caused by a single base substitution at the 6th codon of the HBB gene. A thymine is switched to an adenine, resulting in a glutamate (hydrophilic) being replaced with a valine (hydrophobic) (9, 10). This mutation, under hypoxic conditions, causes the red blood cells to have their characteristic rigid sickle shape, allowing them to stick to vessel walls and clump together (sickling). This sickling can lead to vaso-occlusive crises, also known as pain crises (6). SCD refers to the full range of potential genotypes involving the mutated HBB gene, not to be confused with sickle cell anemia which refers specifically to being homozygous for this mutated HBB gene.

While SCD is caused by a single point mutation, β-thalassemia has over 300 different reported mutations. Most of these mutations are single point mutations in different functionally important regions of the HBB gene. The frequency of these different mutations varies by population (11, 12). The severity of the illness caused by these respective mutations also varies (13). β-thalassemia is broken up into two types, minor and major. Carriers of a single mutated allele are considered to have β-thalassemia minor. People who are homozygous for the mutated alleles are said to have β-thalassemia major. The end result of these differing mutations is a decreased production of the β-globin chain (5).

Treatment for these disorders typically involves frequent blood transfusions. However, transfusion dependency has many associated complications such as infection or iron overload (12). Other treatments involve the use of pharmacological agents to raise the levels of fetal hemoglobin (HbF), which increases total hemoglobin production while also avoiding the use of the mutated β-globin chain in SCD (12). The effects of these treatments, however, are only transient. However, a number of genes have been identified that can control HbF expression (**Figure 1**). Thus, gene editing technologies allow the opportunity to create a more lasting treatment. In this paper, we will look at the use of CRISPR-Cas9 in inducing long-term expression of HbF, which has been shown to lower morbidity and mortality in β-hemoglobinopathies (14, 15).

The B-cell lymphoma/leukemia 11A gene (BCL11A) and promoters of the gamma globin genes (HBG1 and HBG2) have been largely studied for inducing long-term expression of HbF (16, 17). CRISPR studies involving these genes have had promising results thus far (18, 19). However, Krueppel-like factor 1 (KLF1), zinc-finger protein 410 (ZNF410), ATF4, and Speckle-type POZ protein (SPOP) have all shown to play respective roles in the switch from HbF to HbA and merit further studies using CRISPR (20, 21).

BCL11A encodes a zinc-finger protein which mostly functions as a repressor and is involved in fetal-to-adult hemoglobin switching (16). By directly binding to a TGACCA motif at −115 bp of the *γ-globin* promoter it plays a crucial rule in silencing expression of the γ-globin genes (22). This promotes the formation of adult hemoglobin (HbA) over HbF (23). An interesting extension of this is KLF1, which also plays a role in fetal-to-adult hemoglobin switching by activating BCL11A as well as promoting HBB directly (24).

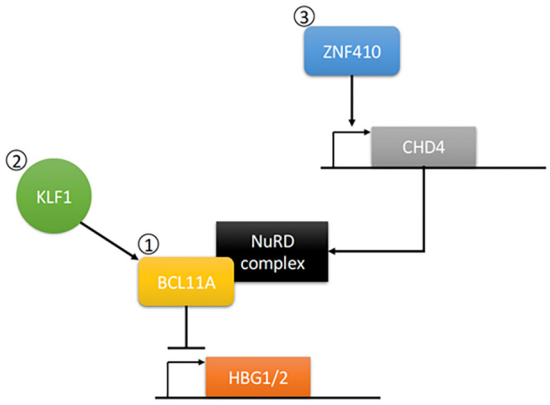

FIGURE 1
Control of Gamma Globin Gene Transcription. Gamma globin production for the generation of fetal hemoglobin (HbF) occurs through the transcription of the HBG1/2 genes. (1) This can be negatively regulated through expression of B-cell lymphoma/leukemia 11A gene (BCL11A), which has been extensively studied in CRISPR knockout models for the induction of gamma globin expression (Frangoul et al. (13), Lamsfus-Calle et al. (25), Khosravi et al. (18), Wu et al. (17). (2) Kruppel-like factor 1 (KLF1) can directly activate gamma globin gene repressors such as BCL11A to play a role in switch from gamma to beta globin. CRISPR knockout studies of KLF1 have showed induction of gamma globin expression [Shariati et al. (21)]. (3) Zinc finger protein 410 (ZNF410) can regulate BCL11A repression of gamma globin gene expression *via* induction of the Nucleosome Remodeling Deacetylase (NuRD) complex component CHD4. BCL11A contains a motif that can interact with a variety of NuRD-associated molecules, thus allowing for activation and then subsequent repression of gamma globin gene expression. CRISPR knockout studies of ZNF410 have identified its role as a possible gene target for induction of gamma globin expression [Lan et al. (20)]. Speckle-type POZ protein (SPOP) not included in the pathway.

HBG1 and HBG2 encode for the γ-chain of HbF, they are homologous except for some differences in the upstream region of their respective distal promoters (25). In order to promote the transcription of these genes, CRISPR/Cas9 was used to knockout parts of the promoter region where repressors would bind. The inspiration for this method came from nature in the form of hereditary persistence of fetal hemoglobin (HPFH), a benign genetic condition in which HbF production persists into adulthood (26).

ZNF410 does not directly bind to HBG1/2, instead, it binds the Chromodomain-helicase-DNA-binding protein 4 (CHD4) gene. In turn activating the CHD4/NuRD (nucleosome remodeling and deacetylase) complex which is used in the repression of HBG1/221) What makes ZNF410 unique to some of the other HBG1/2 repressors is that it is highly specific for the CHD4 gene, to such a degree that it's two binding site clusters are not found elsewhere in the genome (20, 27).

SPOP is a substrate adaptor of the CUL3 ubiquitin ligase complex (28). It acts independently of BCL11A in suppressing HBG1/2 and when depleted was shown to amplify the effects of hydroxyurea and pomalidomide, pharmacological inducers of HbF (29). It is suspected that SPOP-CUL3 promotes the ubiquitination and degradation of transcription factors that activate HBG1/2 (29).

As CRISPR research continues to advance, the number of publications available can be daunting to read through. A meta-analysis allows us to take the data from multiple publications and create a clearer image of the results. Even among studies which may seem conflicting a meta-analysis can yield statistically significant results, said results will also have a better ability to be extrapolated to the larger population.

This meta-analysis would allow us to obtain a larger view of the effects that CRISPR targeting these genes can have on HbF production. While also comparing the potential weaknesses of the different targets, such as the likelihood of producing off-target mutations. This will help to create a clearer path to guide future research in regard to which genes merit further research and which should be discounted.

Materials and methods

Identification of eligible studies

A comprehensive examination of all peer-reviewed studies published through June of 2021 was performed using PubMed. Combinations of the search terms "β-Thalassemia," "beta-thal" "sickle cell disease," "SCD," and "CRISPR" were used to screen for studies for potential inclusion. The searches were completed with no language restrictions and no additional search engine filters.

Inclusion and exclusion criteria

The following exclusion criteria were applied: non-primary studies like reviews, letters to the editor, conference abstracts, studies that did not contain specific CRISPR gene edits, studies without available full-texts, and studies that did not measure any post-CRISPR edit cellular changes. Final inclusion criteria included: assessment of HbF protein levels following CRISPR edit compared to control cells and studies that included CRISPR editing of either or both of BCL11A and HBG1/2. A detailed PRISMA diagram is included in **Figure 2**.

Data extraction

Hemoglobin expression, n number, and corresponding SD values were collected from texts and/or figure legends of studies where included. Studies that did not explicitly indicate mean and/or SD values were extrapolated *via* pixel analysis of relevant graphs using ImageJ. Pixel sizes were determined using two

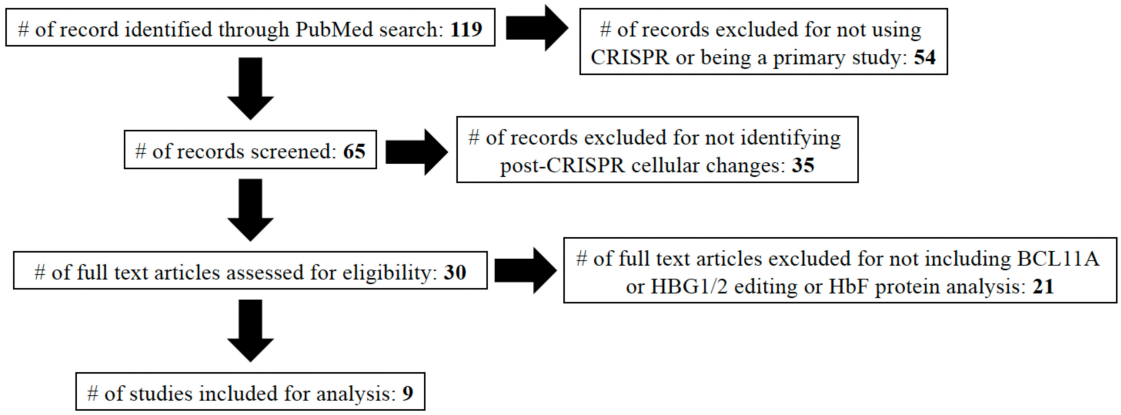

FIGURE 2
PRISMA diagram. In total, 119 studies were identified using the search terms: "β-Thalassemia," "beta-thal," "sickle cell disease," "SCD," and "CRISPR." Studies were then included/excluded based on described characteristics. The nine studies included in the study are referenced (13, 17–19, 25, 30–33).

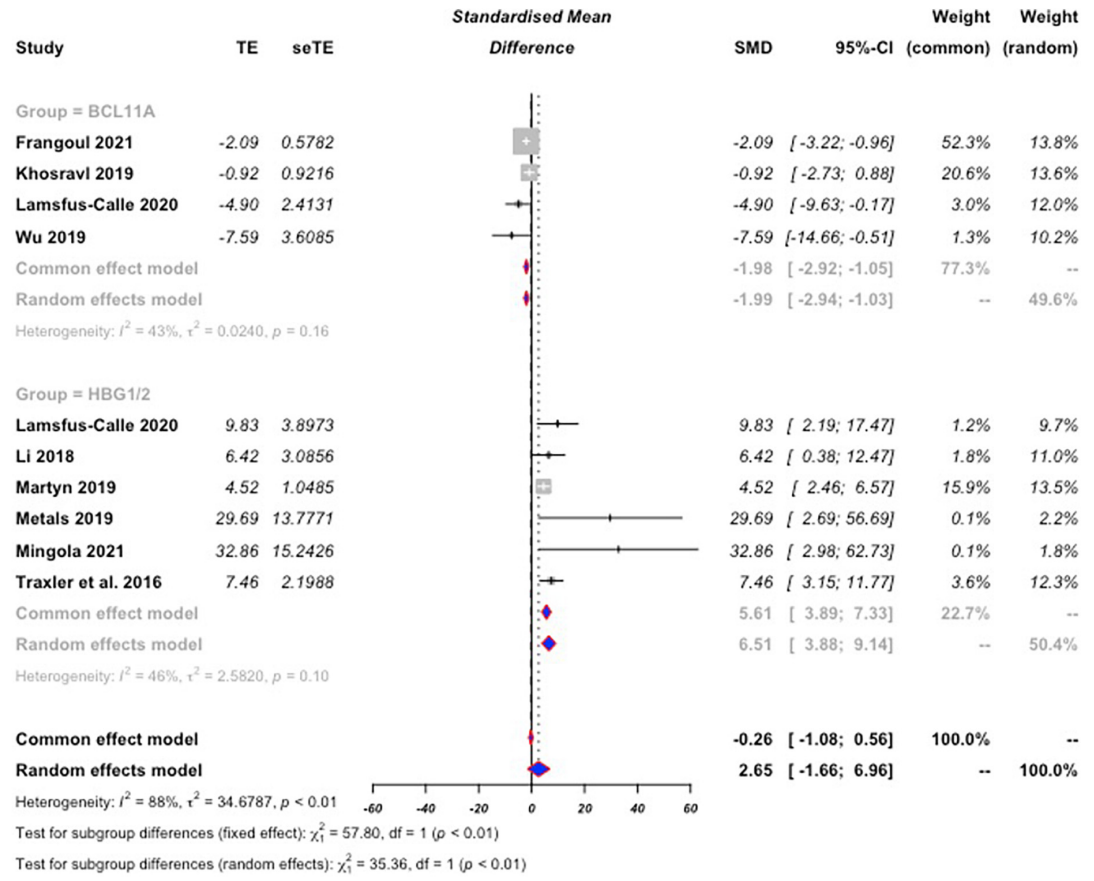

FIGURE 3
Forest plot for BCL11A and HBG1/2. Standard mean difference (SMD) values were calculated for both BCL11A, and HBG1/2 based on percent HbF expression as a total of hemoglobin expression following CRISPR editing compared to baseline. SMD values are negative for BCL11A not as an indication that expression levels dropped, but as a differentiator from the positive values of HBG1/2. The calculated SMD values from the collection of BCL11A studies was then compared to the calculated values from HBG1/2 for comparison of efficacy of HbF expression induction. The random effects model was used due to significant heterogeneity between the included studies. Editing of HBG1/2 significantly increased HbF expression by comparison to BCL11A editing (p < 0.01).

known points (e.g., the distance between 0 and 10% on the *y*-axis is 200, then 1 pixel = 0.05%). Then the pixel distance between a zero value and an unknown value was determined and multiplied by the pixel size (e.g., the distance between 0% and the unknown mean was 50,000, 50,000/200 = 250; 250*0.05% = 12.5%). This method was performed on the following studies: Wu et al. (17), Khosravi et al. (18), Traxler et al. (30), Martyn et al. (31), Li et al. (32), Lamsfus-Calle et al. (25).

Statistical analysis

The standardized mean difference (SMD) was used to quantify effect sizes of combined studies using a random effects model. For this study SMD score of 0.2–0.5 were considered small, 0.5−0.8 medium, and values > 0.8 were considered large. Nevertheless, we conducted and compared both common-and-random-effect models. We employed exact computations for the bias factor to evaluate the studies for heterogeneity, chi-square tests and I^2 statistics were utilized. A value of 0.05 was considered statistically significant for the chi-square tests and I^2 values $\geq$ 75% was indicative of high heterogeneity. Subgroup analysis was performed between BCL11A and HBG1/2 genes. R (version 4.1.2) was employed for all data analysis.

Results

Analysis of literature search

Following application of all exclusion criteria, 30 studies remained that analyzed post-CRISPR edit cellular changes. In an effort to focus on clinical significance of changes, we chose to focus only on studies that included HbF protein expression analysis over those that looked at only mRNA expression. Multiple genes including BCL11A, HBG1/2, and KLF1 had two or more studies analyzing HbF protein expression. However, we opted to only include BCL11A and HBG1/2 for analysis since the lack of studies including KLF1 posed a significant obstacle to sufficient comparative analysis (**Figure 2**).

Post-CRISPR editing HbF values from the included studies came from a variety of *in vitro*, *in vivo*, and *ex vivo* sources. Analysis of protein levels was determined *via* high-performance liquid chromatography in all studies except for Khosravi et al. (18) (western blot) and Mingoia et al. (33) (flow cytometry). HbF values were determined as a percentage of HbF of total hemoglobin production. In order to collect a sufficient amount of data, all studies regardless of cellular source were included. To control sample source variability, a random effects model was applied during the meta-analysis and forest plot generation.

Meta-analysis results

There was significant heterogeneity between the studies (I2 = 87.6% [95% CI: 79.1%,92.6%], $p < 0.01$); therefore, we used a random-effect model to estimate mean differences. Statistical results reveal that both the BCL11A and HBG1/2 showed significant increases in HbF protein expression post-CRISPR editing compared to controls with standardized mean differences of −1.98 [95% CI of −2.94, −1.02] and 6.51 [95% CI of 3.87,9.14], respectively (**Figure 3**). This indicates that a significant effect in HbF induction was achieved by comparison to baseline expression levels. A significant difference was also found between BCL11A and HBG1/2 ($p < 0.01$), suggesting that CRISPR editing of HBG1/2 may have more effect on the induction of HbF protein expression than BCL11A.

Conclusion

In this study, we considered 119 studies for inclusion for the study. Following the screening based on the exclusion and inclusion criteria, the protein expression of HbF in 9 individual studies utilizing CRISPR editing of either or both of BCL11A and HBG1/2 were analyzed. It was determined that CRISPR editing of both BCL11A, and HBG1/2 significantly induces HbF expression by comparison to control values. It was also identified that HBG1/2 may have a more potent induction of this HbF protein expression by comparison to BCL11A ($p < 0.01$) (**Figure 3**). One limitation of this study was the sheer lack of available CRISPR data for SCD and β-Thalassemia. Due to CRISPR technologies being less than a decade old, much is yet to still be discovered and investigated with its use. More studies on these and other genes in the treatment/management of SCD and β-Thalassemia are required to further elucidate the true significance of CRISPR therapy.

While this analysis may have identified that HBG1/2 CRISPR editing is more effective than BCL11A editing, it is important to note that CRISPR editing of BCL11A is the only therapy that has progressed to human trials. Frangoul et al. (13) examined the use of BCL11A editing in two separate patients (one with SCD and one with β-Thalassemia). Both patients were transfusion dependent prior to receiving the therapy, however, following treatment, both patients had successful elevation of their long-term hemoglobin levels to become transfusion independent. Additionally, the SCD patient reported zero vaso-occlusive events over the 16-month period of the study after averaging 7 such events in previous years. The findings from this study highlight the potential efficacy of CRISPR gene editing in the treatment of SCD and β-Thalassemia, as well as underscore the importance of expanding on HBG1/2 gene editing in the future.

As mentioned above, there are multiple other genes that have been and/or are under investigation for potential efficacy of CRISPR editing in SCD and β-Thalassemia. More investigation of these genes such as KLF, ZNF410, ATF4, and SPOP and how CRISPR editing can affect HbF protein levels needs to be performed to elucidate additional targets that may be more efficient or effective than BCL11A or HBG1/2. Additionally, the gold standard for treatment and cure of SCD and β-Thalassemia is likely to be successful editing and correction of defective beta globin genes. While there are a few studies that have begun to investigate this possibility, it is still in its infancy and very few post-edit findings are currently available.

A major concern about the efficacy of CRISPR gene editing is the balance of effective gene editing with cellular toxicity. One possible way to mitigate these concerns is the utilization of nanoparticles for CRISPR delivery. Cruz et al. (34) was able to maintain a high level of CRISPR editing (elevation of gamma globin mRNA levels to 47–69% from 15 to 16%) while significantly reducing cellular toxicity. Increased efficiency of delivery *via* nanoparticle technologies could be critical to increasing overall efficiency of CRISPR gene editing in a clinical setting.

Additional benefits to increasing efficiency of CRISPR editing and reduced cellular toxicity could include the ability to therapeutically target multiple genes simultaneously. As mentioned above, there are multiple genes that play a role in HbF protein expression. Therefore, it is possible that by CRISPR editing multiple genes simultaneously, there could be a synergistic induction of HbF expression that could have an added clinical benefit. Thus, targeting multiple genes concurrently requires investigation at the pre-clinical level for efficacy.

In addition to studying CRISPR-complex delivery mechanisms for gene editing efficacy, it is also important to consider varying patient genetics and how they can have an effect as well. Modari et al. (35) identified that even within the same patient samples, variations in homology directed repairs can cause wide variations in responses to CRISPR directed gene editing. Therefore, significant study into how patient genetics can play a role in their response to CRISPR editing is required in order to improve overall efficacy of therapies.

Though in its infancy, CRISPR editing in the treatment of diseases has already shown excellent progress and effectiveness. In this meta-analysis, it was identified that CRISPR editing of HBG1/2 offers a possible benefit over BCL11A for induction of HbF expression for the management of SCD and β -Thalassemia.

Advancements in CRISPR gene editing technologies highlight a promising movement into the future of medicine. However, they do not come without significant ethical challenges and concerns. Brokowski and Adli (36) sought to discuss some of these ethical issues by focusing on four key aspects: (1) the extent to which CRISPR use should be permitted; (2) access to CRISPR applications; (3) what regulatory frameworks should be put into place for clinical research; and (4) what international regulations can be employed to govern the inappropriate utilization of CRISPR. They concluded that moral decisions and governing bodies regarding CRISPR utilization should be constantly evolving with the changing scientific landscape. Currently, governing bodies on the use of CRISPR technologies are still limited, and those that do exist like the International Commission on the Clinical Use of Human Germline Genome Editing are still hesitant on the application of this technology for therapeutic use. Continued discussion on the ethical issues and the balance of therapeutic applications of CRISPR should remain continuous as the technology becomes more expansive.

Data availability statement

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

Author contributions

AQ analyzed the initial manuscripts, performed the analysis, and wrote the manuscript. DA wrote the manuscript. PH performed the analysis and wrote the manuscript. SP conceptualized the study, performed the analysis, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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.

Publisher's note

References

1. Wiedenheft B, Sternberg SH, Doudna JA. RNA-guided genetic silencing systems in bacteria and archaea. *Nature.* (2012) 482:331–8. doi: 10.1038/nature10886

2. Terns MP, Terns RM. CRISPR-based adaptive immune systems. *Curr Opin Microbiol.* (2011) 14:321–7. doi: 10.1016/j.mib.2011.03.005

3. Bhaya D, Davison M, Barrangou R. CRISPR-Cas systems in bacteria and archaea: versatile small RNAs for adaptive defense and regulation. *Annu Rev Genet.* (2011) 45:273–97. doi: 10.1146/annurev-genet-110410-132430

4. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science.* (2012) 337:816–21. doi: 10.1126/science.1225829

5. Needs T, Gonzalez-Mosquera LF, Lynch DT. Beta Thalassemia. In *StatPearls [Internet]*. Treasure Island, FL: StatPearls Publishing (2022).

6. Sedrak A, Kondamudi NP. Sickle Cell Disease. In *StatPearls [Internet]*. Treasure Island, FL: StatPearls Publishing (2022).

7. Bunn HF. Pathogenesis and treatment of sickle cell disease. *N Engl J Med.* (1997) 337:762–9. doi: 10.1056/NEJM199709113371107

8. Giardine B, van Baal S, Kaimakis P, Riemer C, Miller W, Samara M, et al. HbVar database of human hemoglobin variants and thalassemia mutations: 2007 update. *Hum Mutat.* (2007) 28:206. doi: 10.1002/humu.9479

9. Titus HJ, Huisman, Marianne FH, Carver, Erol B. *A Syllabus of Thalassemia Mutations*. Augusta, GA: The Sickle Cell Anemia Foundation (1997).

10. Riemer C, ElSherbini A, Stojanovic N, Schwartz S, Kwitkin PB, Miller W. A database of experimental results on globin gene expression. *Genomics.* (1998) 53:325–37. doi: 10.1006/geno.1998.5524

11. Galanello R, Origa R. Beta-thalassemia. *Orphanet J Rare Dis.* (2010) 21:11. doi: 10.1186/1750-1172-5-11

12. Prati D. Benefits and complications of regular blood transfusion in patients with beta-thalassaemia major. *Vox Sang.* (2000) 79:129–37. doi: 10.1159/000031230

13. Frangoul H, Altshuler D, Cappellini MD, Chen YS, Domm J, Eustace BK, et al. CRISPR-Cas9 Gene Editing for Sickle Cell Disease and β-Thalassemia. *N Engl J Med.* (2021) 384:252–60. doi: 10.1056/NEJMoa2031054

14. Platt OS, Brambilla DJ, Rosse WF, Milner PF, Castro O, Steinberg MH, et al. Mortality in sickle cell disease. Life expectancy and risk factors for early death. *N Engl J Med.* (1994) 330:1639–44. doi: 10.1056/NEJM199406093302303

15. Miller ST, Sleeper LA, Pegelow CH, Enos LE, Wang WC, Weiner SJ, et al. Prediction of adverse outcomes in children with sickle cell disease. *N Engl J Med.* (2000) 342:83–9. doi: 10.1056/NEJM200001133420203

16. Yin J, Xie X, Ye Y, Wang L, Che F. BCL11A: a potential diagnostic biomarker and therapeutic target in human diseases. *Biosci Rep.* (2019) 39:BSR20190604. doi: 10.1042/BSR20190604

17. Wu Y, Zeng J, Roscoe BP, Liu P, Yao Q, Lazzarotto CR, et al. Highly efficient therapeutic gene editing of human hematopoietic stem cells. *Nat Med.* (2019) 25:776–83. doi: 10.1038/s41591-019-0401-y

18. Khosravi MA, Abbasalipour M, Concordet JP, Berg JV, Zeinali S, Arashkia A, et al. Targeted deletion of BCL11A gene by CRISPR-Cas9 system for fetal hemoglobin reactivation: A promising approach for gene therapy of beta thalassemia disease. *Eur J Pharmacol.* (2019) 5:398–405. doi: 10.1016/j.ejphar.2019.04.042

19. Métais JY, Doerfler PA, Mayuranathan T, Bauer DE, Fowler SC, Hsieh MM, et al. Genome editing of HBG1 and HBG2 to induce fetal hemoglobin. *Blood Adv.* (2019) 3:3379–92. doi: 10.1182/bloodadvances.2019000820

20. Lan X, Ren R, Feng R, Ly LC, Lan Y, Zhang Z, et al. ZNF410 Uniquely Activates the NuRD Component CHD4 to Silence Fetal Hemoglobin Expression. *Mol Cell.* (2021) 81:239–54. doi: 10.1016/j.molcel.2020.11.006

21. Shariati L, Khanahmad H, Salehi M, Hejazi Z, Rahimmanesh I, Tabatabaiefar MA, et al. Genetic disruption of the KLF1 gene to overexpress the γ-globin gene using the CRISPR/Cas9 system. *J Gene Med.* (2016) 18:294–301. doi: 10.1002/jgm.2928

22. Martyn GE, Wienert B, Yang L, Shah M, Norton LJ, Burdach J, et al. Natural regulatory mutations elevate the fetal globin gene via disruption of BCL11A or ZBTB7A binding. *Nat Genet.* (2018) 50:498–503. doi: 10.1038/s41588-018-0085-0

23. Sankaran VG, Xu J, Orkin SH. Transcriptional silencing of fetal hemoglobin by BCL11A. *Ann N Y Acad Sci.* (2010) 1202:64–8. doi: 10.1111/j.1749-6632.2010.05574.x

24. Zhou D, Liu K, Sun CW, Pawlik KM, Townes TM. KLF1 regulates BCL11A expression and gamma- to beta-globin gene switching. *Nat Genet.* (2010) 42:742–4. doi: 10.1038/ng.637

25. Lamsfus-Calle A, Daniel-Moreno A, Antony JS, Epting T, Heumos L, Baskaran P, et al. Comparative targeting analysis of KLF1, BCL11A, and HBG1/2 in CD34+ HSPCs by CRISPR/Cas9 for the induction of fetal hemoglobin. *Sci Rep.* (2020) 10:10133. doi: 10.1038/s41598-020-66309-x

26. Sharma DC, Singhal S, Woike P, Rai S, Yadav M, Gaur R. Hereditary persistence of fetal hemoglobin. *Asian J Transfus Sci.* (2020) 14:185–6. doi: 10.4103/ajts.AJTS_71_16

27. Vinjamur DS, Yao Q, Cole MA, McGuckin C, Ren C, Zeng J, et al. ZNF410 represses fetal globin by singular control of CHD4. *Nat Genet.* (2021) 53:719–28. doi: 10.1038/s41588-021-00843-w

28. Chen HY, Chen RH. Cullin 3 ubiquitin ligases in cancer biology: functions and therapeutic implications. *Front Oncol.* (2016) 6:113. doi: 10.3389/fonc.2016.00113

29. Lan X, Khandros E, Huang P, Peslak SA, Bhardwaj SK, Grevet JD, et al. The E3 ligase adaptor molecule SPOP regulates fetal hemoglobin levels in adult erythroid cells. *Blood Adv.* (2019) 3:1586–97. doi: 10.1182/bloodadvances.2019032318

30. Traxler EA, Yao Y, Wang YD, Woodard KJ, Kurita R, Nakamura Y, et al. A genome-editing strategy to treat β-hemoglobinopathies that recapitulates a mutation associated with a benign genetic condition. *Nat Med.* (2016) 22:987–90. doi: 10.1038/nm.4170

31. Martyn GE, Wienert B, Kurita R, Nakamura Y, Quinlan KGR, Crossley M. A natural regulatory mutation in the proximal promoter elevates fetal globin expression by creating a de novo GATA1 site. *Blood.* (2019) 133:852–6. doi: 10.1182/blood-2018-07-863951

32. Li C, Psatha N, Sova P, Gil S, Wang H, Kim J, et al. Reactivation of γ-globin in adult β-YAC mice after ex vivo and in vivo hematopoietic stem cell genome editing. *Blood.* (2018) 131:2915–28. doi: 10.1182/blood-2018-03-838540

33. Mingoia M, Caria CA, Ye L, Asunis I, Marongiu MF, Manunza L, et al. Induction of therapeutic levels of HbF in genome-edited primary β0 39-thalassaemia haematopoietic stem and progenitor cells. *Br J Haematol.* (2021) 192:395–404. doi: 10.1111/bjh.17167

34. Cruz LJ, van Dijk T, Vepris O, Li TMWY, Schomann T, Baldazzi F, et al. PLGA-nanoparticles for intracellular delivery of the crispr-complex to elevate fetal globin expression in erythroid cells. *Biomaterials.* (2021) 268:120580. doi: 10.1016/j.biomaterials.2020.120580

35. Modarai SR, Kanda S, Bloh K, Opdenaker LM, Kmiec EB. Precise and error-prone CRISPR-directed gene editing activity in human CD34+ cells varies widely among patient samples. *Gene Ther.* (2021) 28:105–13. doi: 10.1038/s41434-020-00192-z

36. Brokowski C, Adli M. CRISPR ethics: Moral considerations for applications of a powerful tool. *J Mol Biol.* (2019) 431:88–101. doi: 10.1016/j.jmb.2018.05.044

frontiers | Frontiers in Medicine

TYPE Mini Review
PUBLISHED 24 November 2022
DOI 10.3389/fmed.2022.1055540

OPEN ACCESS

EDITED BY
Hollie Marie Reeves,
University Hospitals Cleveland Medical Center, United States

REVIEWED BY
Ioanna Sakellari,
G. Papanikolaou General Hospital, Greece

*CORRESPONDENCE
Ljiljana V. Vasovic
ljv9004@med.cornell.edu

†These authors have contributed equally to this work

SPECIALTY SECTION
This article was submitted to Hematology, a section of the journal Frontiers in Medicine

RECEIVED 27 September 2022
ACCEPTED 04 November 2022
PUBLISHED 24 November 2022

CITATION
Tanhehco YC, Nathu G and Vasovic LV (2022) Development of curative therapies for sickle cell disease. *Front. Med.* 9:1055540. doi: 10.3389/fmed.2022.1055540

Development of curative therapies for sickle cell disease

Yvette C. Tanhehco[1†], Ghazala Nathu[2†] and Ljiljana V. Vasovic[3*†]

[1]Department of Pathology and Cell Biology, Columbia University Irving Medical Center, New York, NY, United States, [2]Department of Clinical Pathology, Bassett Healthcare Network—Cobleskill Regional Hospital, Cobleskill, NY, United States, [3]Department of Pathology and Laboratory Medicine, Weill Cornell Medicine, New York, NY, United States

Recent advances in managing Sickle Cell Disease (SCD) significantly improved patient survival and quality of life. Disease-modifying drug therapies such as hydroxyurea, L-glutamine, voxelotor, and crizanlizumab reduce pain crises and severe complications. Allogeneic hematopoietic stem cell transplantation using matched-sibling donors is currently the only standard curative option; however, only a small proportion of patients have such donors. Cord blood and haploidentical transplantation with a modified conditioning regimen have expanded the allogeneic donor pool, making the therapy available to more patients. Gene therapy is a promising cure that is currently undergoing clinical trials and different approaches have demonstrated efficacy. Multidisciplinary expertise is needed in developing the best treatment strategy for patients with SCD.

KEYWORDS

Sickle Cell Disease (SCD), gene therapy, hematopoietic stem cell transplantation (HSCT), disease modifying drugs, lentiviral vectors (LVS)

Introduction

Sickle Cell Disease (SCD) is caused by a single point mutation in the hemoglobin beta gene that codes for a valine in the sixth amino acid position instead of the wild type glutamic acid creating hemoglobin S (HbS) (1). Under deoxygenated conditions, HbS polymerizes causing erythrocytes to assume a sickle shape (2). These HbS-containing erythrocytes are insufficient for transporting oxygen. The conformational change of erythrocytes and increased surface expression of adhesion molecules can lead to blood vessel occlusion, painful vaso-occlusive crises (VOC), and long-term damage to tissues and organs (3).

SCD is among the most prevalent inherited monogenic disorders worldwide. It affects Black Americans more than any other racial group. It is believed that more than 20 million people worldwide and 80,000–100,000 Americans battling SCD, with an average of 3,000 children being born with it annually in the United States (US) (4). Newborn screening programs, early interventions, and preventive care improved pediatric patients' survival. Nevertheless, the life span of patients with SCD is about 20 years shorter than the general population. The National Institutes of Health Cure Sickle Cell Initiative established a collaborative, patient-centric research program aimed at discovering effective genetic treatments for SCD. This is a systematic effort to support

the development of curative therapies and empower understanding of basic research, clinical trials, and patient support which have wide implications for educational and community engagement activities (5).

Traditional therapies for SCD include pain and symptom management. In certain cases, physicians might recommend a blood transfusion in order to instill an appropriate amount of hemoglobin A containing erythrocytes, prevent strokes, and treat anemia (6, 7). Simple transfusion, chronic transfusion or red blood cell exchange protocols have been utilized. As with any transfusion, there is a risk for alloimmunization due to incomplete phenotypic matches, iron accumulation, and other transfusion-related adverse events.

The first disease modifying drug therapy that was used to reduce complications is hydroxyurea (Table 1). Subsequently, the US Food and Drug administration (FDA) approved L-glutamine, voxelotor, and crizanlizumab-tmca to reduce pain crises, sickling, and VOC which have all significantly improved the quality of life of patients with SCD (7).

Thus, far, the only curative approach to SCD has been a hematopoietic stem cell (HSC) transplantation. Allogeneic stem cells lacking the genetic mutation will yield healthy erythrocytes in the recipient (8). However, the potential complications of an allogenic transplant, such as rejection or graft-vs. host disease (GVHD), are considerable potentially causing morbidity and death. The major hurdle of procuring a fully matched HLA donor can be circumvented by using cord blood and haploidentical donors.

There is a new treatment paradigm on the horizon that aims to cure SCD by correcting the core issue (i.e., the genetic mutation) through gene therapy. Gene therapy approaches are variable and may involve introducing a new gene into the patient to be integrated and expressed or to modify the native gene itself through gene editing. All of these methodologies require that the HSCs, the targets of these therapies, be collected and harvested from the patient. Genetic modification then ensues *in-vitro* followed by transplantation back to the patient after the residual marrow population is ablated using chemotherapy (9).

This minireview discusses the available therapeutic modalities for SCD including gene therapy.

Disease modifying drug therapies

Hydroxyurea

Hydroxyurea (HU) is a disease modifying drug (DMD) therapy that was approved by the US FDA in 1998 for the treatment of adults (>18 years) with SCD who have had at least 3 painful crises in the past year (10). HU increases the level of fetal hemoglobin (HbF) and reduces the tendency for HbS to polymerize, preventing RBCs from sickling and causing VOC (11). HU also reduces circulating leukocytes and inflammatory mediators. A double-blind, randomized clinical trial enrolled 148 men and 151 women at least 18 years of age with sickle cell anemia at 21 clinics. Study subjects must have had at least 3 crises in the year before entry into the study. Study results showed that the 152 patients who received HU treatment had lower annual rates of crises than the 147 patients who received placebo (median, 2.5 vs. 4.5 crises per year, $P < 0.001$). The median times to the first crisis (3.0 vs. 1.5 months, $P = 0.01$) and the second crisis (8.8 vs. 4.6 months, $P < 0.001$) were longer with HU treatment. Fewer patients who were treated with HU had chest syndrome (25 vs. 51, $P < 0.001$), and fewer received transfusions (48 vs. 73, $P = 0.001$). No significant adverse effects were observed but its use must be carefully monitored. The beneficial effects of HU did not manifest for several months and the maximally tolerated doses of HU may not be necessary to achieve a therapeutic effect (12). HU was subsequently approved in 2017 for the reduction in the frequency of painful crises and the need for blood transfusions in pediatric patients (>2 years) with SCD with recurrent moderate to severe painful crises.

L-glutamine

1In 2017, L-glutamine received approval from the FDA for the treatment of SCD. L-glutamine is an amino acid required to synthesize nicotinamide adenine dinucleotide (NAD+) and its reduced form NADH. NAD+ is an important antioxidant in RBCs, so the rationale for using L-glutamine in patients with SCD is to improve the redox ratio to reduce oxidative stress, RBC sickling and hemolysis, and decrease the frequency of VOC. A multicenter, randomized, placebo-controlled, double-blind, phase 3 trial enrolled patients with sickle cell anemia (HbSS or HbSβ^0-thalassemia) at least 5 years old at 31 sites across the United States who had a history of at least 2 pain crises during the previous year (13). This clinical trial studied the efficacy of pharmaceutical-grade L-glutamine (0.3 per kilogram of body weight per dose) administered twice daily compared to placebo in reducing the incidence of pain crises. Patients who were receiving HU at least 3 months prior to screening continued that therapy throughout the 48-week treatment period. Two hundred and thirty patients (age range, 5–58 years; 53.9% female) were randomly assigned in a 2:1 ratio to receive L-glutamine ($n = 152$) or placebo ($n = 78$). Patients receiving L-glutamine had significantly fewer pain crises than those receiving placebo (median, 3 vs. 4, $P = 0.005$) and fewer hospitalizations (2 vs. 3, $P = 0.005$). The adverse effects of low-grade nausea, non-cardiac chest pain, fatigue, and musculoskeletal pain occurred more frequently in the L-glutamine group compared to the placebo group (13).

TABLE 1 Sickle cell disease therapeutic options.

Therapeutic options	Mechanism of action
Disease modifying drug therapies	
Hydroxyurea	Increase HbF
L-glutamine	Increase NAD and NADH, preventing VOD
Crizanlizumab	Monoclonal antibody to P-selectin that blocks the adhesion of activated erythrocytes, neutrophils and platelets, preventing VOD
Voxelotor	Increase oxygen affinity, stabilize oxygenated HbS, inhibit HbS polymerization
Complement inhibitors	Improvement in intravascular hemolysis, anemia, transfusion independence, and thrombotic events
Allogeneic hematopoietic stem cell transplantation (HSCT)	
Matched related donor transplant	Establishing donor-derived erythropoiesis
Cord blood transplant	Establishing donor-derived erythropoiesis
Haploidentical familial donor transplant	Establishing donor-derived erythropoiesis
Alternate donor sources	Establishing donor-derived erythropoiesis
Gene therapy/autologous HSCT	
Gene addition (Antisickling Gene)	Lentiviral vector with T87Q mutation (HbA^{T87Q}), leading to steric inhibition of HbS polymerization
Gene editing (HbF Induction)	Upregulating HbF expression with CRISPR Cas-9 disruption of BCL11a gene

VOD, Vaso-occlusive crises; HSCT, hematopoietic stem cell transplantation.

Crizanlizumab

Crizanlizumab is an anti-P selectin inhibitor that decreases the adhesion of white blood cells and RBCs to the endothelium that was approved by the FDA in 2019 based on a phase 2 randomized, double-blind, placebo-controlled trial. Patients with sickle cell disease (HbSS, HbSC, $HbS\beta^0$-thalassemia, $HbS\beta^+$-thalassemia, and other genotypes) who were between 16 and 65 years of age and had 2–10 sickle cell-related pain crises in the 12 months prior to enrollment received low-dose crizanlizumab (2.5 mg per kg body weight), high-dose crizanlizumab (5 mg/kg) or placebo, administered intravenously 14 times over 52 weeks. One hundred and ninety-eight patients were enrolled across 60 sites. The median rate of crises per year was significantly lower with high-dose crizanlizumab compared to placebo (median, 1.63 vs. 2.98, $P = 0.01$). The median time to first and the second crisis was significantly longer with high-dose crizanlizumab compared to placebo (first crisis, median, 4.07 vs. 1.38 months, $P = 0.001$; second crisis, median, 10.32 vs. 5.09 months, $P = 0.02$). The median rate of uncomplicated crises per year was lower with high-dose crizanlizumab compared with placebo (median, 1.08 vs. 2.91, $P = 0.02$). Adverse events such as arthralgia, diarrhea, pruritus, vomiting, and chest pain were observed (14).

Voxelotor

Voxelotor is another DMD that was approved by the FDA in 2019. It inhibits HbS polymerization by binding covalently to the N-terminal valine of the β-globin chain in Hb to stabilize the oxygenated HbS (11). A phase 3, multicenter, double-blind, randomized, placebo-controlled trial (HOPE trial), compared the efficacy and safety of 2 dose levels of voxelotor (1500 mg and 900 mg) with placebo in patients aged 12–65 years old with SCD (HbSS, HbSC, HbSβ-thalassemia, and other variants) of different genotypes (15). Participants ($n = 274$) were randomly assigned in a 1:1:1 ratio to receive a once-daily oral dose of 1500 mg of voxelotor, 900 mg of voxelotor, or placebo. Study results showed a significantly higher percentage of study subjects with a Hb response in the 1500 mg voxelotor group (51%; 95% confidence interval [CI], 41–61) compared to the 900 mg voxelotor group (33%; 95% CI, 23–42) and placebo group (7%; 95% CI, 1–12) at 24 weeks. The annualized adjusted incidence rate of VOC was 2.77, 2.76, and 3.19 in the 1500 mg, 900 mg and placebo groups, respectively. Furthermore, there was a trend toward reduced incidence of crises over time with voxelotor compared to placebo. Most adverse events were concluded to be unrelated to voxelotor or placebo. There was no significant difference of SCD-related adverse events among the 3 groups (76% 1500 mg voxelotor, 73% 900 mg voxelotor, and 73% placebo group) (15).

Complement inhibitors

One area of growing interest in SCD pathogenesis is complement activation, the protein-based arm of the innate immune system. The first report on complement activation in SCD was published in 1967 by Francis and Womack in which they discovered high levels of serum complement markers in patients with SCD (16). Alternative pathway (AP) of complement activation may be implicated in SCD;

anaphylatoxins C3a and C5a were determined to be increased during a VOC (17, 18). In adults with SCD levels of sC5b-9, a marker of complete complement activation, was significantly elevated compared to those without SCD (19, 20). Of note is that hydroxyurea reduces the degree of complement activation: 61% of patients with SCD who did not take hydroxyurea had elevated levels of sC5b-9 compared to those on the treatment (19). To date, no complement inhibitors have been specifically FDA approved within the context of SCD nor have any large-scale clinical trials been undertaken. Nonetheless, case reports of small-scale administration of certain complement inhibitors (e.g., eculizumab and ravulizumab) within certain contexts of SCD (e.g., pregnancy, delayed hemolytic transfusion reaction, VOC) have been published (21). Further research is needed to elucidate the thorough relationship between complement and SCD.

Allogeneic hematopoietic stem cell transplantation

A significant expertise is needed to render peri-transplant care for SCD patients. In general, pediatric patients have better outcomes. Adult patients with SCD who have acquired significant organ damage due to chronic inflammation and transplant related toxicity potentially prolonging recovery. Delayed immune reconstitution with infectious complications remain significant problems. In addition, SCD patients are at risk of rejection due to immunological responses. Blood transfusion support during the recovery period might require extensive RBC antigen matching. Targeting the bone marrow microenvironment pharmacologically or by modifying the effect of mesenchymal stromal cells might improve durable engraftment with a high degree of chimerism (22).

Allogeneic hematopoietic stem cell transplantation from related donors

Allogeneic hematopoietic stem cell transplantation (HSCT) using matched sibling donors is the only standard curative option for SCD and offers a >90% cure rate (23). Unfortunately, <20% of patients have HLA-matched donors. In the absence of a matched sibling donor, HLA-matched unrelated donors and HLA-identical sibling cord blood (CB) donors are alternatives (24).

Unrelated cord blood transplantation

Unrelated CB transplantation (CBT) significantly expanded the donor pool for patients with severe SCD. These transplants are associated with better outcomes and low incidences and severity of GVHD. In a study of pediatric patients, a reduced intensity regimen supported donor engraftment in the majority of patients with 100% 1 year overall survival and 78% disease-free survival (25). Historically, a major limitation associated with CBT in adults was a high rate of graft failure due to inadequate cell dose. Therefore, transplantation with double cord blood units is increasingly used in adults who lack a matched related or unrelated donor.

An *ex vivo* expanded HSC from a single umbilical cord blood unit, Omidubicel, demonstrated faster hematopoietic recovery with no differences in GVHD incidences compared to the standard myeloablative CBT ($p < 0.001$) in a phase 3 randomized clinical study (26).

Haploidentical donor transplant

Recent data of haploidentical familial donor transplants are encouraging with high overall survival, limited toxicities, and reduced rates of acute and chronic GVHD with up to 60 months of follow-up. Expanding the donor pool to haploidentical donors such as parents, children, siblings, half-siblings, and extended family donors is a promising therapeutic option for patients with SCD. To minimize rejection and GVHD, T-cell depletion of donor grafts is performed either *ex vivo* using CD34+ positive cell selection methods or i*n vivo* with high-dose post-transplant cyclophosphamide (PTCy) (27). PTCy targeted depletion of alloreactive T cells is superior to anti-thymocyte globulin or alemtuzumab rendered non-selective T-cell depletion. Increasing total body irradiation in the preparative regimen decreased the rejection rate from 43 to 6%, with most of patients achieving full donor chimerism, and 100% survival (median follow-up 705 days) (28). Addition of thiotepa in haploidentical HSCT showed an improvement in engraftment, 20% acute GVHD rates, and 100% survival after a median follow-up of 13 months (29).

Gene therapy

Autologous HSCT offers several advantages over allogeneic HSCT: lowering risk of serious complications, increasing donor availability, absence of GVHD and graft rejection, and strengthened rapid immune reconstitution. Granulocyte colony-stimulating factor is an effective mobilization agent but is contraindicated in SCD, because it triggers severe adverse events. The CXCR4 antagonist, plerixafor, has been successful in gene therapy trials for SCD (30, 31). However, the degree of mobilization in patients with SCD is often suboptimal, so the patient may require multiple cycles of mobilization and apheresis collection. As a pretransplant preparative regimen, RBC exchange transfusions is performed to prevent the occurrence of SCD-related morbidities associated with the

HSC mobilization and procurement. Gene modification of the autologous stem cells is required to reverse the effects of HbS and sustain hematopoietic engraftment. To that end, the following include potential gene therapy avenues: HbS gene correction, hemoglobin F induction, and modified HbA gene addition. Toxicities may be associated with the conditioning chemotherapy and lentiviral vector; namely, insertional mutagenesis leading to secondary malignancies.

Antisickling gene therapy

The first gene therapy treatment of SCD was reported in 2017 (32). LentiGlobin BB305 is a lentiviral vector that encodes a modified β-globin gene, HbA^{T87Q}. HbA^{T87Q} is a modified adult hemoglobin with an amino acid substitution at position 87 from threonine to glutamine, which results in anti-sickling properties due to steric inhibition of polymerization of HbS. As of February 2021, in Group C of phase 1–2 studies, all patients ($n = 35$) who received LentiGlobin infusion engrafted at a median follow-up of 17.3 months (range 3.7 to 37.6 months) (33). The median total hemoglobin level increased from 8.5 g/dL at baseline to ≥11 g/dL from 6–36 months after infusion. HbA^{T87Q} accounted for at least 40% of total Hb and lower levels of hemolysis were observed. Severe vaso-occlusive events resolved in the 25 patients who could be evaluated who experienced a median of 3.5 events per year (range, 2–13.5) in the 24 months before enrollment. Three patients experienced vaso-occlusive events after infusion. The median time to neutrophil engraftment (absolute neutrophil count, ≥500 per microliter for 3 days) was 20 days (range, 12–35) while the median time to platelet engraftment (platelet count, ≥50 x 10^3 per microliter for 3 days without platelet transfusion) was 36 days (range, 18–136). Adverse events such as abdominal pain, opiate withdrawal syndrome, nausea, and vomiting were reported. One case of leukopenia, one of decreased diastolic blood pressure and one of febrile neutropenia were attributed possibly or definitely to LentiGlobin infusion. No cases of hematologic malignancies were reported at 37.6 months of follow-up (33).

Gene editing to induce fetal hemoglobin

Other gene therapy approaches being studied include upregulating the expression of HbF with CRISPR Cas-9 disruption of the BCL11a gene (34), CRISPR-Cas-12 mutation of the HGB 1 and 2 enhancer sites (35, 36), and RNAi-induced suppression of BCL11a mRNA transcription through a short hairpin RNA expressed through a lentiviral-based vector (37). Very promising results of a phase I study involving six patients suggest this is a feasible approach for HbF induction in patients with SCD. All the patients evaluated achieved robust and stable HbF induction (percentage HbF/(F+S) ranged from 20.4 to 41.3%). Clinical manifestations of SCD were reduced or absent during the 6–29 month follow-up period (37).

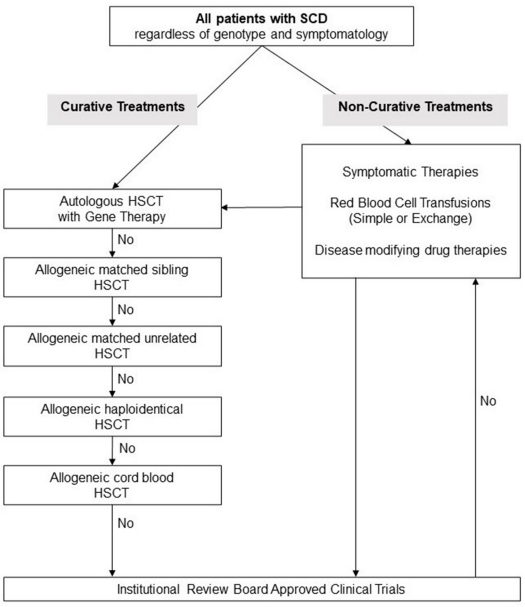

FIGURE 1
Suggested management algorithm for patients with sickle cell disease. SCD, Sickle Cell Disease; HSCT, hematopoietic stem cell transplantation.

Discussion

Number of potentially curative therapies recently become available, see Figure 1 for a suggested algorithm for evaluation of patients with SCD for novel treatments. While gene therapies are promising and may perhaps cure SCD, further research is still needed before they can be utilized on all patients. Lentiviral vectors are currently preferred over gamma retroviral vectors because of their ability to transduce non-dividing primitive cells such as HPCs. A shorter *in-vitro* transduction period with lentiviral vectors is preferred in order to minimize the loss of HSC "stemness." The long-term engraftment durability needs to be evaluated and the long-term risks of conditioning regimens such as infertility and risk of secondary malignancy must be addressed.

The high cost of transplant and gene therapy currently limits the availability of curative treatment to primarily developed countries. The estimated cost of HSCT per patient ranges from $350,000 to $800,000, while costs for gene therapy is estimated to be as high as $1- $2 million, which severely constricting access. However, this upfront cost may be acceptable when compared to the total lifetime cost of managing a patient by age 50 with a chronic disease such as SCD that exceeds $8 million (38).

Future developments of alternative therapies include pharmacologic, biologic and genetic approaches. There has been an expansion of pharmacologic agents that target mediators of inflammation in SCD, such as cellular adhesion molecules, cytokines, complement, leukotrienes, and nuclear signaling factors (39). Development of an *in vivo* gene therapy delivery system that contains nucleases packaged in viral or non-viral vehicles would bypass the need for an autologous transplant and make worldwide application logistics easier. However, reactivation of HbF using pharmacologic approaches such as small molecule regulators targeting BCL11A or other genes are more likely to be broadly implemented (40).

Author contributions

YT, GN, and LV co-wrote the manuscript. All authors contributed to the article and approved the submitted version.

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.

Publisher's note

References

1. Ingram VM. Abnormal human haemoglobins. I. The comparison of normal human and sickle-cell haemoglobins by "fingerprinting". 1958. *Biochim Biophys Acta*. (1989) 1000:151–7.

2. Herrick JB. Peculiar elongated and sickle-shaped red blood corpuscles in a case of severe anemia. *Arch Intern Med.* (1910) 6:517–21. doi: 10.1001/archinte.1910.00050330050003

3. Gardner RV. Sickle cell disease: advances in treatment. *Ochsner J.* (2018) 18:377–89. doi: 10.31486/toj.18.0076

4. Chakravorty S, Williams TN. Sickle cell disease: a neglected chronic disease of increasing global health importance. *Arch Dis Child.* (2015) 100:48–53. doi: 10.1136/archdischild-2013-303773

5. Silberstein LE, Telen MJ. Update on the cure sickle cell initiative. *Hematologist*. The Hematologist Ash Publication. (2021) 18. Available online at: https://ashpublications.org/thehematologist/article/475190/Update-on-the-Cure-Sickle-Cell-Initiative

6. Yawn BP, Buchanan GR, Afenyi-Annan AN, Ballas SK, Hassell KL, James AH, et al. Management of sickle cell disease: summary of the 2014 evidence-based report by expert panel members. *JAMA*. (2014) 312:1033–48. doi: 10.1001/jama.2014.10517

7. NHLBI NIH. *Sickle Cell Disease Treatment [Online Multimedia]*. (2022). Available online at: https://www.nhlbi.nih.gov/health/sickle-cell-disease/treatment (accessed July 15, 2022).

8. Bhatia M, Sheth S. Hematopoietic stem cell transplantation in sickle cell disease: patient selection and special considerations. *J Blood Med.* (2015) 6:229–38. doi: 10.2147/JBM.S60515

9. Tanhehco YC, Bhatia M. Hematopoietic stem cell transplantation and cellular therapy in sickle cell disease: where are we now? *Curr Opin Hematol.* (2019) 26:448–52. doi: 10.1097/MOH.0000000000000541

10. Ault A, US FDA. approves first drug for sickle-cell anaemia. *Lancet.* (1998) 351:809. doi: 10.1016/S0140-6736(05)78941-8

11. Leibovitch JN, Tambe AV, Cimpeanu E, Poplawska M, Jafri F, Dutta D, et al. l-glutamine, crizanlizumab, voxelotor, and cell-based therapy for adult sickle cell disease: Hype or hope? *Blood Rev.* (2022) 53:100925. doi: 10.1016/j.blre.2021.100925

12. Charache S, Terrin ML, Moore RD, Dover GJ, Barton FB, Eckert SV, et al. Effect of hydroxyurea on the frequency of painful crises in sickle cell anemia. Investigators of the multicenter study of hydroxyurea in sickle cell anemia. *N Engl J Med.* (1995) 332:1317–22. doi: 10.1056/NEJM199505183322001

13. Niihara Y, Miller ST, Kanter J, Lanzkron S, Smith WR, Hsu LL, et al. A phase 3 trial of l-glutamine in sickle cell disease. *N Engl J Med.* (2018) 379:226–35. doi: 10.1056/NEJMoa1715971

14. Ataga KI, Kutlar A, Kanter J, Liles D, Cancado R, Friedrisch J, et al. Crizanlizumab for the prevention of pain crises in sickle cell disease. *N Engl J Med.* (2017) 376:429–39. doi: 10.1056/NEJMoa1611770

15. Vichinsky E, Hoppe CC, Ataga KI, Ware RE, Nduba V, El-Beshlawy A, et al. a phase 3 randomized trial of voxelotor in sickle cell disease. *N Engl J Med.* (2019) 381:509–19. doi: 10.1056/NEJMoa1903212

16. Francis WG, Womack CR. Serum complement activity in normal individuals and patients with sickle cell hemoglobin abnormalities. *Am J Med Technol.* (1967) 33:77–86.

17. Yoo JJ, Graciaa SH, Jones JA, Zuo Z, Arthur CM, Leong T, et al. Complement activation during vaso-occlusive pain crisis in pediatric sickle cell disease. *Blood.* (2021) 138:858. doi: 10.1182/blood-2021-154132

18. Conran N, Belcher JD. Inflammation in sickle cell disease. *Clin Hemorheol Microcirc.* (2018) 68:263–99. doi: 10.3233/CH-189012

19. Roumenina LT, Chadebech P, Bodivit G, Vieira-Martins P, Grunenwald A, Boudhabhay I, et al. Complement activation in sickle cell disease: dependence on cell density, hemolysis and modulation by hydroxyurea therapy. *Am J Hematol.* (2020) 95:456–64. doi: 10.1002/ajh.25742

20. Vercellotti GM, Dalmasso AP, Schaid TR. Jr., Nguyen J, Chen C, Ericson ME, et al. Critical role of C5a in sickle cell disease. *Am J Hematol.* (2019) 94:327–37. doi: 10.1002/ajh.25384

21. Varelas C, Tampaki A, Sakellari I. Anagnostopoulos, Gavriilaki E, Vlachaki E. Complement in sickle cell disease: are we ready for prime time? *J Blood Med.* (2021) 12:177–87. doi: 10.2147/JBM.S287301

22. Kharbanda S, Smith AR, Hutchinson SK, McKenna DH, Ball JB, Lamb Jr LS, et al. Unrelated donor allogeneic hematopoietic stem cell transplantation for patients with hemoglobinopathies using a reduced-intensity conditioning regimen and third-party mesenchymal stromal cells. *Biol Blood Marrow Transplant.* (2014) 20:581–6. doi: 10.1016/j.bbmt.2013.12.564

23. Gluckman E, Cappelli B, Bernaudin F, Labopin M, Volt F, Carreras J, et al. Sickle cell disease: an international survey of results of HLA-identical sibling hematopoietic stem cell transplantation. *Blood.* (2017) 129:1548–56. doi: 10.1182/blood-2016-10-745711

24. Kanter J, Liem RI, Bernaudin F, Bolaños-Meade J, Fitzhugh CD, Hankins JS, et al. American society of hematology 2021 guidelines for sickle cell disease: stem cell transplantation. *Blood Advances.* (2021) 5:3668–89. doi: 10.1182/bloodadvances.2021004394C

25. Abraham A, Cluster A, Jacobsohn D, Delgado D, Hulbert ML, Kukadiya D, et al. Unrelated umbilical cord blood transplantation for sickle cell disease following reduced-intensity conditioning: results of a phase I trial. *Biol Blood Marrow Transplant*. (2017) 23:1587–92. doi: 10.1016/j.bbmt.2017.05.027

26. Horwitz ME, Stiff PJ, Cutler C, Brunstein C, Hanna R, Maziarz RT, et al. Omidubicel vs standard myeloablative umbilical cord blood transplantation: results of a phase 3 randomized study. *Blood*. (2021) 138:1429–40. doi: 10.1182/blood.2021011719

27. Bolaños-Meade J, Fuchs EJ, Luznik L, Lanzkron SM, Gamper CJ, Jones RJ, et al. HLA-haploidentical bone marrow transplantation with posttransplant cyclophosphamide expands the donor pool for patients with sickle cell disease. *Blood*. (2012) 120:4285–91. doi: 10.1182/blood-2012-07-438408

28. Bolaños-Meade J, Cooke KR, Gamper CJ, Ali SA, Ambinder RF, Borrello IM, et al. Effect of increased dose of total body irradiation on graft failure associated with HLA-haploidentical transplantation in patients with severe haemoglobinopathies: a prospective clinical trial. *Lancet Haematol*. (2019) 6:e183–e93. doi: 10.1016/S2352-3026(19)30031-6

29. de la Fuente J, Dhedin N, Koyama T, Bernaudin F, Kuentz M, Karnik L, et al. Haploidentical bone marrow transplantation with post-transplantation cyclophosphamide plus thiotepa improves donor engraftment in patients with sickle cell anemia: results of an international learning collaborative. *Biol Blood Marrow Transplant*. (2019) 25:1197–209. doi: 10.1016/j.bbmt.2018.11.027

30. Leonard A, Bonifacino A, Dominical VM, Luo M, Haro-Mora JJ, Demirci S, et al. Bone marrow characterization in sickle cell disease: inflammation and stress erythropoiesis lead to suboptimal CD34 recovery. *Br J Haematol*. (2019) 186:286–99. doi: 10.1111/bjh.15902

31. Naoya U, Alexis L, David S, Sandhya RP, Kamille W, Eoghan M, et al. Safe and efficient peripheral blood stem cell collection in patients with sickle cell disease using plerixafor. *Haematologica*. (2020) 105:e497. doi: 10.3324/haematol.2019.236182

32. Ribeil J-A, Hacein-Bey-Abina S, Payen E, Magnani A, Semeraro M, Magrin E, et al. Gene therapy in a patient with sickle cell disease. *N Engl J Med*. (2017) 376:848–55. doi: 10.1056/NEJMoa1609677

33. Kanter J, Walters MC, Krishnamurti L, Mapara MY, Kwiatkowski JL, Rifkin-Zenenberg S, et al. Biologic and clinical efficacy of lentiglobin for sickle cell disease. *N Engl J Med*. (2022) 386:617–28. doi: 10.1056/NEJMoa2117175

34. Frangoul H, Altshuler D, Cappellini MD, Chen YS, Domm J, Eustace BK, et al. CRISPR-cas9 gene editing for sickle cell disease and β-thalassemia. *N Engl J Med*. (2021) 384:252–60. doi: 10.1056/NEJMoa2031054

35. Métais JY, Doerfler PA, Mayuranathan T, Bauer DE, Fowler SC, Hsieh MM, et al. Genome editing of HBG1 and HBG2 to induce fetal hemoglobin. *Blood Adv*. (2019) 3:3379–92. doi: 10.1182/bloodadvances.2019000820

36. Traxler EA, Yao Y, Wang YD, Woodard KJ, Kurita R, Nakamura Y, et al. A genome-editing strategy to treat β-hemoglobinopathies that recapitulates a mutation associated with a benign genetic condition. *Nat Med*. (2016) 22:987–90. doi: 10.1038/nm.4170

37. Esrick EB, Lehmann LE, Biffi A, Achebe M, Brendel C, Ciuculescu MF, et al. Post-transcriptional genetic silencing of BCL11A to treat sickle cell disease. *N Engl J Med*. (2021) 384:205–15. doi: 10.1056/NEJMoa2029392

38. Leonard A, Tisdale J, Abraham A. Curative options for sickle cell disease: haploidentical stem cell transplantation or gene therapy? *Br J Haematol*. (2020) 189:408–23. doi: 10.1111/bjh.16437

39. Hoppe CC. Inflammatory mediators of endothelial injury in sickle cell disease. *Hematol Oncol Clin North Am*. (2014) 28:265–86. doi: 10.1016/j.hoc.2013.11.006

40. Yu L, Myers G, Engel JD. Small molecule therapeutics to treat the β-globinopathies. *Curr Opin Hematol*. (2020) 27:129–40. doi: 10.1097/MOH.0000000000000579

frontiers | Frontiers in Medicine

TYPE Original Research
PUBLISHED 25 November 2022
DOI 10.3389/fmed.2022.1043686

OPEN ACCESS

EDITED BY
Robert W. Maitta,
Case Western Reserve University,
United States

REVIEWED BY
J. Peter Ray Pelletier,
UF Health Shands Hospital,
United States
Laure Steiner,
University of Rochester Medical
Center, United States

*CORRESPONDENCE
Athena Starlard-Davenport
astarlar@uthsc.edu

†These authors share first authorship

SPECIALTY SECTION
This article was submitted to
Hematology,
a section of the journal
Frontiers in Medicine

RECEIVED 13 September 2022
ACCEPTED 10 November 2022
PUBLISHED 25 November 2022

CITATION
Gu Q, Palani CD, Smith A, Li B,
Amos-Abanyie EK, Ogu U, Lu L,
Pace BS and Starlard-Davenport A
(2022) MicroRNA29B induces fetal
hemoglobin *via* inhibition of the HBG
repressor protein MYB *in vitro* and in
humanized sickle cell mice.
Front. Med. 9:1043686.
doi: 10.3389/fmed.2022.1043686

MicroRNA29B induces fetal hemoglobin *via* inhibition of the HBG repressor protein MYB *in vitro* and in humanized sickle cell mice

Qingqing Gu[1†], Chithra D. Palani[2,3†], Alana Smith[1], Biaori Li[2,3], Ernestine Kubi Amos-Abanyie[1], Ugochi Ogu[4], Lu Lu[1], Betty S. Pace[2,3] and Athena Starlard-Davenport[1,5*]

[1]Department of Genetics, Genomics, and Informatics, College of Medicine, The University of Tennessee Health Science Center, Memphis, TN, United States, [2]Department of Pediatrics, Division of Hematology/Oncology, Augusta University, Augusta, GA, United States, [3]Department of Biochemistry and Molecular Biology, Augusta University, Augusta, GA, United States, [4]Center for Sickle Cell Disease, Department of Medicine-Hematology, The University of Tennessee Health Science Center, Memphis, TN, United States, [5]Center for Sickle Cell Disease, The University of Tennessee Health Science Center, Memphis, TN, United States

Introduction: Therapeutic strategies aimed at reactivating *HBG* gene transcription and fetal hemoglobin (HbF) synthesis remain the most effective strategy to ameliorate the clinical symptoms of sickle cell disease (SCD). We previously identified microRNA29B (MIR29B) as a novel HbF inducer *via* targeting enzymes involved in DNA methylation. We provided further evidence that the introduction of MIR29B into KU812 leukemia cells significantly reduced MYB protein expression. Therefore, the aim of this study was to determine the extent to which MIR29B mediates HbF induction *via* targeting *MYB* in KU812 leukemia cells and human primary erythroid progenitors and to investigate the role of MIR29B in HbF induction *in vivo* in the humanized Townes SCD mouse model.

Materials and methods: Human KU812 were cultured and normal CD34 cells (n = 3) were differentiated using a two-phase erythropoiesis culturing system and transfected with MIR29B (50 and 100 nM) mimic or Scrambled (Scr) control *in vitro*. A luciferase reporter plasmid overexpressing MYB was transfected into KU812 cells. Luciferase activity was quantified after 48 h. Gene expression was determined by quantitative real-time PCR. *In vivo* studies were conducted using Townes SCD mice (6 per group) treated with MIR29B (2, 3, and 4 mg/kg/day) or Scr control by 28-day continuous infusion using subcutaneous mini osmotic pumps. Blood samples were collected and processed for complete blood count (CBC) with differential and reticulocytes at weeks 0, 2, and 4. Flow cytometry was used to measure the percentage of HbF-positive cells.

Results: *In silico* analysis predicted complementary base-pairing between MIR29B and the 3′-untranslated region (UTR) of *MYB*. Overexpression of MIR29B significantly reduced *MYB* mRNA and protein expression in KU812

cells and erythroid progenitors. Using a luciferase reporter vector that contained the full-length *MYB* 3′-UTR, we observed a significant reduction in luciferase activity among KU812 cells that co-expressed MIR29B and the full-length *MYB* 3′-UTR as compared to cells that only expressed *MYB* 3′-UTR. We confirmed the inhibitory effect of a plasmid engineered to overexpress *MYB* on *HBG* activation and HbF induction in both KU812 cells and human primary erythroid progenitors. Co-expression of MIR29B and *MYB* in both cell types further demonstrated the inhibitory effect of MIR29B on *MYB* expression, resulting in *HBG* reactivation by real-time PCR, Western blot, and flow cytometry analysis. Finally, we confirmed the ability of MIR29B to reduce sickling and induce HbF by decreasing expression of *MYB* and *DNMT3* gene expression in the humanized Townes sickle cell mouse model.

Discussion: Our findings support the ability of MIR29B to induce HbF *in vivo* in Townes sickle cell mice. This is the first study to provide evidence of the ability of MIR29B to modulate *HBG* transcription by *MYB* gene silencing *in vivo*. Our research highlights a novel MIR-based epigenetic approach to induce HbF supporting the discovery of new drugs to expand treatment options for SCD.

KEYWORDS

MIR29B, fetal hemoglobin (HbF), MYB, HBG, sickle cell

Introduction

Sickle cell disease (SCD) is a common genetic red blood cell disorder that affects over 20 million individuals worldwide (1). People with SCD have abnormal hemoglobin S (HbS) molecules, resulting from an A to T mutation in codon 6 of the *HBB* gene (2, 3). Under low oxygen conditions, HbS molecules polymerize and erythrocytes sickle, which can result in vaso-occlusion. Therapeutic strategies aimed at reactivating *HBG* gene transcription and fetal hemoglobin (HbF) synthesis remains the most effective strategy to ameliorate the clinical symptoms of SCD, including vaso-occlusive crises (4, 5).

Recently microRNAs (miRNAs) have emerged as a novel class of potential therapeutics due to their ability to restore expression of genes involved in tumor suppression (6), aging (7), and various human diseases (8–11). MiRNAs are endogenous, small (∼22 nt in size) regulatory RNA molecules that function to modulate post-transcriptional gene silencing through complimentary base-pair binding to their target mRNAs (12). MiRNAs are naturally expressed at varying levels in mammalian tissues, including blood plasma, and serum (13). Studies also provide evidence for a role of miRNAs in directly targeting *HBG* gene expression or transcriptional repressors of *HBG* gene expression during hemoglobin switching (14, 15). Thus, miRNAs that target genes involved in regulating *HBG* gene expression may serve as attractive therapeutic candidates for HbF induction.

Previously, we demonstrated that microRNA29B (MIR29B) reactivated *HBG* gene transcription and induced HbF expression *in vitro* by inhibiting the *de novo* DNA methyltransferases, *DNMT3A* and *DNMT3B* (16). Our findings further suggested that MIR29B may also target the *HBG* transcriptional repressor protein MYB in KU812 leukemia cells (16). Here, we have expanded our findings and show for the first time that MIR29B reactivates *HBG* gene transcription and induces HbF expression by silencing the *HBG* repressor protein MYB *in vitro* in KU812 leukemia cells and normal human erythroid progenitors generated from $CD34^+$ stem cells and in the preclinical Townes SCD mouse model. Our preclinical findings highlight the therapeutic potential of MIR29B as a promising treatment for inducing HbF in SCD and other β-hemoglobinopathies.

Materials and methods

In vitro cell culture with microRNA29B and co-transfection with MYB DNA

Human KU812 leukemia cells were cultured in Iscove's Modified Dulbecco Medium with 10% fetal bovine serum as previously published by our lab (16). Cells were harvested for cell count and viability using 0.4% Trypan blue exclusion assay. Cells were seeded at a density of 0.5×10^6 viable cells

per 100 mm plate for different treatments. During log phase growth, KU812 cells were transfected with 50 and 100 nmol/l of pre-MIR29B (Applied Biosystems, Waltham, MA, USA) or Scrambled (Scr) oligonucleotide control (100 nmol/l) for 48 h in three independent replicates using Opti-MEM media (Gibco, Waltham, MA, USA) and Lipofectamine^{TM} 2000 transfection reagent (Invitrogen Carlsbad, CA, USA) according to the manufacturer's instructions, then harvested for subsequent analyses. KU812 cells were also co-transfected with pGL3-*MYB*-3′-untranslated region (UTR) (Addgene plasmid # 25,798, Watertown, MA, USA), which contains the full length *MYB* 3′UTR cloned into the *Xba*I/*Sal*I restriction sites of the pGL3-control luciferase reporter vector (17). The human tagged ORF clone engineered to overexpress c-*MYB* (Origene, Rockville, MD, USA) was used alone or co-transfected with MIR29B.

Erythroid differentiation of human CD34$^+$ stem cells and microRNA29B co-electroporation with MYB DNA

Human bone marrow CD34$^+$ stem cells (ReachBio, Seattle, WA, USA) were cultured in a modified two-phase liquid culture system as previously published (18). During phase I, stem cells were grown in minimum essential medium-α (αMEM) containing AB serum, interleukin-3 (10 ng/ml), stem cell factor (10 ng/ml), and erythropoietin (2 IU/ml). On day 7, cells transitioned to Phase II media where they remained under erythropoietin (2 IU/ml) stimulation. Erythroid progenitors were electroporated on day 8 with human mature MIR29B or Scr mimic or with c-*MYB* overexpression plasmid using the Amaxa® Human CD34$^+$ Cell Nucleofector® Kit. After 48 h, cells were harvested for reverse transcription-quantitative PCR (RT-qPCR), Western blot, and flow cytometry analysis. Giemsa staining was used to monitor cell morphology and cell counts; viability was monitored using 0.4% Trypan blue exclusion assay (Gibco, Carlsbad CA, USA).

RNA isolation and RT-qPCR analysis

Total RNA was isolated as previously published (16). To quantify mRNA levels for *MYB*, *HBG*, and *HBB* and the internal control β-actin, gene specific primers were used (**Supplementary Table 1**). All mRNA levels were normalized to β-actin before analysis. Quantification of MIR29B was performed using the TaqMan miRNA assay (Applied Biosystems, Waltham, MA, USA) according to the manufacturer's instructions and *RNU48* was used as endogenous control. The $2^{-\Delta\Delta Ct}$ method was used for calculating the relative amount of target mRNA. All RT-qPCR reactions were performed in triplicate, repeated at least three times, and always included a no-template sample as a negative control. RT-qPCR results are presented as average fold change of target gene in cells relative to Scr control, which was normalized to one.

Western blot analysis

Total protein was isolated and Western blot analysis was performed as previously published (16). Primary antibodies against MYB (59995S), HbF (39386S), and HbA (84934S) were purchased from Cell Signaling Technology (Danvers, MA, USA) and diluted in the range of 1:250 to 1:2000, incubated overnight and then followed by treatment with secondary antibody. The primary antibody against β-actin (AM4302), the internal control, was purchased from Invitrogen (Waltham, MA, USA).

Animal models and drug treatment

The humanized Townes SCD transgenic mouse (B6; 129-Hbatm1 [HBA] Tow Hbbtm2 [HBG1, HBB*] Tow/Hbbtm3 [HBG1, HBB] Tow/J), which completes hemoglobin switching from human γ-globin to β^s-globin shortly after birth (19), were purchased from Jackson Laboratories. Mice were maintained and genotyping was performed with gene specific primers. All animal studies were approved by the Augusta University Institutional Animal Care and Use Committee. Townes SCD mice ages 4–6 months old, 4–10 mice per group (equal males and females), were treated by 28 days of continuous infusion using surgically implanted subcutaneous Alzet mini-osmotic pumps (DURECT corporation, Alzet Osmotic Pumps, Cupertino, CA, USA). Drug treatment groups in SCD mice included: (1) 2 mg/kg/day Scr control, (2) 2 mg/kg/day MIR29B mimic, (3) 3 mg/kg/day Scr control, (4) 3 mg/kg/day MIR29B mimic, (5) 4 mg/kg/day Scr control, and (6) 4 mg/kg/day MIR29B mimic. MIR29B mimics were purchased from Dharmacon (Lafayette, CO, USA). Mice were weighed at week 0 before and after pump placement and then again at weeks 2 and 4. Blood samples were collected in BD Microtainer Capillary blood collection K2-EDTA tubes at weeks 0, 2, and 4 and were processed for complete blood count (CBC) with differential. The percentage of reticulocytes (acridine orange) was determined by flow cytometry. To quantify MIR29B levels, total RNA was isolated using TRIzol and RT-qPCR analysis was performed.

Alzet mini-osmotic pump implantation

Before surgery, Buprenorphine SR was given for pain and then mice anesthetize with Isoflurane by inhalation. Once the animal was anesthetized, the area where pump was placed was shaved and then cleaned with a surgical scrub consisting of alternating betadine and alcohol wipes. Lidocaine was injected locally followed by a half-inch mid-scapular incision for pump placement on the back of the animal. A hemostat was used to spread the incision and subcutaneous tissue to create a pocket for the pump. Scr control or MIR29B filled Alzet mini-osmotic

pump was inserted with the delivery portal first and wound closed with wound clips. Mice were monitored daily and pain was addressed with an additional dose of Buprenorphine SR. Once the mice wound was healed, clips were removed, and the blood samples were collected at weeks 0, 2, and 4 for analysis.

Complete blood count with differential

Peripheral blood from 4–6 months old mice was collected in BD Microtainer Lithium heparin tubes by tail vein bleeding. Automated CBC and differentials were completed on the Micros 60 CS/CT machine (HORIBA Medical/ABX Diagnostics, Irvine, CA, USA) according to the manufacturer's protocol.

In vitro RBC sickling analysis

In a BD Vacutainer EDTA tube, peripheral blood was collected through tail bleeding and washed with 1X phosphate buffered saline. Cells were suspended 1:300 ratio in Iscove's Modified Dulbecco's Medium containing 10% heat inactivated fetal bovine serum, and incubated at 37°C in normoxic (21%) and hypoxic (1%) conditions for 12 h in a O_2 hypoxic chamber (Coy Laboratory Products, Grass Lake, MI, USA). Subsequently, blood samples were treated with 4% formaldehyde for 10 min and then transferred to room temperature. Using light microscopy, sickling of red blood cells were quantified by changes in cell morphology. 20X magnification of bright field images were attained on an EVOS Cell Imaging systems (Thermo Fisher Scientifics, Waltham, MA, USA).

Flow cytometry analysis

To measure the percentage of HbF positive cells (F-cells), mouse peripheral blood samples were washed using cold PBS containing 0.5% BSA and fixed with 4% paraformaldehyde. Centrifuged to discard the supernatant, cells were washed and permeabilized with 1:1 of Acetone and Methanol and then stained with FITC conjugated sheep anti-human HbF antibody (Cat. #A80-136) purchased from Bethyl Laboratories, Inc. (Montgomery, TX, USA). Sheep IgG Isotype control was used to detect non-specific staining. The cells were washed once with phosphate buffered saline containing 0.5% bovine serum albumin buffer, and F-cells were measured and quantified by flow cytometry (20) using AttuneTM NxT Flow Cytometer and Attune and Novex software (ThermoFisher Scientific, Rockland, IL, USA).

Statistical analysis

Data from at least 3–6 replicates of independent experiments were reported as the mean ± standard error of the mean (SEM). Data was analyzed by ANOVA or by a two-tailed Student's *t*-test to determine statistical significance. Statistical analysis was completed by unpaired student's *t*-test and ANOVA for $N = 6$ mice per group for two independent experiments. $P < 0.05$ was considered statistically significant.

Results

We previously showed that overexpression of MIR29B resulted in a significant decrease in expression of MYB, a known *HBG* repressor protein, in KU812 leukemia cells (16). To determine the effect of exogenous MIR29B expression on *MYB* gene silencing, we initially introduced MIR29B mimic (50 and 100 nM) into KU812 cells, which expresses the *HBG* and *HBB* genes, and measured *MYB* mRNA expression using RT-qPCR (**Figure 1**). We observed that transfection of MIR29B mimic (100 nM) for 48 h significantly decreased *MYB* mRNA expression by 25% as compared to Scr control (100 nM) cells ($p < 0.01$) (**Figure 1A**). Therefore, we utilized KU812 cells to determine whether MIR29B alters *MYB* expression as a mechanism of HbF induction.

Because miRNAs are known to post-transcriptionally regulate expression of their target mRNAs *via* binding to the 3′-UTR to repress protein production, we hypothesized that MIR29B might target the 3′-UTR of *MYB*. Using *in silico* analysis, we discovered that MIR29B has a consensus sequence complimentary to the 3′-UTR of *MYB* (**Figure 1B**). Thus, we wanted to determine whether *MYB* is regulated by MIR29B in KU812 cells. We transfected a luciferase reporter plasmid containing the full-length *MYB* 3′-UTR sequence. The sequence replaces the SV40 enhancer and SV40 poly (A) signal of pGL3-control (17) (**Figure 1C**) into KU812 cells alone or in combination with MIR29B mimic (50 and 100 nM) or Scr control (100 nM) and assessed luciferase activity (**Figure 1D**). In cells that overexpressed the full length *MYB* 3′-UTR alone, luciferase activity was significantly increased ($P < 0.0001$) as compared to Scr control cells (**Figure 1D**). However, when exogenous MIR29B mimic (50 and 100 nM) was co-expressed with the full length *MYB* 3′-UTR, luciferase activity was significantly reduced by up to 16-fold (MIR29B 50 nM, $p < 0.001$) and 80-fold (MIR29B 100 nM, $p < 0.001$) as compared to KU812 cells transfected with the full length *MYB* 3′-UTR alone (**Figure 1D**). Western blot analysis further confirmed the inhibitory effect of introducing MIR29B (100 nM) on MYB protein expression (**Figure 1D**). Specifically, co-transfection of MIR29B mimic (100 nM) and a plasmid engineered to overexpress c-*MYB* into KU812, resulted in a significant decrease in MYB protein expression in KU812 cells (**Figure 1D**). Thus, based on these findings we postulated that MIR29B might activate *HBG* transcription, in part *via* binding the 3′-UTR of *MYB*.

To determine the extent to which MIR29B reactivates *HBG* gene expression *via MYB* gene silencing, we used a human tagged c-*MYB* ORF clone to overexpress *MYB* alone or when co-transfected with MIR29B mimic (50 or 100 nM) or Scr control (100 nM) into KU812 cells (**Figure 2**). Western blot analysis revealed that transfection of MYB plasmid DNA at a concentration of 2 μg resulted in the highest expression of MYB protein and lowest expression of HbF when compared to control

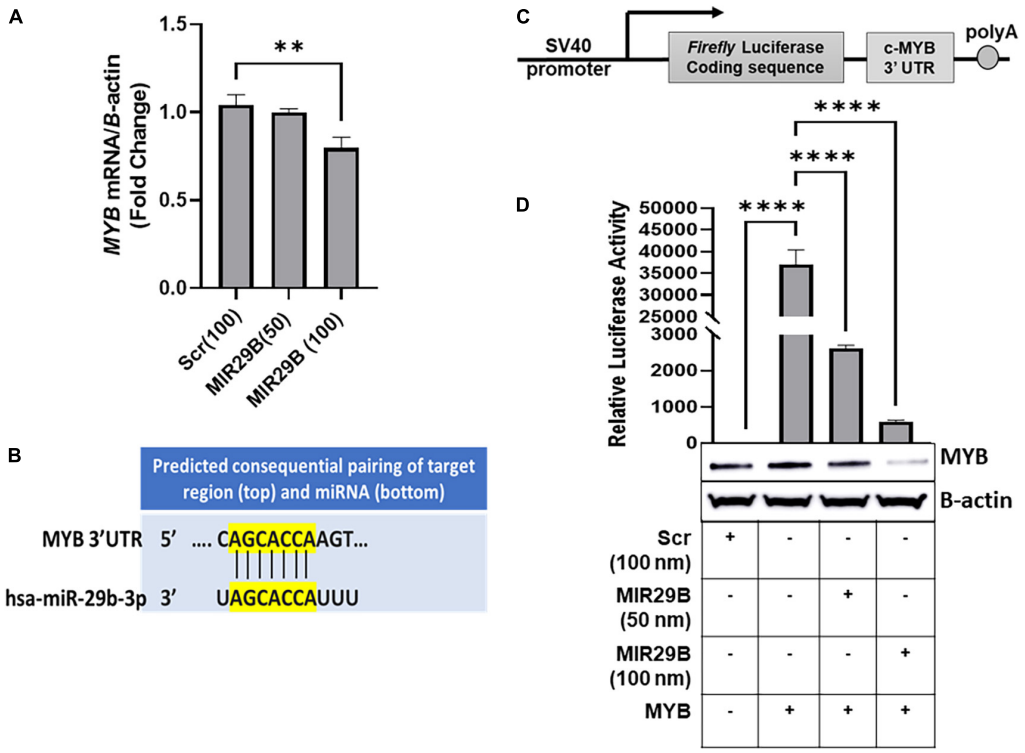

FIGURE 1
MicroRNA29B (MIR29B) targets 3′-untranslated region (UTR) of *MYB* in KU812 cells. **(A)** Real-time PCR analysis of *MYB* mRNA relative to β-actin internal control in KU812 cells transfected with MIR29B mimic (50 and 100 nM) or scrambled (Scr) control (100 nm). **(B)** Complementary sequence for MIR29B and the 3 – UTR of *MYB*. **(C)** Luciferase reporter construct containing the c-*MYB* 3′-UTR. **(D)** Luciferase activity in KU812 cells 48 h after transfection with MIR29B mimic (50 and 100 nM) and *MYB* 3′-UTR reporter plasmid. Accompanied Western blot showing expression of MYB protein in cells co-transfected with MYB overexpression plasmid and MIR29B mimic or Scr control (100 nm). The + and – signs indicate the presence or absence of MYB or MIR29B overexpression in KU812 cells. Data are shown at the mean ± standard error of the mean (SEM). $^{**}P < 0.005$, $^{***}P < 0.0005$, and $^{****}P < 0.0001$ is statistically significant.

cells (Figure 2A). Therefore, we used a concentration of 2 μg of MYB plasmid DNA to overexpress MYB in the remaining experiments. We further confirmed efficient transfection of *MYB* alone or when co-transfected with MIR29B (50 and 100 nM) into KU812 cells after 48 h by RT-PCR (Figure 2). Specifically, in KU812 cells co-transfected with MIR29B (50 and 100 nM) and *MYB*, we observed a significant increase in *MYB* mRNA expression as compared to Scr (100 nM) control cells and cells transfected with MIR29B alone (Figure 2B). Furthermore, we observed a drastic decrease, up to two-fold, in *MYB* mRNA expression in cells that were co-transfected with MIR29B (50 and 100 nM), which further suggests that MIR29B targets *MYB* resulting in its gene silencing.

Therefore, to determine whether MIR29B mediates *HBG* reactivation, in part *via MYB* gene silencing, we next quantified the ratio of *HBG*-to-*HBB* mRNA expression as a function of *HBG*/*HBG* + *HBB* using RT-qPCR analysis (Figure 2D). Based on our previous findings in KU812 cells, we observed a significant, up to a two-fold, increase in the ratio of *HBG*-to-*HBB* mRNA in cells transfected with MIR29B (50 and 100 nM) alone as compared to Scr control cells ($p < 0.001$). Furthermore, there was no significant difference in the ratio of *HBG*-to-*HBB* mRNA in KU812 cells transfected with *MYB* alone as compared to Scr control cells, which further confirms the role of MYB as an *HBG* repressor protein. Interestingly, in cells co-transfected with both MIR29B (100 nM) and *MYB*, the ratio of *HBG*-to-*HBB* mRNA yielded close to a two-fold increase which is similar to that of cells transfected with MIR29B alone (Figure 2D). In support of these findings, we further demonstrated a significant increase in HbF protein expression as a ratio of HbF-to-HbA in KU812 cells transfected with MIR29B (100 nM) alone or in combination with *MYB* as compared to Scr control cells (Figures 2E,F). We also confirmed the positive expression of MYB protein in cells transfected with MYB alone or in combination with MIR29B (50 and 100 nM). Flow cytometry analysis further confirmed the ability of MIR29B (50 and 100 nM) alone and in combination with MYB to increase the% of HbF-positive cells by up to 4.8-fold as compared to Scr control cells (Supplementary Figure 1). These findings further support our hypothesis that MIR29B mediates *HBG* reactivation and HbF induction in part *via MYB* gene silencing.

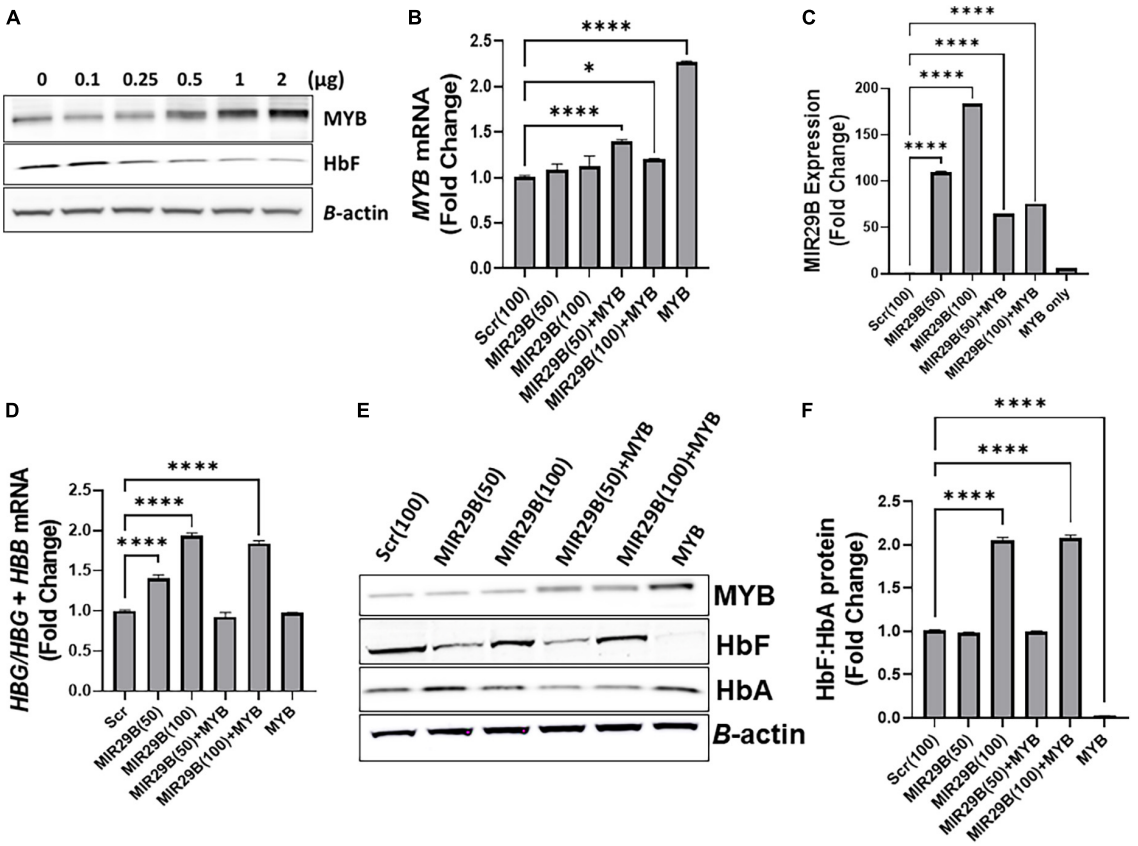

FIGURE 2
MicroRNA29B (MIR29B) mediates *HBG* reactivation *via* inhibiting *MYB* in KU812 cells. **(A)** Western blot analysis of MYB and HbF protein expression relative to β-actin protein in KU812 cells transfected with increasing concentrations of MYB plasmid DNA (μg). Fold change of **(B)** *MYB* mRNA relative to β-actin, **(C)** MIR29B expression relative to RNU48, and **(D)** *HBG*-to-*HBB* mRNA ratio as a function of *HBG*/*HBG* + *HBB* mRNA expression in KU812 cells following transfection for 48 h with MIR29B mimic (50 or 100 nm) alone or in combination with MYB overexpression plasmid vector (2 μg). **(E)** Western blot of MYB, HbF, and HbA protein expression with β-actin internal control. **(F)** Bar graphs showing quantification of the ratio of HbF-to-HbA protein in KU812 cells transfected with MIR29B alone or in combination with MYB. *$P < 0.05$ **$P < 0.005$, ***$P < 0.0005$, and ****$P < 0.0001$ is statistically significant.

We next performed studies under physiological conditions to further determine the extent to which MIR29B mediates *HBG* reactivation *via* inhibiting *MYB* using normal erythroid progenitors generated from human CD34^{+} stem cells in a liquid culture system as previously described (16, 21). Cell morphology examination by Giemsa staining confirmed erythroid lineage commitment by day 7 followed by the appearance of mature red blood cells by day 12 (Figure 3A). To compliment cell morphology, we also measured expression of the erythroid differentiation markers CD235a and CD71 by flow cytometry (Supplementary Figures 2A,B) to gain a better understanding of the effect of ectopic expression of MIR29B and *MYB* on erythroid maturation. The expression of CD71 did not significantly change following introduction of MIR29B alone; however, levels of CD71 decreased in cells that expressed *MYB* as compared to Scr control (Supplementary Figure 2A). Conversely, the percentage of CD235a positive cells significantly increased following MIR29B alone or in combination with *MYB* when compared to Scr control cells (Supplementary Figure 2B). Together, these data suggest that elevated levels of MIR29B and *MYB* may enhance erythroid maturation.

To further determine whether MIR29B and *MYB* were effectively delivered into the cells by electroporation, we measured MIR29B and *MYB* mRNA expression by RT-qPCR (Figures 3B,C). We observed a significant seven-fold increase in erythroid progenitors electroporated with *MYB* alone as compared to Scr control cells (Figure 3B). *MYB* mRNA expression was also confirmed in erythroid progenitors following co-electroporation with MIR29B (50 and 100 nM). Co-electroporation of erythroid progenitors with MIR29B (100 nM) and *MYB* significantly decreased *MYB* mRNA expression by 2.3-fold when compared to *MYB* alone (Figure 3B). In addition, we observed a dose-dependent increase up to 400-fold in MIR29B expression in cells transfected with MIR29B (50 and 100 nM) alone as compared to Scr control cells ($p < 0.0001$)(Figure 3C). This finding

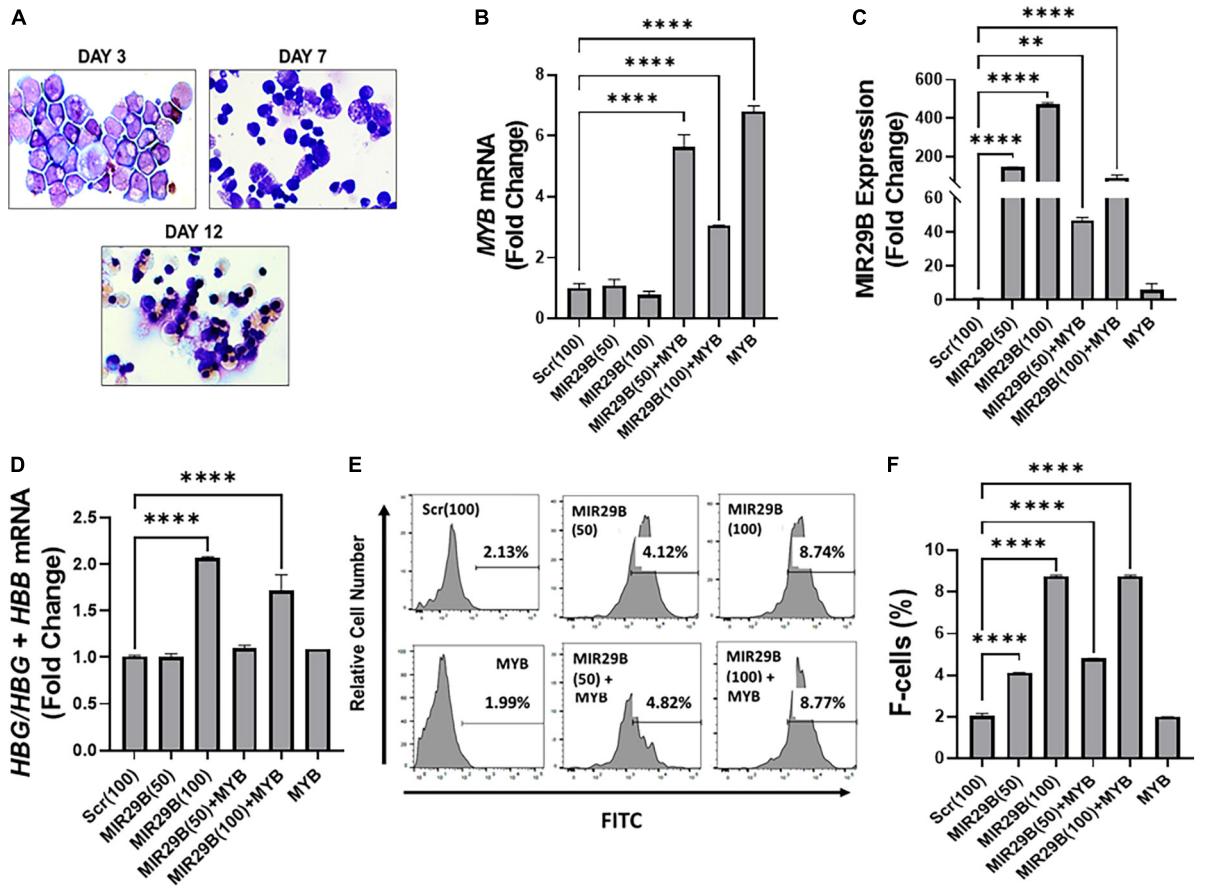

FIGURE 3
MicroRNA29B (MIR29B) mediates *HBG* reactivation *via MYB* gene silencing in normal human erythroid progenitors. **(A)** Wright-Giemsa-stained images of human erythroid progenitors (n = 3) undergoing differentiation at day 3, day 7, and day 12 *in vitro*. Quantitative RT-qPCR analysis of **(B)** *MYB* mRNA relative to β-actin, **(C)** MIR29B relative to RNU48, and **(D)** *HBG*-to-*HBB* mRNA ratio as a function of *HBG*/*HBG* + *HBB*, **(E)** Representative histograms from flow cytometry analysis of the percentage of HbF-positive cells following transfection of MIR29B alone or in combination with *MYB* into primary erythroid progenitors (n = 3) stained with FITC anti-HbF antibody. **(F)** Bar graphs showing quantification of the ratio of HbF-to-HbA in erythroid progenitors (n = 3) transfected with MIR29B alone or in combination with *MYB*. *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$, and ****$P < 0.0001$ is statistically significant.

further supports the role of MIR29B in inhibiting *MYB* gene expression. This difference in expression of MIR29B between cells electroporated with MIR29B alone or co-electroporated with *MYB* is likely due to overexpression of *MYB*.

We further determined whether MIR29B mediates *HBG* activation *via* silencing *MYB* under physiological conditions. We co-expressed MIR29B (50 and 100 nM) and *MYB* into normal erythroid progenitors and measured the expression of *HBG*-to-*HBB* mRNA by RT-qPCR (Figure 3D). Real-time PCR analysis revealed a significantly higher *HBG*-to-*HBB* mRNA ratio by up to two-fold in erythroid progenitors electroporated with MIR29B (100 nM) alone or up to 1.5-fold when co-expressed with *MYB* as compared to Scr control cells (Figure 3D). Furthermore, there was no change in the ratio of *HBG*-to-*HBB* mRNA in erythroid progenitors electroporated with *MYB* alone when compared to Scr control cells (Figure 3D). In support of these findings, we further demonstrated by flow cytometry analysis the inhibitory effect of overexpressing MIR29B on MYB function (Figure 3E). Specifically, we confirmed a significant, up to four-fold, increase ($P < 0.0001$) in the percentage of HbF-positive cells among erythroid progenitors whether they overexpressed MIR29B alone (50 and 100 nM) or in combination with *MYB* ($P < 0.0001$) (Figures 3E,F). Furthermore, the percentage of HbF-positive cells in erythroid progenitors electroporated with *MYB* alone was similar to that of Scr control cells (Figures 3E,F). These studies support an important role for MIR29B in *MYB* and *HBG* gene regulation *in vitro*.

To advance the field and move novel small molecules from bench to bedside requires evidence of *in vivo* efficacy of HbF induction. Therefore, our final preclinical studies evaluated the potential of MIR29B to induce HbF using the humanized sickle cell mouse model. Townes sickle cell

mice are an excellent model since they express human α-globin, γ-globin, and β^S-globin genes and exhibit phenotypes including chronic hemolysis and sickling similar to human SCD. Mice 4–6 months old were administered MIR29B (2, 3, and 4 mg/kg/day) or Scr control (same concentration) to establish optimal dosing by continuous 4 weeks infusion using subcutaneous mini-osmotic pumps with six mice per treatment group (**Figure 4A**). At week 0, 2, and 4, mice were weighed and blood samples collected by tail bleed for automated CBC and reticulocyte percent, percentage of F-cells by flow cytometry. Over 4 weeks of treatment, no drug toxicity occurred or death (**Supplementary Figure 3**). Scr control mice had no significant change in blood counts over the 4 weeks treatment period, except for platelets at 3 mg/kg after 2 weeks (**Supplementary Figure 3A**). Treatment of mice with MIR29B (2–4 mg/kg/day) after 2 weeks caused a significant increase in the number of platelets 1.6-fold ($p < 0.0005$) and reticulocytes 1.7-fold ($p < 0.05$), suggesting that MIR29B stimulated erythropoiesis. By contrast, levels of total hemoglobin, hematocrit, and lymphocytes did not significantly change after MIR29B treatment (**Supplementary Figures 3B,C**). During MIR29B treatment, all mice exhibited normal behavior and had steady weight gain (**Supplementary Figure 3D**). At the end of treatment, pumps were removed to confirm that all medications were delivered.

We next analyzed the ability of MIR29B to induce HbF expression *in vivo*. As shown in **Figure 4B**, the percentage of F-cells significantly increased 2.1 and 3.4-fold in mice treated with 2 and 3 mg/kg MIR29B, respectively. We further demonstrated the anti-sickling effect mediated by MIR29B under hypoxic conditions (**Figure 4C**). Sickle erythroid precursors from Townes mice were incubated in 1% hypoxia conditions overnight, fixed with formaldehyde and examined by light microscopy. As shown in **Figure 4D**, MIR29B reduced the percentage of sickled erythroid precursors by up to 68% ($p < 0.0005$) after 4 weeks treatment, supporting the anti-sickling effects mediated by MIR29B. These findings support the ability of MIR29B to induce HbF *in vivo* in preclinical Townes sickle cell mice.

We previously showed that MIR29B functions as a DNA methyltransferase inhibitor by targeting *DNMT* gene expression *in vitro* (16). Since treatment of mice with 3 and 4 mg/kg MIR29B at 4 weeks significantly reduced the percentage of sickle cells, we measured mRNA expression from mice spleen tissue at week 0 and week 4 (**Figure 4D**). We confirmed a significant increase up to 3.8-fold in MIR29B expression in mice treated with 3 and 4 mg/kg MIR29B, respectively, compared to Scr control. Treatment of mice with 3 and 4 mg/kg MIR29B resulted in up to a 68% significant decrease in MYB mRNA expression ($p < 0.005$) and up to 76% for DNMT3B (**Figure 4D**). Similarly, treatment of mice with 3 mg/kg significantly decreased DNMT1 mRNA up to 70% and DNMT3A mRNA expression by 90%, while *HBG* mRNA shown as a function of *HBG*-to-*HBB* mRNA ratio significantly increased (**Figure 4D**), suggesting that MIR29B reactivated *HBG* gene expression in part *via* inhibiting *DNMT3* and *MYB* gene expression. Collectively, our findings support the ability of MIR29B to function as an HbF inducer for the treatment of β-hemoglobinopathies in part *via* targeting *MYB*.

Discussion

MicroRNAs represent a novel class of small molecules that have gained much attention for their diagnostic and therapeutic potential to treat a wide variety of clinical diseases *via* targeting oncogenes and their gene products (22–25). MiRNAs are short (∼22 nucleotides) non-coding RNA molecules that associate with the miRNA-induced silencing complex (mRISC) and guide mRISC to silence specific mRNA in the cytoplasm (26). MicroRNAs facilitate mRNA degradation or suppression of translation by base-pairing to complementary sequences in the 3′-UTR of target mRNA (12, 26). We previously showed that MIR29B functions as an HbF inducer in KU812 cells and normal human erythroid progenitors by targeting *DNMT3* gene silencing (16). The discovery of novel small miRNA molecules, such as MIR29B, that target genes involved in *HBG* gene silencing to induce HbF levels will expand strategies to develop therapeutic options for SCD and other β-hemoglobinopathies.

Indeed, several miRNAs have been associated with HbF induction. In earlier studies by Bianchi et al. (27) they reported the upregulation of MIR210 in erythroid precursors of a thalassemia patient with high HbF levels. Additionally, they demonstrated that mithramycin targeted MIR210 as a mechanism of *HBG* activation in KU812 cells (27). In a subset of patients with SCD treated with hydroxyurea, expression of MIR26B, and MIR-151-3p was associated with HbF levels at maximum tolerated doses (28). Sangokoya et al. (29) were the first to report a correlation between MIR144 expression and the level of anemia in sickle cell patients and oxidative stress in sickle red blood cells due to reduced levels of the transcription factor NRF2. Following genome-wide miRNA expression profiling using reticulocytes isolated from SCD patients with extremes of HbF levels, our group confirmed increased MIR144 and reduced NRF2 levels in SCD patients with low HbF levels (21). Our findings provided additional supporting evidence for indirect mechanisms of HbF regulation by miRNAs (21). Previous studies by our group also demonstrated that MIR34A mediated HbF induction in KU812 cells by repression of STAT3 expression, another known repressor of *HBG* (30). Previously, Lee et al. (31) confirmed overexpression of LIN28B decreased miR-Let7 expression and increased HbF levels in primary erythroid cells. As further evidence of the therapeutic potential of miRNAs, MIR-486-3p induced HbF in adult erythroid progenitors by inhibiting BCL11A expression (32). MIR15A and

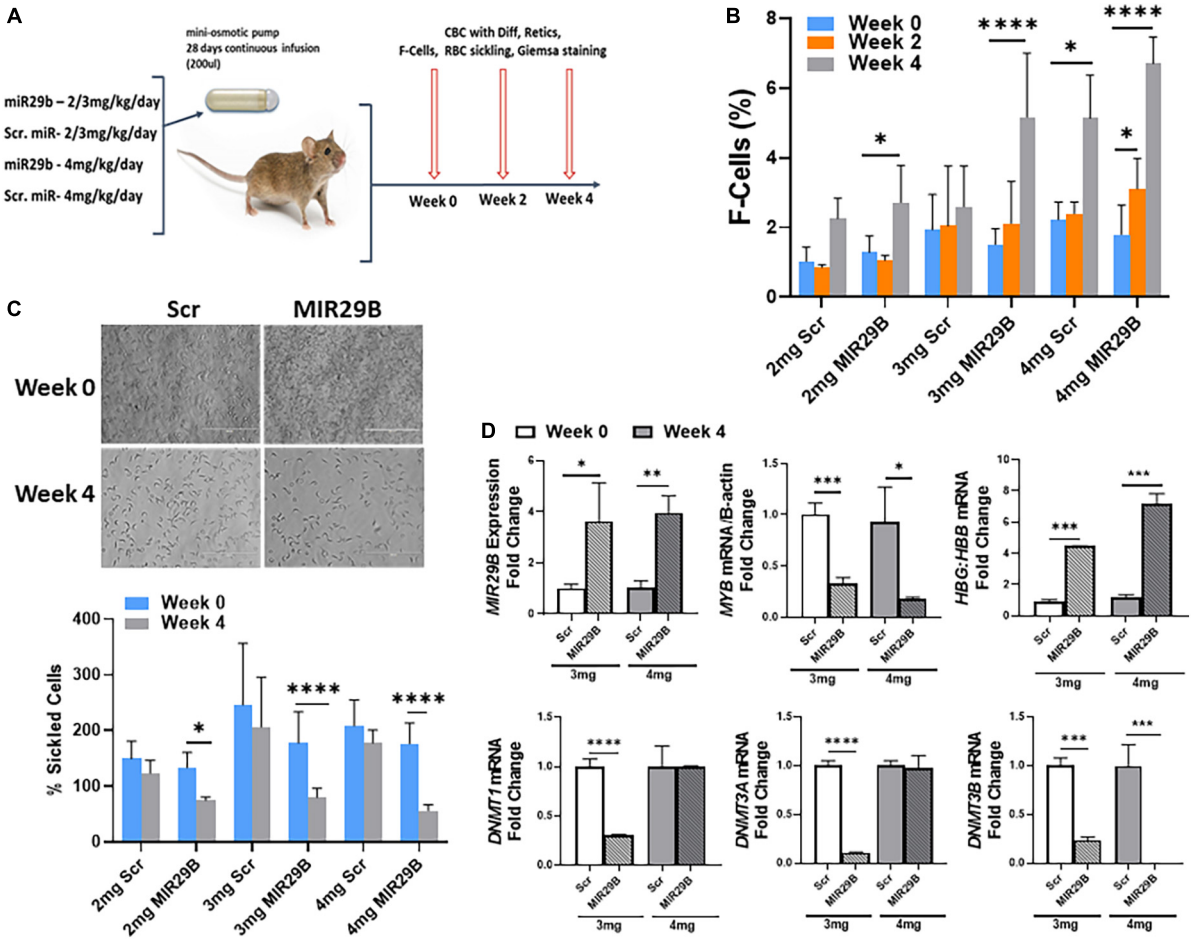

FIGURE 4
MicroRNA29B (MIR29B) induces HbF in humanized Townes sickle cell mice. **(A)** Townes SCD mice, which express human α-globin, γ-globin, and βS-globin genes and exhibit phenotypes including chronic hemolysis and sickling similar to human SCD, were surgically implanted with a mini-osmotic pump (Alzet) that continuously released MIR29B mimic at the concentration shown over 28 days. Age-and sex-matched mice (n = 4–10 per group) were treated and blood was collected at weeks 0, 2, and 4 for complete blood counts (CBC) with differential, reticulocytes, %F-cells, and red blood cell sickling under hypoxia conditions. Treatments consisted of 4 mice per group (2 males and 2 females) for MIR29B or SCR mimic at 2, 3, and 4 mg/kg/day and water vehicle and HU (100 mg/kg/day) controls. Tail–vein blood samples were collected in EDTA blood tubes at week 0, 2, and 4 along with weights. Evaluation of the effects of MIR29B on bone marrow hematopoiesis, were conducted by **(B)** Shown are bar graphs of the percentage of HbF-positive cells (F–cells) in erythroid progenitors isolated from Townes SCD mice and stained with fluorescein isothiocyanate-labeled anti-HbF antibody under the different treatment conditions. **(C)** Shown are bar graphs of the percentage of sickled cells obtained from freshly collected EDTA blood from sickle mice incubated under 1% hypoxia conditions for 12 h following the treatment conditions. **(D)** RT-qPCR analysis of MIR29B, *MYB*, *DNMT* gene expression and the *HBG*-to-*HBB* mRNA ratio as a function of *HBG*/*HBG* + *HBB* in mice treated with MIR29B or Scr control; *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$, and ****$P < 0.0001$ is statistically significant.

MIR16-1 also induced HbF by targeting the *HBG* repressor MYB in infants with human trisomy 13 (33). Our recently published work demonstrated that MIR29B functions as an HbF inducer in KU812 cells and normal human erythroid progenitors by targeting *DNMT3* gene silencing (16). This is the first study to provide evidence of a miRNA that targets DNA methylation machinery as a mechanism of HbF induction. In that same study, we showed that introduction of MIR29B into KU812 cells further decreased MYB protein expression (16).

MYB is a well-characterized oncogene protein that also functions as an *HBG* repressor protein (34). In a genome-wide association study consisting of SCD and β-Thalassemia patients, the *HBS1L-MYB* intergenic region was associated with ∼17% of the inherited HbF variance in those patients (35). The transcriptional activator MYB is known to be essential for definitive hematopoiesis (36) and is highly expressed in immature hematopoietic cells and downregulated during erythropoiesis (37). Overexpression of MYB has been demonstrated to reduce HbF levels in KU812 cells (34). Knockdown of MYB in primary human erythroid progenitors

has been reported to also induce HbF expression (33). Moreover, MYB regulates HbF expression in quantitative trait locus studies and functional assays (20, 28, 29, 33–35). MYB also indirectly silences *HBG* expression by activating KLF1 (Krüppel-like factor 1), a transcription factor that directly binds the *HBB* gene and BCL11A promoters during adult erythropoiesis (38).

Considering the function of MYB as an *HBG* repressor protein and our published study suggesting that MIR29B targets *MYB* in KU812 cells, we hypothesized that overexpression of MIR29B to silence *MYB* would mediate HbF induction. Using *in silico* analysis, we discovered that MIR29B has a consensus sequence complimentary to the 3′-UTR of *MYB*. We confirmed the ability of MIR29B to interact with the 3′-UTR of *MYB* using a luciferase reporter construct that expresses the *MYB* 3′-UTR. Introduction of MIR29B into KU812 cells and erythroid progenitors resulted in decreased MYB mRNA and protein, accompanied by an increase in HbF expression. Our findings support the ability of MIR29B to silence *MYB* through 3′UTR interactions. Collectively, our published studies support an essential role of MIR29B in modulating *HBG* transcription by two mechanisms involving DNA methylation and *MYB* gene silencing.

An excellent candidate for clinical development is MIR29B since it is a well-characterized tumor suppressor gene that functions as a DNMT inhibitor. To our knowledge, the only miRNA mimic that has previously undergone investigation in clinical trials was for liposomal injection of MIR34A mimic MRX43 for the treatment of advanced melanoma cancer (ClinicalTrials.gov Identifier: NCT01829971). Although this phase I clinical trial has now been terminated due to five immune related serious adverse events, the authors provided proof-of-concept for a miRNA-based therapy for cancer (39). MIR34A, similarly to MIR29B, regulates a broad number of genes involved in proliferation, metastasis, and chemo-resistance (40). Moreover, small RNA inhibitor molecules and antisense oligonucleotides, such as Patisiran (41) and Nusinersen (42), have been used in clinical trials for the treatments of hereditary transthyretin amyloidosis and infantile Spinal Muscular Atrophy, respectively. In the present study, we demonstrated for the first time the ability of MIR29B to increase HbF levels *via* inhibiting MYB in KU812 cells and normal human erythroid progenitors. We further showed the ability of MIR29B to induce HbF and reduce red blood cell sickling without producing adverse side effects in Townes sickle mice. Our findings further support the development of MIR29B as an HbF inducer in preclinical sickle transgenic mice.

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by the Augusta University Institutional Animal Care and Use Committee.

Author contributions

AS-D designed the research study. QG, AS, EA-A, LL, UO, and BL performed the research. AS-D and BP contributed essential reagents and tools. AS-D, QG, AS, CP, and BL analyzed the data. AS-D and BP wrote several drafts of the manuscript. All authors reviewed the final draft of the manuscript.

Funding

This work was funded through an NIH grant R01HL144641 (AS-D) and R01HL069234 (BP) and The University of Tennessee Health Science Center Office of Research CORNET award (AS-D).

Acknowledgments

We thank Yu Yao and Mitch Weiss at the St. Jude Children's Research Hospital in Memphis, TN for providing the research support.

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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.1043686/full#supplementary-material

References

1. Weatherall DJ. The role of the inherited disorders of hemoglobin, the first "molecular diseases," in the future of human genetics. *Annu Rev Genomics Hum Genet.* (2013) 14:1–24. doi: 10.1146/annurev-genom-091212-153500

2. Rees DC, Gibson JS. Biomarkers in sickle cell disease. *Br J Haematol.* (2012) 156:433–45. doi: 10.1111/j.1365-2141.2011.08961.x

3. Stuart MJ, Nagel RL. Sickle-cell disease. *Lancet.* (2004) 364:1343–60. doi: 10.1016/S0140-6736(04)17192-4

4. Powars DR, Weiss JN, Chan LS, Schroeder WA. Is there a threshold level of fetal hemoglobin that ameliorates morbidity in sickle cell anemia? *Blood.* (1984) 63:921–6. doi: 10.1182/blood.V63.4.921.bloodjournal634921

5. Estepp JH, Smeltzer MP, Kang G, Li C, Wang WC, Abrams C, et al. A clinically meaningful fetal hemoglobin threshold for children with sickle cell anemia during hydroxyurea therapy. *Am J Hematol.* (2017) 92:1333–9. doi: 10.1002/ajh.24906

6. Starlard-Davenport A, Kutanzi K, Tryndyak V, Word B, Lyn-Cook B. Restoration of the methylation status of hypermethylated gene promoters by microRNA-29b in human breast cancer: a novel epigenetic therapeutic approach. *J Carcinog.* (2013) 12:15. doi: 10.4103/1477-3163.115720

7. Jaber VR, Zhao Y, Sharfman NM, Li W, Lukiw WJ. Addressing Alzheimer's Disease (AD) Neuropathology Using Anti-microRNA (AM) strategies. *Mol Neurobiol.* (2019) 56:8101–8. doi: 10.1007/s12035-019-1632-0

8. Wang V, Wu W. MicroRNA-based therapeutics for cancer. *BioDrugs.* (2009) 23:15–23. doi: 10.2165/00063030-200923010-00002

9. Shah V, Shah J. Restoring ravaged heart: molecular mechanisms and clinical application of miRNA in heart regeneration. *Front Cardiovasc Med.* (2022) 9:835138. doi: 10.3389/fcvm.2022.835138

10. Sobolewski C, Dubuquoy L, Legrand N. MicroRNAs, tristetraprolin family members and HuR: a complex interplay controlling cancer-related processes. *Cancers.* (2022) 14:3516. doi: 10.3390/cancers14143516

11. Contiliani DF, Ribeiro YA, de Moraes VN, Pereira TC. MicroRNAs in prion diseases-from molecular mechanisms to insights in translational medicine. *Cells.* (2021) 10:1620. doi: 10.3390/cells10071620

12. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell.* (2004) 116:281–97. doi: 10.1016/S0092-8674(04)00045-5

13. Duttagupta R, Jiang R, Gollub J, Getts RC, Jones KW. Impact of cellular miRNAs on circulating miRNA biomarker signatures. *PLoS One.* (2011) 6:e20769. doi: 10.1371/journal.pone.0020769

14. Starlard-Davenport A, Gu Q, Pace BS. Targeting genetic modifiers of HBG gene expression in sickle cell disease: the miRNA option. *Mol Diagn Ther.* (2022) 26:497–509. doi: 10.1007/s40291-022-00589-z

15. Starlard-Davenport A, Fitzgerald A, Pace BS. Exploring epigenetic and microRNA approaches for gamma-globin gene regulation. *Exp Biol Med.* (2021) 246:2347–57. doi: 10.1177/15353702211028195

16. Starlard-Davenport A, Smith A, Vu L, Li B, Pace BS. MIR29B mediates epigenetic mechanisms of HBG gene activation. *Br J Haematol.* (2019) 186:91–100. doi: 10.1111/bjh.15870

17. Navarro F, Gutman D, Meire E, Caceres M, Rigoutsos I, Bentwich Z, et al. miR-34a contributes to megakaryocytic differentiation of K562 cells independently of p53. *Blood.* (2009) 114:2181–92. doi: 10.1182/blood-2009-02-205062

18. Li B, Ding L, Yang C, Kang B, Liu L, Story MD, et al. Characterization of transcription factor networks involved in umbilical cord blood CD34+ stem cells-derived erythropoiesis. *PLoS One.* (2014) 9:e107133. doi: 10.1371/journal.pone.0107133

19. Wu LC, Sun CW, Ryan TM, Pawlik KM, Ren J, Townes TM. Correction of sickle cell disease by homologous recombination in embryonic stem cells. *Blood.* (2006) 108:1183–8. doi: 10.1182/blood-2006-02-004812

20. Kodeboyina S, Balamurugan P, Liu L, Pace BS. cJun modulates Ggamma-globin gene expression *via* an upstream cAMP response element. *Blood Cells Mol Dis.* (2010) 44:7–15. doi: 10.1016/j.bcmd.2009.10.002

21. Li B, Zhu X, Ward CM, Starlard-Davenport A, Takezaki M, Berry A, et al. MIR-144-mediated NRF2 gene silencing inhibits fetal hemoglobin expression in sickle cell disease. *Exp Hematol.* (2019) 70:85–96.e5. doi: 10.1016/j.exphem.2018.11.002

22. Winkle M, El-Daly SM, Fabbri M, Calin GA. Noncoding RNA therapeutics – challenges and potential solutions. *Nat Rev Drug Discov.* (2021) 20:629–51. doi: 10.1038/s41573-021-00219-z

23. Fyfe I. MicroRNAs – diagnostic markers in Parkinson disease? *Nat Rev Neurol.* (2020) 16:65. doi: 10.1038/s41582-019-0305-y

24. Bhatnagar B, Garzon R. Clinical applications of MicroRNAs in acute myeloid leukemia: a mini-review. *Front Oncol.* (2021) 11:679022. doi: 10.3389/fonc.2021.679022

25. Geisler L, Mohr R, Lambrecht J, Knorr J, Jann H, Loosen SH, et al. The role of miRNA in the pathophysiology of neuroendocrine tumors. *Int J Mol Sci.* (2021) 22:8569. doi: 10.3390/ijms22168569

26. Kim VN. Small RNAs: classification, biogenesis, and function. *Mol Cells.* (2005) 19:1–15.

27. Bianchi N, Zuccato C, Lampronti I, Borgatti M, Gambari R. Expression of miR-210 during erythroid differentiation and induction of gamma-globin gene expression. *BMB Rep.* (2009) 42:493–9. doi: 10.5483/BMBRep.2009.42.8.493

28. Walker AL, Steward S, Howard TA, Mortier N, Smeltzer M, Wang YD, et al. Epigenetic and molecular profiles of erythroid cells after hydroxyurea treatment in sickle cell anemia. *Blood.* (2011) 118:5664–70. doi: 10.1182/blood-2011-07-368746

29. Sangokoya C, Telen MJ, Chi JT. microRNA miR-144 modulates oxidative stress tolerance and associates with anemia severity in sickle cell disease. *Blood.* (2010) 116:4338–48. doi: 10.1182/blood-2009-04-214817

30. Ward CM, Li B, Pace BS. Original research: stable expression of miR-34a mediates fetal hemoglobin induction in K562 cells. *Exp Biol Med.* (2016) 241:719–29. doi: 10.1177/1535370216636725

31. Lee YT, de Vasconcellos JF, Yuan J, Byrnes C, Noh SJ, Meier ER, et al. LIN28B-mediated expression of fetal hemoglobin and production of fetal-like erythrocytes from adult human erythroblasts *ex vivo*. *Blood.* (2013) 122:1034–41. doi: 10.1182/blood-2012-12-472308

32. Lulli V, Romania P, Morsilli O, Cianciulli P, Gabbianelli M, Testa U, et al. MicroRNA-486-3p regulates gamma-globin expression in human erythroid cells by directly modulating BCL11A. *PLoS One.* (2013) 8:e60436. doi: 10.1371/journal.pone.0060436

33. Sankaran VG, Menne TF, Scepanovic D, Vergilio JA, Ji P, Kim J, et al. MicroRNA-15a and –16-1 act *via* MYB to elevate fetal hemoglobin expression in human trisomy 13. *Proc Natl Acad Sci U.S.A.* (2011) 108:1519–24. doi: 10.1073/pnas.1018384108

34. Jiang J, Best S, Menzel S, Silver N, Lai MI, Surdulescu GL, et al. cMYB is involved in the regulation of fetal hemoglobin production in adults. *Blood.* (2006) 108:1077–83. doi: 10.1182/blood-2006-01-008912

35. Thein SL, Menzel S, Peng X, Best S, Jiang J, Close J, et al. Intergenic variants of HBS1L-MYB are responsible for a major quantitative trait locus on chromosome 6q23 influencing fetal hemoglobin levels in adults. *Proc Natl Acad Sci U.S.A.* (2007) 104:11346–51. doi: 10.1073/pnas.0611393104

36. Sheiness D, Gardinier M. Expression of a proto-oncogene (proto-myb) in hemopoietic tissues of mice. *Mol Cell Biol.* (1984) 4:1206–12. doi: 10.1128/mcb.4.7.1206-1212.1984

37. Vegiopoulos A, Garcia P, Emambokus N, Frampton J. Coordination of erythropoiesis by the transcription factor c-Myb. *Blood.* (2006) 107:4703–10. doi: 10.1182/blood-2005-07-2968

38. Tallack MR, Perkins AC. Three fingers on the switch: kruppel-like factor 1 regulation of gamma-globin to beta-globin gene switching. *Curr Opin Hematol.* (2013) 20:193–200. doi: 10.1097/MOH.0b013e32835f59ba

39. Hong DS, Kang YK, Borad M, Sachdev J, Ejadi S, Lim HY, et al. Phase 1 study of MRX34, a liposomal miR-34a mimic, in patients with advanced solid tumours. *Br J Cancer.* (2020) 122:1630–7. doi: 10.1038/s41416-020-0802-1

40. Saki N, Abroun S, Hajizamani S, Rahim F, Shahjahani M. Association of chromosomal translocation and MiRNA expression with the pathogenesis of multiple myeloma. *Cell J.* (2014) 16:99–110.

41. Adams D, Gonzalez-Duarte A, O'Riordan WD, Yang CC, Ueda M, Kristen AV, et al. Patisiran, an RNAi therapeutic, for hereditary transthyretin amyloidosis. *N Engl J Med.* (2018) 379:11–21.

42. Finkel RS, Mercuri E, Darras BT, Connolly AM, Kuntz NL, Kirschner J, et al. Nusinersen versus sham control in Infantile-Onset spinal muscular atrophy. *N Engl J Med.* (2017) 377:1723–32. doi: 10.1056/NEJMoa1702752

frontiers | Frontiers in Medicine

TYPE Case Report
PUBLISHED 29 November 2022
DOI 10.3389/fmed.2022.1048571

OPEN ACCESS

EDITED BY
Magali J. Fontaine,
University of Maryland, Baltimore, United States

REVIEWED BY
Efthymia Vlachaki,
Aristotle University of Thessaloniki, Greece
Mervat Mattar,
Cairo University, Egypt

*CORRESPONDENCE
Awni Alshurafa
dr.a.shurafa@gmail.com

SPECIALTY SECTION
This article was submitted to Hematology, a section of the journal Frontiers in Medicine

RECEIVED 19 September 2022
ACCEPTED 02 November 2022
PUBLISHED 29 November 2022

CITATION
Alshurafa A and Yassin MA (2022) Short- and long-term follow-up and additional benefits in a sickle cell disease patient experienced severe crizanlizumab infusion-related vaso-occlusive crisis: A case report. *Front. Med.* 9:1048571. doi: 10.3389/fmed.2022.1048571

Short- and long-term follow-up and additional benefits in a sickle cell disease patient experienced severe crizanlizumab infusion-related vaso-occlusive crisis: A case report

Awni Alshurafa* and Mohamed A. Yassin

Hamad Medical Corporation, Doha, Qatar

Sickle cell disease is an autosomal recessive disorder characterized by the presence of sickle hemoglobin that leads to chronic hemolysis and vaso-occlusive crisis. After decades of limited therapy options, crizanlizumab is a humanized monoclonal antibody approved by the Food and Drug Administration (FDA) in 2019 for sickle cell-related pain crises for patients 16 years of age and above. Although rare, infusion-related reactions, including painful crises, occurred in 3% as per the package insert. However, the data on how to deal with such reactions and about further treatment outcomes are limited as most patients stopped crizanlizumab after the reaction. Herein, we report the good outcome of 13 doses of crizanlizumab in a 19-year-old female patient with sickle cell disease on hydroxyurea, despite experiencing a severe infusion-related painful crisis during the second infusion. Additional benefits of crizanlizumab, in this case, were preventing new episodes of acute chest syndrome, quitting chronic narcotics use, and a remarkable improvement in quality of life and overall performance.

KEYWORDS

sickle cell disease, crizanlizumab, vaso-occlusive crisis, hemolytic anemia, infusion-related reactions

Introduction

Sickle cell disease (SCD) is an autosomal recessive disorder characterized by the presence of sickle hemoglobin, leading to chronic hemolysis, and vaso-occlusive events. Worldwide, an estimated 20–25 million people suffer from homozygous SCD. Acute pain crisis, acute chest syndrome, hepatic sequestration, and splenic sequestration are the most common vaso-occlusive-related events in SCD patients (1–3). Vaso-occlusive complications in SCD are thought to result from microvascular obstruction

by increased adhesion of sickle blood cells to the endothelium that may induce vascular changes, including endothelial damage, vasoconstriction, and inflammation that lead to vaso-occlusion (4).

P-Selectin is a cyto-adhesion molecule in resting platelets and endothelial cell granules, and it is expressed on the cell membrane once activated by inflammation and trauma. The upregulation of P-selectin initiates and maintains the binding of sickle red blood cells and leukocytes to the vessel wall, which contributes to the process of vaso-occlusive crises (5, 6).

Crizanlizumab is a humanized monoclonal antibody approved by the Food and Drug Administration (FDA) in 2019 for sickle cell-related pain crises for patients above 16 years of age (7). It binds to P-selectin, which, in turn, will block the interaction between sickle erythrocytes, leukocytes, platelets, and endothelial cells, leading to improved microvascular blood flow (8, 9). However, the package insert notes that 3% of patients who received crizanlizumab had infusion-related reactions but does not provide management guidance. Moreover, most of the reported cases stopped crizanlizumab after the infusion reaction. Herein, we report the short- and long-term outcomes and other benefits in a case of severe infusion-related painful crisis during the second crizanlizumab infusion in a 19-year-old female patient with SCD.

Case report

A 19-year-old female patient known case of sickle cell disease (hemoglobin SS) with a history of recurrent vaso-occlusive crisis requiring emergency visits and hospital admissions and a history of acute chest syndrome twice in the last 3 years. She was started on hydroxyurea 5 years ago and maintained a dose of 1,500 mg daily. However, it does not reduce the vaso-occlusive crisis severity and frequency and it does not prevent the occurrence of acute chest syndrome despite being a complaint of hydroxyurea.

The patient and family were counseled and agreed to start crizanlizumab in an attempt to reduce pain frequency and severity. She was started on 29 March 2021 with a dose of 5 mg per kg as a loading dose followed by another 5 mg/kg after 2 weeks then monthly 5 mg/kg. The last dose was cycle 13 on 29 May 2022 then stopped as she became pregnant.

The first infusion was uneventful and well tolerated. The patient came to the second loading infusion after 2 weeks in her baseline clinical status. By the end of the 2nd infusion, she experienced body pain mainly in the back and legs, rated as 9 out of 10 in severity. She was hemodynamically stable when checked with serial vital signs monitoring. There was no fever, skin rash, wheezes, or shortness of breath. Laboratory tests showed mild leukocytosis but hemoglobin level and hemolysis markers were in the baseline (Table 1). She was given subcutaneous 5 mg morphine but did not improve. Another 5 mg subcutaneous was added with no benefit. So the patient was admitted for treatment of vaso-occlusive crisis with patient-controlled analgesia (PCA) for 3 days. The patient and family were counseled about the event and they decided to continue crizanlizumab. In the subsequent infusions, she had premedication with acetaminophen and the infusion was extended to 1 h. It was well tolerated without any pain events during or within 24 h of the infusion.

During 13 doses of crizanlizumab plus hydroxyurea 1,500 mg daily, she visited the emergency department three times and was hospitalized once for 5 days due to a painful crisis (Table 2). Other additional benefits after starting crizanlizumab were preventing new episodes of acute chest syndrome, quitting narcotics, and improving quality of life and overall performance.

TABLE 1 Changes in laboratory results throughout admission.

Laboratory value	Before infusion	After infusion	Day of discharge
WBC (×109/L)	6.1	14.4	5.6
Hemoglobin (g/dl)	11	10.9	10.4
Reticulocytes count (×109/L)	236	249	263
Reticulocytes %	6.4	6.2	6.9
Platelets (×109/L)	217	208	213
Total bilirubin (mg/dl)	17	18	15

TABLE 2 Frequency of emergency department visits (ED), hospitalization, and total days of hospitalization before and during crizanlizumab use.

	Before crizanlizumab		During crizanlizumab
	Dec 2018–Jan 2020 (14 months)	Feb 2020–March 2021 (14 months)	April 2021–May 2022 (14 months)
ED visits	5	6	3
Hospitalization	3	4	1
Total days of hospitalization	21	27	5

Discussion

Crizanlizumab is a P-selectin inhibitor approved to reduce the frequency of vaso-occlusive pain crises in sickle cell disease patients. It received FDA approval following SUSTAIN study, a multicenter, randomized, double-blind, placebo-controlled, phase 2 trial, which showed that crizanlizumab therapy resulted in a significantly decreased sickle cell-related pain episodes by 45% and increased the median time to the first and second vaso-occlusive pain events. It is administered intravenously over 30 min, once every 2 weeks for 2 doses then every 4 weeks thereafter (10, 11). The most commonly reported side effects are nausea, abdominal pain, arthralgia, headache, back pain, and fever (12).

Post marketing, cases of severe infusion-related reactions, including severe pain events, have been reported, especially during the first and second infusions, some cases even required hospitalizations. The management of infusion-related pain events in the reported case was to hold infusion, and to give paracetamol, non-steroidal anti-inflammatory drugs, opioids, intravenous hydration, and/or oxygen supply (13–15).

The mechanism of crizanlizumab infusion-related pain crisis is not clear yet. Karkoska et al. suggested that this reaction may be a complement activation-related pseudoallergy (CARPA) as a work-up revealed a mild increase in sC5b-9 level in a 17-year-old male who experienced severe pain in his back and lower limbs within 10 min of the infusion, later he developed fever and hospitalized for 7 days to control his pain then patient and the family asked to stop further crizanlizumab doses (15). Li et al. hypothesized that crizanlizumab-associated painful febrile reaction may be IgG-mediated based on two cases that had reactions after a first uneventful infusion without evidence of mast cell activation or increased IgE production (14).

A systematic evaluation of post-marketing reports of pain events during or after crizanlizumab infusion showed that most affected patients (86%) initially developed infusion-related pain events at the first or second infusion, 71% required hospitalization, and the majority recovered within 3 days; however, crizanlizumab was stopped in 82% of patients after pain reaction, so the data about the long-term outcomes are lacking (13). In our case, the patient developed a severe reaction during the second infusion, which required hospitalization for pain management, then further infusions were uneventful and she had a good outcome. Clinicians need to be aware of rare infusion-related reactions to crizanlizumab even after the first uneventful dose. Additional benefits in this case after starting crizanlizumab were no episodes of acute chest syndrome, quitting chronic narcotics use and there was remarkable improvement in quality of life and overall performance, so, this reflects the importance of reporting the real-world data for novel therapeutic agents in SCD (16, 17). To our knowledge, this is the first case to report the long-term outcome of crizanlizumab after a severe infusion-related painful crisis.

In conclusion, considering the limited data, this case report indicates that patients who had crizanlizumab infusion-related pain reactions may benefit from further infusions and have good long-term outcomes; however, further studies are required to confirm this finding.

Data availability statement

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

Ethics statement

The case was approved by Hamad Medical Corporation Medical Research Center and the patient signed a written informed consent was obtained from the individual for the publication of any potentially identifiable images or data included in this article.

Author contributions

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

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.

Publisher's note

References

1. Borhade MB, Kondamudi NP. *Sickle Cell Crisis*. Treasure Island, FL: StatPearls Publishing (2022).

2. Wali Y, Kini V, Yassin MA. Distribution of sickle cell disease and assessment of risk factors based on transcranial Doppler values in the Gulf region. *Hematol Amst Neth.* (2020) 25:55–62. doi: 10.1080/16078454.2020.1714113

3. Yassin M, Soliman A, De Sanctis V, Nashwan A, Abusamaan S, Moustafa A, et al. Liver Iron Content (LIC) in Adults with Sickle Cell Disease (SCD): correlation with serum ferritin and liver enzymes concentrations in Trasfusion Dependent (TD-SCD) and Non-Transfusion Dependent (NT-SCD) Patients. *Mediterr J Hematol Infect Dis.* (2017) 9:e2017037. doi: 10.4084/MJHID.2017.037

4. Turhan A, Weiss LA, Mohandas N, Coller BS, Frenette PS. Primary role for adherent leukocytes in sickle cell vascular occlusion: a new paradigm. *Proc Natl Acad Sci USA.* (2002) 99:3047–51. doi: 10.1073/pnas.052522799

5. Man Y, Goreke U, Kucukal E, Hill A, An R, Liu S, et al. Leukocyte adhesion to P-selectin and the inhibitory role of Crizanlizumab in sickle cell disease: a standardized microfluidic assessment. *Blood Cells Mol Dis.* (2020) 83:102424. doi: 10.1016/j.bcmd.2020.102424

6. Frenette PS. Sickle cell vaso-occlusion: multistep and multicellular paradigm. *Curr Opin Hematol.* (2002) 9:101–6. doi: 10.1097/00062752-200203000-00003

7. Ali MA, Ahmad A, Chaudry H, Aiman W, Aamir S, Anwar MY, et al. Efficacy and safety of recently approved drugs for sickle cell disease: a review of clinical trials. *Exp Hematol.* (2020) 92:11–8.e1. doi: 10.1016/j.exphem.2020.08.008

8. Karki NR, Kutlar A. P-selectin blockade in the treatment of painful vaso-occlusive crises in sickle cell disease: a spotlight on crizanlizumab. *J Pain Res.* (2021) 14:849–56. doi: 10.2147/JPR.S278285

9. Adel AA, Abushanab D, Hamad A, Al-Badriyeh D, Yassin MA. L-glutamine and crizanlizumab for adults with sickle cell disease (SCD) in qatar: a cost effectiveness analysis. *Blood.* (2021) 138(Suppl 1):4945. doi: 10.1182/blood-2021-144636

10. Ataga KI, Kutlar A, Kanter J, Liles D, Cancado R, Friedrisch J, et al. Crizanlizumab for the prevention of pain crises in sickle cell disease. *N Engl J Med.* (2017) 376:429–39. doi: 10.1056/NEJMoa1611770

11. Delgado J, Voltz C, Stain M, Lapveteläinen T, Urach S, Lähteenvuo J, et al. The European medicines agency review of crizanlizumab for the prevention of recurrent vaso-occlusive crises in patients with sickle cell disease. *HemaSphere.* (2021) 5:e604. doi: 10.1097/HS9.0000000000000604

12. Riley TR, Riley TT. Profile of crizanlizumab and its potential in the prevention of pain crises in sickle cell disease: evidence to date. *J Blood Med.* (2019) 10:307–11. doi: 10.2147/JBM.S191423

13. Kanter J, Shah A, Joshi V, Mehta H, Levine M, Arunagiri U, et al. Rare cases of infusion-related reactions (IRRs) presenting as pain events during or after crizanlizumab infusion in patients (Pts) with Sickle Cell Disease (SCD): a systematic evaluation of post-marketing (PM) reports. *Blood.* (2021) 138:3112–21. doi: 10.1182/blood-2021-152362

14. Li VJ, Adesina OO, Fertrin KY. Crizanlizumab-associated painful febrile reaction in sickle cell disease patients. *Blood.* (2021) 138(Suppl 1):4186. doi: 10.1182/blood-2021-154355

15. Karkoska K, Quinn CT, Clapp K, McGann PT. Severe infusion-related reaction to crizanlizumab in an adolescent with sickle cell disease. *Am J Hematol.* (2020) 95:E338–9. doi: 10.1002/ajh.26002

16. Alshurafa A, Yassin MA. Case report: safety and efficacy of voxelotor in a patient with sickle cell disease and stage IV chronic kidney disease. *Front Med.* (2022) 9:931924. doi: 10.3389/fmed.2022.931924

17. Elenga N, Loko G, Etienne-Julan M, Al-Okka R, Adel AM, Yassin MA. Real-World data on the efficacy of L-glutamine in preventing sickle cell disease-related complications in pediatric and adult patients. *Front Med.* (2022) 9:931925. doi: 10.3389/fmed.2022.931925

frontiers | Frontiers in Medicine

TYPE Case Report
PUBLISHED 30 November 2022
DOI 10.3389/fmed.2022.1063255

OPEN ACCESS

EDITED BY
Magali J. Fontaine,
University of Maryland, Baltimore, United States

REVIEWED BY
Aloukick Kumar Singh,
Center for Stem Cell Research (CSCR), India
Giuseppe Lassandro,
University of Bari Aldo Moro, Italy

*CORRESPONDENCE
Awni Alshurafa
dr.a.shurafa@gmail.com

SPECIALTY SECTION
This article was submitted to Hematology, a section of the journal Frontiers in Medicine

RECEIVED 06 October 2022
ACCEPTED 08 November 2022
PUBLISHED 30 November 2022

CITATION
Alshurafa A, Elhissi M and Yassin MA (2022) Complete resolution of stage II avascular necrosis affecting three joints by hyperbaric oxygen in a patient with sickle cell disease: A case report.
Front. Med. 9:1063255.
doi: 10.3389/fmed.2022.1063255

Complete resolution of stage II avascular necrosis affecting three joints by hyperbaric oxygen in a patient with sickle cell disease: A case report

Awni Alshurafa[1]*, Mohammad Elhissi[2] and Mohamed A. Yassin[1]

[1]Department of Hematology, Hamad Medical Corporation, Doha, Qatar, [2]Clinical Imaging Department, Hamad Medical Corporation, Doha, Qatar

Avascular necrosis (AVN) or joint osteonecrosis is a debilitating complication of sickle cell disease, increasing the disease burden on both patients and healthcare systems. AVN can be radiologically categorized into early and late stages depending on the extent of the disease. Management of AVN is challenging and controversial. Generally, it includes conservative measures for early disease to preserve the joint for as long as possible and surgical management for more advanced diseases. Hyperbaric oxygen (HBO) therapy can be used as primary or adjunctive therapy for different medical disorders. Currently, the main rule of HBO in AVN is an adjunctive therapy to control symptoms and improve the quality of life of a patient; however, the concept of using HBO as a primary treatment choice for AVN in patients with sickle cell disease is not well evaluated yet. In this case study, we reported a 15-year-old boy with sickle cell disease who was suffering from stage II AVN in bilateral femoral and right shoulder joints. A total of 57 sessions of HBO resulted in the complete resolution of AVN in post-treatment MRI.

KEYWORDS

sickle cell disease, avascular necrosis, hyperbaric oxygen, osteonecrosis, vaso-occlusive crisis

Introduction

Sickle cell disease is an inherited disorder of the globulin chains and the most common genetic disorder in the United States (1). Patients with sickle cell disease (SCD) are at a high risk of a variety of serious organ system complications. Recurrent vaso-occlusive crisis, chronic hemolysis, hypercoagulability, and fat emboli are the main mechanisms

of end-organ injury in SCD (2–5). Avascular necrosis (AVN) is a well-reported complication that affects around 10% of patients with SCD (6). It can result in severe pain and loss of joint function affecting the patient's quality of life. AVN can be radiologically categorized into early and late stages depending on the extent of the disease (7).

The management of AVN is challenging and controversial. Generally, it includes conservative measures for early disease to preserve the joint for as long as possible, while surgical options are usually kept for more advanced diseases (8). Conservative measures may include physical therapy, offloading as tolerated, and pain management. Low-molecular-weight heparin may decrease the progression rate of idiopathic osteonecrosis from the pre-collapse to the collapsed stage (9, 10).

Hyperbaric oxygen (HBO) is a treatment modality in which individuals breathe 100 % of oxygen which may improve AVN by increasing the oxygen supply to joints. Currently, its main role in AVN is to control joint pain, improve the range of motion, and delay joint loss (11, 12). Some studies reported radiographic improvement in AVN stages I and II with HBO (13). However, the complete resolution of stage II AVN by HBO has not been reported before.

In this case study, we reported a 15-year-old boy with SCD, who was suffering from stage II avascular necrosis in both hips and right shoulder joints. A total of Fifty-seven sessions of HBO resulted in the complete resolution of AVN in post-treatment MRI.

Case report

We report a 15-year-old boy who was suffering from a known case of non-transfusion-dependent SCD (hemoglobin SS) and was on hydroxyurea of 1,000 mg daily. His disease course was complicated by a chronic aching pain and a limited range of motion in both hip and right shoulder joints. An MRI showed geographic subarticular high-signal intensities in STIR sequences with no femoral or humeral head collapse of both hip joints and right shoulder joint in keeping with a stage II AVN of both femurs and right shoulder (**Figures 1A**, **2A**, **3A**). Apart from SCD, there were no other risk factors for AVN.

Conservative measures were tried with pain management, offloading, and physiotherapy without significant clinical improvement. Thus, the patient and family were given counseling about HBO to control the joint pain and reduce analgesic use and they agreed to the treatment.

A total of 57 sessions of HBO therapy was given; it was five sessions per week that lasted for 2 h with a pressure of 2.5 pounds per square inch. The treatment was well-tolerated, and the course was uneventful of any side effects. The number of sessions was guided by clinical improvement.

The patient showed significant clinical improvement in the form of pain resolution and range of motion improvement. Post-treatment MRI showed total resolution of the previously noted geographic subarticular high-signal intensities of both hip joints and right shoulder joint denoting complete resolution (**Figures 1B**, **2B**, **3B**) of the AVN in affected joints.

Discussion

Avascular necrosis or osteonecrosis is a debilitating complication of SCD, increasing the disease burden on both patients and healthcare systems. Radiologically, AVN can be divided into pre-collapse and post-collapse of the subchondral

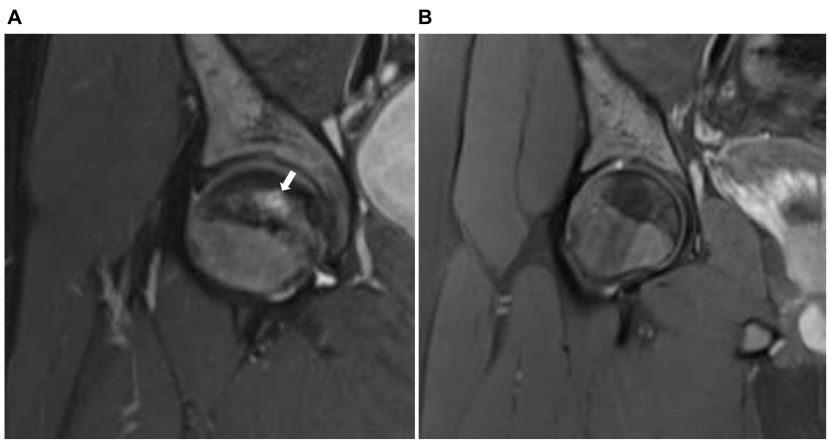

FIGURE 1
(A) Coronal STIR (short T1 inversion recovery) of the right hip before treatment with HBO showing geographic subarticular area of high-signal intensity (arrow) with no collapse of the head of the femur (stage II). **(B)** Coronal STIR (short T1 inversion recovery) of the right hip after treatment showing complete resolution of the previously seen subarticular high-signal intensity suggests complete radiological resolution of avascular necrosis.

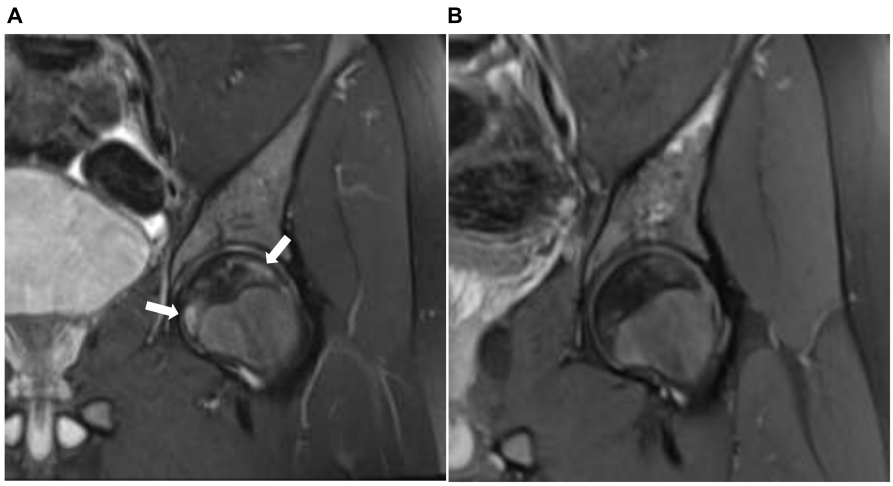

FIGURE 2
(A) Coronal STIR (short T1 inversion recovery) of the left hip before treatment with HBO showing geographic subarticular area of high-signal intensity (arrows) with no collapse of the head of the femur (stage II). **(B)** Coronal STIR (short T1 inversion recovery) of the left hip after treatment showing complete resolution of the previously seen subarticular high-signal intensity suggests complete radiological resolution of avascular necrosis.

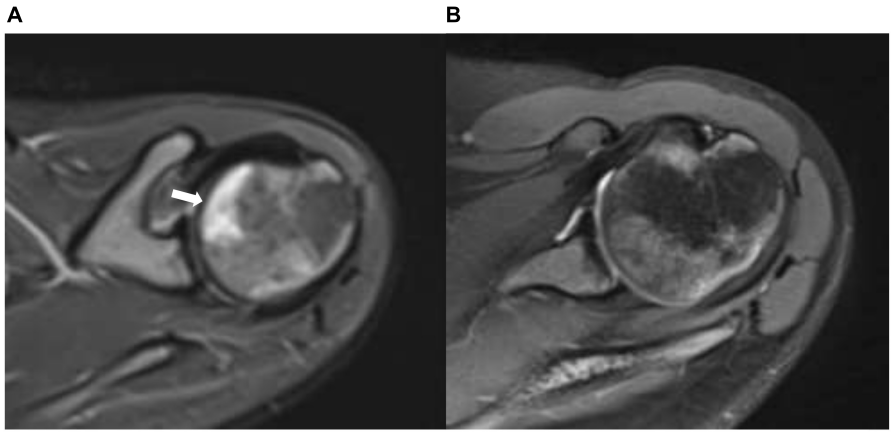

FIGURE 3
(A) Axial STIR (short T1 inversion recovery) of the right shoulder before treatment with HBO showing geographic subarticular area of high-signal intensity (arrow) with no collapse of the head of the femur (stage II). **(B)** Axial STIR (short T1 inversion recovery) of the right shoulder after treatment showing complete resolution of the previously seen subarticular high-signal intensity suggests complete radiological resolution of avascular necrosis.

surface. Although the management of AVN in patients with SCD is controversial, initially, conservative measures are usually tried because it is less invasive and may be effective for some individuals. Patients and their families are increasingly involved in making treatment decisions (14, 15).

Hyperbaric oxygen therapy is a treatment modality that can be used as primary or adjunctive therapy for different medical disorders. Although the therapy is not approved worldwide, the main rule of HBO in AVN is an adjunctive treatment to control symptoms and to improve the quality of life of a patient; however, the concept of using HBO as a primary treatment choice for AVN in patients with SCD is not well established yet (13, 16).

In a recently published systematic review and meta-analysis assessing the use of HBO in femoral head AVN, regardless of the underlying etiology, Paderno et al. showed that patients with femoral head AVN managed with HBO can achieve a significant clinical improvement in the form of pain reduction and improvement in the range of hip motion; however, the radiological outcome was not described (13). In a double-blind, randomized, controlled, prospective study evaluating HBO therapy in 20 patients with femoral head necrosis,

Camporesi et al. showed that besides clinical improvement, there was a continuous radiological improvement in most studied patients at 7 years of follow-up; however, most of the radiographic changes were seen between the baseline MRI and the 12-month post-treatment follow-up images (17). In an SCD-related AVN, Shier et al. reported three cases of pre-collapse femoral head AVN. Of the three cases, two cases showed significant clinical and radiological improvement with HBO (11).

The exact mechanism of the therapeutic effects of HBO in AVN is not yet fully understood. HBO acts by giving oxygen at high atmospheres of pressure, resulting in an increased level of dissolved oxygen in the plasma, and in turn, more oxygen reaches the tissues (18). At the molecular level, the main players in bone turnover are specific cytokines osteoprotegerin (OPG), receptor activator of NF-kB (RANK), and its ligand (RANKL). Any change in the OPG/RANKL/RANK interaction will lead to a shift either to bone formation or resorption. Previous studies showed that there were interactions between inflammatory factors such as interleukin-1 beta (IL-1b), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-a), and the OPG/RANK/RANKL. Bosco et al. showed a significant decrease in TNF-a and IL-6 plasma levels with HBO therapy over time. This decrease in inflammatory markers corresponded to reductions in joint pain and bone marrow edema on imaging (19–21).

Hyperbaric oxygen therapy is generally well-tolerated, and most side effects are mild and reversible. Pressure is usually maintained between 2.5 and 3.0 atm, while session number and duration are usually decided according to the indication; one or two sessions may be needed in acute settings, while more extended treatment is usually required for chronic medical conditions (22). The role of novel therapies in the sickle cell-related AVN is still not clear (23–25). In our case, the patient was complaining of chronic aching pain in three joints and requiring frequent analgesic use. HBO was started to control pain and delay joint loss. However, clinical and radiological follow-up showed a complete resolution of previously reported stage II AVN.

In conclusion, HBO therapy may be used as a primary treatment modality in an SCD-related AVN in both controlling symptoms and ameliorating radiological findings; however, further studies are needed to confirm these findings.

Data availability statement

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

Ethics statement

Written informed consent was obtained from the individual for the publication of any potentially identifiable images or data included in this article.

Author contributions

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

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.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

1. Khamees I, Ata F, Choudry H, Soliman AT, De Sanctis V, Yassin MA. Manifestations of HbSE sickle cell disease: a systematic review. *J Trans Med.* (2021) 19:262. doi: 10.1186/s12967-021-02931-1

2. Yassin M, Soliman A, De Sanctis V, Nashwan A, Abusamaan S, Moustafa A, et al. Liver iron content (LIC) in adults with sickle cell disease (SCD): correlation with serum ferritin and liver enzymes concentrations in trasfusion dependent (TD-SCD) and non-transfusion dependent (NT-SCD) patients. *Mediterr J Hematol Infect Dis.* (2017) 9:e2017037. doi: 10.4084/MJHID.2017.037

3. Wali Y, Kini V, Yassin MA. Distribution of sickle cell disease and assessment of risk factors based on transcranial doppler values in the Gulf region. *Hematol Amst Neth.* (2020) 25:55–62. doi: 10.1080/16078454.2020.1714113

4. Soliman AT, Yasin M, El-Awwa A, Abdelrahman MO, De Sanctis V. Does blood transfusion affect pituitary gonadal axis and sperm parameters in young males with sickle cell disease? *Indian J Endocrinol Metab.* (2013) 17:962–8. doi: 10.4103/2230-8210.122599

5. Yassin MA, Soliman AT, De Sanctis V, Abdula MAJ, Riaz LM, Ghori FF, et al. Statural growth and prevalence of endocrinopathies in relation to liver iron content (LIC) in adult patients with beta thalassemia major (BTM) and sickle cell disease (SCD). *Acta Bio Med Atenei Parm.* (2018) 89:33–40. doi: 10.23750/abm.v89i2-S.7085

6. Vanderhave KL, Perkins CA, Scannell B, Brighton BK. Orthopaedic manifestations of sickle cell disease. *J Am Acad Orthop Surg.* (2018) 26:94–101. doi: 10.5435/JAAOS-D-16-00255

7. Lee G-C, Khoury V, Steinberg D, Kim W, Dalinka M, Steinberg M. How do radiologists evaluate osteonecrosis? *Skeletal Radiol.* (2014) 43:607–14. doi: 10.1007/s00256-013-1803-4

8. Yawn BP, Buchanan GR, Afenyi-Annan AN, Ballas SK, Hassell KL, James AH, et al. Management of sickle cell disease: summary of the 2014 evidence-based report by expert panel members. *JAMA.* (2014) 312:1033–48. doi: 10.1001/jama.2014.10517

9. Chotanaphuti T, Thongprasert S, Laoruengthana A. Low molecular weight heparin prevents the progression of precollapse osteonecrosis of the hip. *J Med Assoc Thail Chotmaihet Thangphaet.* (2013) 96:326–1330.

10. Molinari AC, Banov L, Bertamino M, Barabino P, Lassandro G, Giordano P. A practical approach to the use of low molecular weight heparins in VTE treatment and prophylaxis in children and newborns. *Pediatr Hematol Oncol.* (2015) 32:1–10. doi: 10.3109/08880018.2014.960119

11. Shier A, Abdelrazek M, Soliman A, De Sanctis V, Elsayed A, Abdulla M, et al. Short-term outcome and mri changes in three adult patients with sickle cell disease and aseptic osteonecrosis after treatment with hyperbaric oxygen therapy: a preliminary report. *Case Rep Oncol.* (2020) 13:365–72. doi: 10.1159/000506330

12. Salameh M, Moghamis IS, Kokash O, Ahmed GO. Hyperbaric oxygen therapy for the treatment of steinberg I and II avascular necrosis of the femoral head: a report of fifteen cases and literature review. *Int Orthop.* (2021) 45:2519–23. doi: 10.1007/s00264-021-05120-3

13. Paderno E, Zanon V, Vezzani G, Giacon TA, Bernasek TL, Camporesi EM, et al. Evidence-supported HBO therapy in femoral head necrosis: a systematic review and meta-analysis. *Int J Environ Res Public Health.* (2021) 18:2888. doi: 10.3390/ijerph18062888

14. Aguilar C, Vichinsky E, Neumayr L. Bone and joint disease in sickle cell disease. *Hematol Oncol Clin North Am.* (2005) 19:929–41. doi: 10.1016/j.hoc.2005.07.001

15. Giordano P, Urbano F, Lassandro G, Faienza MF. Mechanisms of bone impairment in sickle bone disease. *Int J Environ Res Public Health.* (2021) 18:1832. doi: 10.3390/ijerph18041832

16. Gill AL, Bell CNA. Hyperbaric oxygen: its uses, mechanisms of action and outcomes. *QJM Mon J Assoc Phys.* (2004) 97:385–95. doi: 10.1093/qjmed/hch074

17. Camporesi EM, Vezzani G, Bosco G, Mangar D, Bernasek TL. Hyperbaric oxygen therapy in femoral head necrosis. *J Arthr.* (2010) 25:118–23. doi: 10.1016/j.arth.2010.05.005

18. Mathieu D, Marroni A, Kot J. Tenth european consensus conference on hyperbaric medicine: recommendations for accepted and non-accepted clinical indications and practice of hyperbaric oxygen treatment. *Diving Hyperb Med.* (2017) 47:24–32. doi: 10.28920/dhm47.1.24-32

19. Bosco G, Vezzani G, Mrakic Sposta S, Rizzato A, Enten G, Abou-Samra A, et al. Hyperbaric oxygen therapy ameliorates osteonecrosis in patients by modulating inflammation and oxidative stress. *J Enzyme Inhib Med Chem.* (2018) 33:1501–5. doi: 10.1080/14756366.2018.1485149

20. Vezzani G, Quartesan S, Cancellara P, Camporesi E, Mangar D, Bernasek T, et al. Hyperbaric oxygen therapy modulates serum OPG/RANKL in femoral head necrosis patients. *J Enzyme Inhib Med Chem.* (2017) 32:707–11. doi: 10.1080/14756366.2017.1302440

21. Kurokouchi K, Kambe F, Yasukawa K, Izumi R, Ishiguro N, Iwata H, et al. TNF-alpha increases expression of IL-6 and ICAM-1 genes through activation of NF-kappaB in osteoblast-like ROS17/2.8 cells. *J Bone Miner Res Off J Am Soc Bone Miner Res.* (1998) 13:1290–9. doi: 10.1359/jbmr.1998.13.8.1290

22. Camporesi EM, Bosco G. Mechanisms of action of hyperbaric oxygen therapy. *Undersea Hyperb Med J Undersea Hyperb Med Soc Inc.* (2014) 41:247–52.

23. Alshurafa A, Yassin MA. Case report: safety and efficacy of voxelotor in a patient with sickle cell disease and stage IV chronic kidney disease. *Front Med.* (2022) 9:2022. doi: 10.3389/fmed.2022.931924

24. Elenga N, Loko G, Etienne-Julan M, Al-Okka R, Adel AM, Yassin MA. Real-world data on efficacy of L-glutamine in preventing sickle cell disease-related complications in pediatric and adult patients. *Front Med.* (2022) 9:931925. doi: 10.3389/fmed.2022.931925

25. Adel AA, Abushanab D, Hamad A, Al-Badriyeh D, Yassin MA. L-glutamine and crizanlizumab for adults with sickle cell disease (SCD) in qatar: a cost effectiveness analysis. *Blood.* (2021) 138:4945. doi: 10.1182/blood-2021-144636

frontiers | Frontiers in Medicine

TYPE Mini Review
PUBLISHED 09 January 2023
DOI 10.3389/fmed.2022.1065377

OPEN ACCESS

EDITED BY
Robert W. Maitta,
Case Western Reserve University,
United States

REVIEWED BY
Ciaran Michael Lee,
University College Cork, Ireland
Alireza Paikari,
Baylor College of Medicine,
United States

*CORRESPONDENCE
Toni Cathomen
✉ toni.cathomen@
uniklinik-freiburg.de

SPECIALTY SECTION
This article was submitted to Hematology,
a section of the journal
Frontiers in Medicine

RECEIVED 09 October 2022
ACCEPTED 14 December 2022
PUBLISHED 09 January 2023

CITATION
Zarghamian P, Klermund J and Cathomen T (2023) Clinical genome editing to treat sickle cell disease—A brief update.
Front. Med. 9:1065377.
doi: 10.3389/fmed.2022.1065377

Clinical genome editing to treat sickle cell disease—A brief update

Parinaz Zarghamian[1,2,3], Julia Klermund [1,2] and Toni Cathomen[1,2]*

[1]Institute for Transfusion Medicine and Gene Therapy, Medical Center — University of Freiburg, Freiburg, Germany, [2]Center for Chronic Immunodeficiency (CCI), Faculty of Medicine, University of Freiburg, Freiburg, Germany, [3]Ph.D. Program, Faculty of Biology, University of Freiburg, Freiburg, Germany

Sickle cell disease (SCD) is one of the most common hemoglobinopathies. Due to its high prevalence, with about 20 million affected individuals worldwide, the development of novel effective treatments is highly warranted. While transplantation of allogeneic hematopoietic stem cells (HSC) is the standard curative treatment approach, a variety of gene transfer and genome editing strategies have demonstrated their potential to provide a prospective cure for SCD patients. Several stratagems employing CRISPR-Cas nucleases or base editors aim at reactivation of γ-globin expression to replace the faulty β-globin chain. The fetal hemoglobin (HbF), consisting of two α-globin and two γ-globin chains, can compensate for defective adult hemoglobin (HbA) and reverse the sickling of hemoglobin-S (HbS). Both disruption of *cis*-regulatory elements that are involved in inhibiting γ-globin expression, such as BCL11A or LRF binding sites in the γ-globin gene promoters (*HBG1/2*), or the lineage-specific disruption of BCL11A to reduce its expression in human erythroblasts, have been demonstrated to reestablish HbF expression. Alternatively, the point mutation in the *HBB* gene has been corrected using homology-directed repair (HDR)-based methodologies. In general, genome editing has shown promising results not only in preclinical animal models but also in clinical trials, both in terms of efficacy and safety. This review provides a brief update on the recent clinical advances in the genome editing space to offer cure for SCD patients, discusses open questions with regard to off-target effects induced by the employed genome editors, and gives an outlook of forthcoming developments.

KEYWORDS

base editing, clinical trial, CRISPR-Cas, γ-globin, gene editing, *HBB* gene, HbF

Introduction

Sickle cell disease (SCD) is one of the most common hemoglobinopathies, which comprises a group of disorders that are characterized by faulty hemoglobin production (1, 2). Hemoglobin, a two-way respiratory carrier in red blood cells (RBCs), is responsible for transporting oxygen to tissues and returning carbon dioxide to the lung. This tetrameric metalloprotein is composed of two α-subunits, two non-α-subunits, hem groups, and four iron atoms, giving hemoglobin the capacity for binding oxygen (3). For congenital forms of anemia, SCD and thalassemia have the highest incidence (4). According to the European Medicines Agency (EMA) and the U.S. Center for Disease Control and Prevention (CDC), approximately 20 million people worldwide, including 52,000 people in Europe and 100,000 Americans, are affected by SCD. These patients suffer from anemia as well as progressive and fatal cardiovascular, renal, and eye complications due to the abnormal sickling shape of the RBCs that causes clogging of capillaries (1, 2). To alleviate morbidity, current treatment options include regular blood transfusions and the application of drugs that prevent vaso-occlusive crisis (VOC) or that reduce erythrocyte sickling. Still, life expectancy is reduced due to progressive organ dysfunction (1, 2). The only approved curative option for SCD is allogeneic hematopoietic stem cell (HSC) transplantation, which requires the availability of "healthy" blood stem cells of siblings or non-related donors with matched human leukocyte antigen (HLA). Unfortunately, the difficulty of finding suitable donors early in childhood and the high risk of graft-vs.-host-disease limit the option of bone marrow transplantation for SCD patients (5, 6). One way to overcome this limitation is the use of autologous HSCs that are corrected *ex vivo* using gene therapy strategies to restore functional hemoglobin expression. Because of its genetics, SCD represents an ideal target for gene therapy in general and for genome editing in particular.

Hemoglobin expression

The two non-α-subunits of hemoglobin are encoded by five different genes located within the β-globin locus on chromosome 11 (**Figure 1A**). The respective genes, *HBE* (coding for ε-globin), *HBG2* and *HBG1* (γ-globin), *HBD* (δ-globin) and *HBB* (β-globin), are expressed in a developmental stage-specific manner in erythroid cells (7). A single locus control region (LCR) and specific enhancers are responsible for their sequential activation during development. In the early stage embryonic yolk sac, *HBE* is expressed. Later, hematopoiesis shifts to the liver and the *HBG1/HBG2* genes (which are the result of a gene duplication and produce proteins that only differ in one amino acid) are activated to produce fetal hemoglobin (HbF, $\alpha_2\gamma_2$). Shortly after birth, hematopoiesis relocates to the bone marrow, and *HBD* and *HBB* are expressed, leading to an almost complete replacement of HbF by adult hemoglobin HbA (>95% $\alpha_2\beta_2$, 1.5–3.5% $\alpha_2\delta_2$; with 0.6–1% HbF persisting) (8). The γ-globin to β-globin switch is mediated by different transcription factors that repress *HBG1/HBG2* expression, such as BCL11A and LRF (9, 10). Worthy of note, healthy individuals with a benign genetic condition called hereditary persistence of fetal hemoglobin (HPFH) exhibit persistent production of functional HbF even after birth. The molecular basis of HPFH are large deletions in the *HBD* and *HBB* genes, which increase interactions between the LCR and the *HBG1/HBG2* promoters (11), or alternatively mutations in the *cis*-regulatory elements of the *HBG* genes, which are bound by the transcriptional repressors BCL11A and LRF (9, 10). If these repressors can no longer bind to the said *cis*-regulatory elements, *HBG* expression—and hence HbF production—persists (12).

Sickle cell disease arises as a result of a homozygous mutation in the *HBB* gene, in which a single point mutation leads to a codon change from gAg to gTg, resulting in a valine to glutamic acid substitution on the protein level (2). This swap in position six affects the hydrophobic characteristics of hemoglobin, converting HbA into the so-called sickle hemoglobin (HbS, $\alpha_2\beta^S{}_2$)—a term deduced from the sickle-like shape of the RBCs upon polymerization of HbS into fibers under deoxygenated conditions. The kinetic of hemoglobin polymerization is sensitive to the concentration of the HbS. Of note in this context, SCD patients with HPFH mutations present with mild clinical manifestations because *HBG* reactivation enables the formation of $\alpha_2\gamma_2$ and $\alpha_2\gamma\beta^S$ on top of $\alpha_2\beta^S{}_2$. Furthermore, the glutamine at position 87 (Q87) of γ-globin was shown to inhibit HbS polymerization and increase HbS solubility under deoxygenated conditions, so adding to the anti-sickling activity.

Gene therapy for SCD

The earliest attempts to genetically treat SCD were based on lentiviral (LV) transfer of a functional *HBB* copy to autologous HSCs (13). Bluebird Bio initiated first phase I/II gene therapy clinical trials in 2013 in France with seven patients (4 transfusion-dependent β-thalassemia, TDT, 3 SCD; HGB-205, NCT02151526) and in 2014 in the U.S. with 50 SCD patients (HGB-206, NCT02140554). The clinical product, LentiGlobin BB305 (**Figure 1B**), entails autologous HSCs transduced with an LV that encodes an anti-sickling variant of β-globin, known as βA-T87Q (mimicking the inhibitory effect of HbF on HbS polymerization). The recently published results confirmed stable βA-T87Q expression upon engraftment as well as reduced hemolysis, absence of VOC, and transfusion-independency (13, 14). A phase III clinical study (NCT04293185) with 35 SCD patients as well as a long-term follow-up study (NCT04628585) were opened in 2020. Based on these pivotal studies (15, 16),

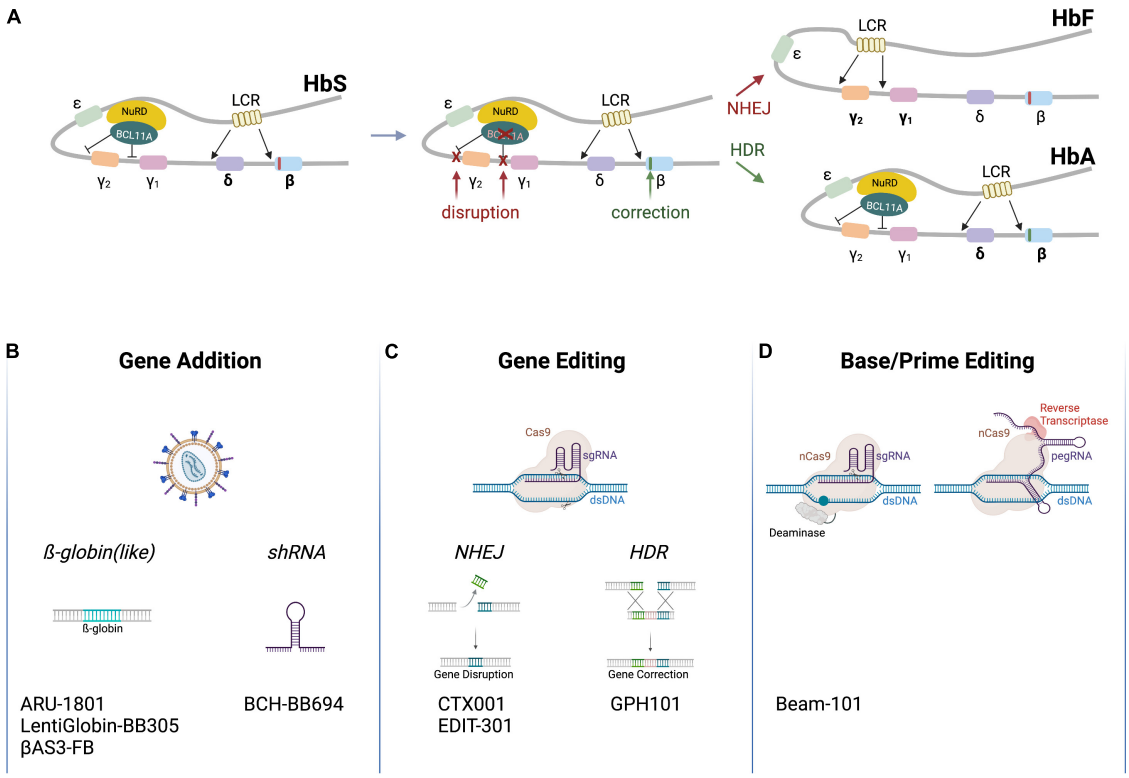

FIGURE 1
Schematic of clinical genome editing approaches for SCD. **(A)** The β-globin locus. The locus encompasses *HBE* (encoding ε-globin), *HBG2* and *HBG1* (γ-globin), *HBD* (δ-globin) and *HBB* (β-globin). A locus control region (LCR) and various factors (depicted are BCL11A and NuRD) regulate developmental stage-specific expression of the hemoglobin genes. A point mutation in *HBB* (red box) leads to expression of HbS. Therapeutic gene editing strategies aim at correcting *HBB via* HDR (green box) or at disrupting *cis*-regulatory elements in the *HBG2/HBG1* promoters or in *BCL11A via* NHEJ to re-activate γ-globin expression (red Xs), resulting in the expression of HbA ($\alpha_2\beta_2$) or HbF ($\alpha_2\gamma_2$), respectively. **(B–D)** Platform technologies used for the treatment of SCD. Clinically employed are **(A)** lentiviral vectors to transfer β-globin like genes or an shRNA targeting BCL11A mRNA, **(B)** genome editors to disrupt *cis*-regulatory elements by NHEJ or correct *HBB* by HDR, or **(C)** base and prime editors to disrupt *cis*-regulatory elements or off-set the mutation in *HBB*. The respective autologous, genome-engineered cell products are listed on the bottom (Created with BioRender.com).

BB305 received marketing authorization from the EMA (17) and the FDA (18) under the trade name Zynteglo® for the treatment of transfusion-dependent β-thalassemia (TDT). Of note, two patients from the phase I/II BB305 study (NCT02140554) were diagnosed with acute myeloid leukemia (AML) 2 years post-infusion (19), but AML development was not linked to insertional mutagenesis. The chosen conditioning regimen and/or the proliferative stress on HSCs upon switching from homeostatic to regenerative hematopoiesis might have played a role in AML induction and/or progression (14, 19).

Because high HbF expression ameliorates symptoms associated with SCD (20), efforts to develop LV-based approaches to increase γ-globin expression have been undertaken. This includes an LV expressing a γ-globin^{G16D} variant that was shown to have increased affinity to α-globin (21). Clinical data (NCT02186418) showed long-lasting engraftment with potentially curative HbF levels (21). The Boston Children's Hospital initiated a phase I clinical study with 10 patients in 2018 (NCT03282656) using autologous HSCs that were transduced with an LV (BCH-BB694) encoding a short-hairpin micro-RNA (shmiR) targeting the BCL11A mRNA (22). The six patients with long-term follow-up (7–29 months) showed high levels of HbF, mild clinical disease manifestation and no SAEs, prompting a phase II trial (NCT05353647) with 25 participants in 2022. Despite these successes, the high manufacturing costs of LV vectors (23), their potential of instigating abnormally spliced transcripts (24), as well as the risk of genotoxicity due to semi-random integration (25), limit the application of LV-based therapies.

Genome editing to treat SCD

Genome editing enables the site-specific modification of the human genome in order to correct or offset mutations underlying genetic disorders (26). Genome modification typically ensues from DNA double strand breaks (DSBs) that are introduced by programmable designer nucleases, such as

zinc finger nucleases (ZFNs) (27), transcription activator-like effector (TALE) nucleases (TALENs) (28, 29), or the CRISPR-Cas system (30). Other than the entirely protein-based ZFNs and TALENs, CRISPR-Cas nucleases contain an engineered guide (gRNA) that is complementary to the desired target sequence and that directs the Cas protein to the chosen genomic locus to induce a DSB (**Figure 1C**). Non-homologous end joining (NHEJ) and HDR are the two major repair pathways triggered by DSB formation (31). NHEJ is a fast but error-prone pathway, leading to insertions and deletions at the break site. NHEJ is hence typically employed to disrupt genes or *cis*-regulatory elements with high efficacy, reaching editing frequencies of over 90% in HSCs (26). In contrast, HDR is a slow but precise DNA repair pathway that uses a co-introduced DNA fragment as a template to correct disease underlying mutations *inter alia*. In HSCs, the HDR template is typically delivered by vectors based on adeno-associated virus (AAV) (32) or in the form of single-stranded or double-stranded oligonucleotides (ODNs) (33). However, because HDR is restricted to the S/G2 phase of the cell cycle, achieving gene targeting frequencies that exceed 20% in mainly quiescent long-term repopulating HSCs remains challenging (34).

Due to the genotoxic potential arising from DSB formation (see below), alternative platforms to edit the genome have been sought for **Figure 1D**. Such strategies are mostly based on CRISPR-Cas nickases that cleave only one DNA strand (35–37). This family includes base editors (BEs) (38, 39) and prime editors (PEs) (40). A Cas9 nuclease is converted to a Cas9 nickase by introducing mutations in one of the two catalytic domains of Cas9 (36). BEs were developed by fusing a deaminase domain to a Cas nickase (38). There are two types of BEs: cytosine base editors (CBEs) convert a C•G base pair (bp) into a T•A while adenine base editors (ABEs) convert an A•T to a G•C bp. BEs can be employed to correct point mutations, to introduce stop codons, or to disrupt *cis*-regulatory elements. PEs consist of a Cas9 nickase coupled to an engineered reverse transcriptase, which transcribes a section of the pegRNA (prime editing gRNA) into DNA to introduce the desired changes, such as base conversions or insertions/deletions of up to 80 bp (40).

Genome editing clinical trials for SCD

In the last 4 years, seven clinical trials using gene editing technologies to treat SCD have been initiated (**Table 1**). In all of them the editing agents are delivered *ex vivo* to autologous HSCs. Five of these therapeutic approaches attempt to reactivate γ-globin expression, either by preventing *BCL11A* expression in the erythroid lineage through disruption of enhancer elements or by mutating the BCL11A binding sites in the *HBG* promoters (**Figure 1A**). Two alternative approaches aim to correct the disease-causing mutation in the *HBB* locus using HDR (**Figure 1A**).

The most advanced product, CTX001, was developed by CRISPR Therapeutics and Vertex Pharmaceuticals. It is currently being tested in CLIMB-121, a phase II/III clinical trial (NCT03745287) that was started in 2018 with 45 SCD patients. CTX001 is administered as an autologous HSC product edited with CRISPR-Cas9 to disrupt the lineage-specific enhancer in the *BCL11A* gene. This alteration reduces BCL11A expression in erythroid cells, which in turn reactivates γ-globin expression. Published clinical data from the first two patients (one SCD and one TDT patient) demonstrated a high level of edited alleles in the stem cell compartment (69% and 80%). At 15 months post-transplantation, HbF levels in the SCD patient rose from 9.1 to 43.2%, while HbS levels were reduced from 74.1 to 52.3%. Patients were reported to be transfusion-independent and free of VOC. A recent update from infusion of CTX001 in 44 TDT and 31 SCD patients confirmed the overall positive response: All patients presented a sustained increase in HbF (39.6–49.6%), improvement in mean total Hb level (>11 g/dl) after 3 months, as well as elimination of VOC. Bone marrow analyses (>12 months follow-up) confirmed durable effects of this therapy over time with > 80% edited alleles. On the other hand, several severe adverse events (SAEs) were observed in patients upon infusion of the edited cells, such as VOC liver disease, sepsis, cholelithiasis, and hemophagocytic lymphohistiocytosis (HLH). Non-serious lymphopenia was also reported, most likely due to a delay in lymphocyte recovery (41, 42).

In 2019, Sangamo Therapeutics started a phase I/II clinical trial (NCT03653247) for eight SCD patients to assess the safety and efficacy of BIVV003, *ex vivo* manufactured autologous HSCs that were edited with ZFN technology to disrupt the *BCL11A* erythroid-specific enhancer. Data from week 26 post-transplantation of four patients showed increased HbF levels (14–39%) and F-cells raised to 48–94%. VOC was reported in one patient with a low level of HbF (14%). BIVV003 was well tolerated without the need for transfusions post-transplantation in all four patients (43). Besides adverse events related to plerixafor-based mobilization of $CD34^+$ cells and busulfan conditioning, no SAEs related to the treatment were reported (43).

Conversely, it was reported that editing of the *BCL11A* erythroid enhancer can result in reduced erythroid output, which was not observed when the binding site of BCL11A in the *HBG* promoters was disrupted (44). Editas Medicine initiated in 2021 the phase I/II RUBY clinical trial (NCT04853576) with almost 40 participants to evaluate the efficacy and safety of EDIT-301, a product based on autologous HSCs in which the *HBG1/2* promoter regions are disrupted using CRISPR-Cas12a. In preclinical mouse models, long-term engraftment of *HBG1/2*-edited HSCs was observed. The ∼90% edited target alleles went along with a high-level of HbF induction in cells from healthy

TABLE 1 Gene editing clinical trials for sickle cell disease.

Clinical trial	Phase	Year started	Treatment name	Target gene	Delivery mode	Designer nuclease	Donor template	Sponsors	Location	Status
NCT05329649	III	2022	CTX001	*BCL11A*	RNP electroporation	CRISPR-Cas9	–	Vertex Pharmaceuticals, CRISPR Therapeutics	United States, Italy	Recruiting
NCT05477563	III	2022	CTX001	*BCL11A*	RNP electroporation	CRISPR-Cas9	–	Vertex Pharmaceuticals, CRISPR Therapeutics	United States	Recruiting
NCT04774536	I/II	2022	CRISPR-SCD001	*HBB*	RNP electroporation	CRISPR-Cas9	ssODN	University of California	United States	Not yet recruiting
NCT05456880	I/II	2022	BEAM-101	*HBG1/HBG2*	RNA electroporation	ABE base editor	–	Beam Therapeutics	United States	Not yet recruiting
NCT05145062 (long-term follow up)	N/A	2021	BIVV003	*BCL11A*	mRNA electroporation	Zinc finger nuclease	–	Sangamo Therapeutics	United States	Recruiting
NCT04208529 (long-term follow up)	N/A	2021	CTX001	*BCL11A*	RNP electroporation	CRISPR-Cas9	–	Vertex Pharmaceuticals, CRISPR Therapeutics	United States, Canada, Germany, Italy, UK	Enrolling by invitation
NCT04819841	I/II	2021	GPH101	*HBB*	RNP electroporation	CRISPR-Cas9	rAAV6	Graphite Bio	United States	Recruiting
NCT04853576	I/II	2021	EDIT-301	*HBG1/HBG2*	RNP electroporation	CRISPR-Cas12a	–	Editas Medicine	United States, Canada	Recruiting
NCT04443907	I/II	2020	OTQ923	*BCL11A*	Unknown	CRISPR-Cas9	–	Novartis Pharmaceuticals, Intellia Therapeutics	United States, Italy	Recruiting
NCT03653247	I/II	2019	BIVV003	*BCL11A*	mRNA electroporation	Zinc finger nuclease	–	Sangamo Therapeutics	United States	Recruiting
NCT03745287	II/III	2018	CTX001	*BCL11A*	RNP electroporation	CRISPR-Cas9	–	Vertex Pharmaceuticals, CRISPR Therapeutics	United States, UK, Canada, France, Italy, Belgium, Germany	Active, not recruiting

10.3389/fmed.2022.1065377

donors (43%) and SCD patients (54%) with no detectable off-target effects (16, 44).

The 2021 initiated CEDAR trial (NCT04819841) is a phase I/II clinical study sponsored by Graphite Bio. As opposed to the previously described products, GPH101 is based on HDR and relies on a high-fidelity CRISPR-Cas9 system in combination with an AAV6-based HDR template. The goal is to correct the SCD-underlying point mutation in *HBB*. In preclinical mouse studies, almost 20% of HSCs harbored a corrected *HBB* locus (32), resulting in 90% of RBCs with normal HbA. The preclinical safety data revealed no evidence of abnormal hematopoiesis as well as absence of detectable off-target activity or chromosomal translocations. Graphite Bio recently announced the enrollment of the first patient, with up to 15 patients following at multiple sites in the U.S. Initial data from the CEDAR trial are expected for mid 2023.

Beam Therapeutics started a phase I/II clinical trial with 15 enrolled SCD patients in 2022 (NCT05456880). In BEAM-101, γ-globin expression is activated through a base swap in the *HBG1/2* promoters using base editing to generate an HPFH genotype variant in autologous HSCs. Based on preclinical mouse data, > 90% of target sites in xenotransplanted HSCs were stably edited, resulting in high levels of γ-globin expression (>65% HbF) (45). Furthermore, an investigational new drug application was filed for BEAM-102, which was designed to change the point mutation in *HBB* from gTg to gCg. The result is a switch from glutamic acid to alanine in position 6, which converts HbS into a better tolerated HbG-Makassar (46).

Technical challenges of *ex vivo* genome editing approaches in HSCs are similar to those in LV-based approaches and comprise to reach a sufficient number of mobilized CD34 + cells as a starting material, sufficient editing efficacy in the LT-HSC compartment, a lower level of engraftment of *ex vivo* edited cells along with reduced stemness of edited HSCs (47–49).

Off-target effects

Similar to insertional mutagenesis associated with integrating vector systems, inadvertent on-target and off-target effects evoked by the genome editing tools represent a major concern when applied in patient cells, particularly in highly proliferating multipotent stem cells. On the one hand, cleavage by CRISPR-Cas nucleases can trigger undesired effects on the target chromosome (50, 51), such as large deletions and inversions (52–54), chromosomal truncations (55), chromothripsis (56), aneuploidy (57), loss of heterozygosity (58), and loss of imprinting (58). On the other hand, unintentional activity at so-called off-target sites, that is sequences with high homology to the intended target site, triggers NHEJ-mediated insertion/deletion mutations at off-target sites and, potentially, comparable structural aberrations as described for the on-target site. Moreover, concomitant insertions of DSBs at multiple sites in the genome elicit translocations between those cleaved sites (54, 59). Several methods to predict or detect off-target activity and/or gross chromosomal rearrangements have been developed. They include deep sequencing of *in silico* predicted off-target sites as well as experimental procedures that detect off-target activity *in vitro* and in cell-based systems. Commonly used *in vitro* methods include CIRCLE-Seq (60), ONE-Seq (61) and NucleaSeq (62), while GUIDE-Seq (63), DISCOVER-Seq (64) and CAST-Seq (54) are prevalently used cell-based approaches. Noteworthy, CAST-Seq not only nominates off-target sites but also detects chromosomal rearrangements at the on-target site as well as induced chromosomal translocations with off-target sites (54).

The gene-edited products that are currently employed in clinical trials typically underwent several genotoxicity tests as part of the preclinical risk assessment. For instance, off-target activities of the CRISPR-Cas nucleases used in CTX001 and GPH101 were profiled by GUIDE-Seq, CIRCLE-Seq, and targeted amplicon next-generation sequencing (Amp-Seq) of *in silico* predicted off-target sites. Similarly, the safety of EDIT301 was investigated with GUIDE-Seq and Amp-Seq of *in silico* predicted off-target sites. Given that translocations are a hallmark of leukemic cells (65, 66) and since they can be rather frequent outcomes of genome editing (54, 59, 67, 68), there is a growing interest in detecting gross structural rearrangements, such as large chromosomal deletions, inversions, truncations, and translocations, too. To our knowledge, many of the above-mentioned products did not undergo a genome-wide and sensitive analysis of induced chromosomal rearrangements. Against the backdrop of the high sequence similarities within the β-globin locus (*HBG1* vs. *HBG2* or *HBB* vs. *HBD*), the potential for off-target editing as well as homology-mediated recombination between two respective paralogous genes is high (69). Indeed, rearrangements between *HBB* and *HBD* were confirmed in *HBB*-edited cell, in addition to translocations between *HBB* and an off-target site (54, 70, 71). In addition, CRISPR-Cas nucleases targeting either *HBB*, *HBD* or *HBG1/HBG2* can lead to complete loss of the distal chromosome 11p arm in HSCs (58). Furthermore, the simultaneous disruption of the BCL11 binding sites in *HBG1* and *HBG2* was reported to result in deletion of the 4.9 kb region between the two target sites, eliminating *HBG2* in 5–30% of cells (72–74).

To avoid this loss of the *HBG2* gene, BEs were employed to introduce HPFH-like mutations in the *HBG1/HBG2* promoters (75). Because single-strand nicks are repaired by the high-fidelity base-excision repair pathway, BEs have been claimed to reduce on-target and off-target effects (36). However, recent data demonstrated deletion of a 4.9 kb region after base editing of the *HBG1/HBG2* promoters, indicating that also base editing can induce structural variations (76). Furthermore, bystander editing effects (77) and gRNA-independent off-target activities

on both DNA and RNA (78, 79) have been described for both ABEs and CBEs. Hence, additional efforts are needed to characterize BE-associated off-target effects as well as to identify gross chromosomal rearrangements triggered by editing tools in HSCs of SCD (and TDT) patients. This also includes the evaluation of the biological long-term effects of genotoxicity in transplanted patients as well as the development of strategies to mitigate the observed off-target effects.

Future developments for SCD-directed genome editing

Genotoxic conditioning regimens still pose a major barrier to the adoption of autologous HSC transplantation in SCD (80, 81). To overcome this problem, Beam Therapeutic, among others, is developing a new approach termed "engineered stem cell antibody paired evasion," in which a BE-introduced epitope switch in CD117 enables those CD117-edited HSCs to selectively escape CD117-directed antibody-based conditioning. Such a strategy can be easily applied to BEAM-101 by targeting *CD117* and the *HBG1/2* promoters simultaneously (82).

Are there additional transcription factors that could be targeted to upregulate γ-globin expression? MYB is a transcription factor that regulates fetal hemoglobin expression at multiple levels, including upregulation of BCL11A expression (83). ATF4 is further upstream and regulates the expression of MYB. It has been recently shown that knockout of *ATF4* lowered MYB—and hence BCL11A—expression, and could thus potentially re-activate γ-globin expression (84). However, it must be noted that MYB and ATF4 have multiple functions outside of HbF regulation in non-erythroid cells (85, 86), highlighting the need to identify erythroid-specific regulation.

Given the constraints of off-target effects associated with all genome editing platforms, the question is whether alternatives to genome editing are available. Several studies deciphered the epigenetic regulation of the β-globin locus during development, including the interaction between epigenetic and transcriptional regulation leading to repression of γ-globin expression (87, 88). This knowledge opened up the idea to modify the epigenome in a targeted fashion for the treatment of SCD. While epigenetic approaches to promote γ-globin re-expression were described before (89, 90), more specific approaches are needed for clinical translation. Designer epigenome modifiers based on the TALE or CRISPR-dCas9 platforms create an opportunity to manipulate the epigenetic marks specifically and without the necessity to induce breaks in the genome (91, 92), e.g., by rewriting the epigenetic code in order to re-activate *HBG* expression or to silence *BCL11A* in a lineage-specific manner. Epigenome modifiers might therefore have less deleterious effects in a cell. On the other hand, the challenge of maintaining long-lasting effects over several cell cycles and throughout lineage differentiation has not been solved yet and it will be interesting to see whether the potential of designer epigenome modifiers can be harnessed for the treatment of SCD in the near future (93, 94).

Author contributions

All authors contributed to the writing and proofreading of the manuscript.

Funding

This research projects on hemoglobinopathies were supported by the European Commission (HORIZON-RIA EDITSCD grant no. 101057659 to TC) and the German Academic Exchange Service (DAAD fellowship grant no. 91764871 to PZ). We further acknowledge support by the Open Access Publication Fund of the University of Freiburg.

Acknowledgments

We would like to thank the members of our department for thriving discussions on the presented topic.

Conflict of interest

TC had a sponsored research collaboration with Cellectis and was an advisor to Bird B., Cimeio Therapeutics, Excision BioTherapeutics, and Novo Nordisk. He holds several patents in the field, including patents on CAST-Seq (US11319580B2) and epigenome modifiers (US11072782B2).

The remaining 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.

Publisher's note

References

1. Kohne E. Hemoglobinopathies: clinical manifestations, diagnosis, and treatment. *Dtsch Arztebl Int.* (2011) 108:532–40. doi: 10.3238/arztebl.2011.0532

2. Brandow A, Liem R. Advances in the diagnosis and treatment of sickle cell disease. *J Hematol Oncol.* (2022) 15:20. doi: 10.1186/s13045-022-01237-z

3. Marengo-Rowe A. Structure-function relations of human hemoglobins. *Proc.* (2006) 19:239–45. doi: 10.1080/08998280.2006.11928171

4. Anurogo D, Yuli Prasetyo Budi N, Thi Ngo M, Huang Y, Pawitan J. Cell and gene therapy for anemia: hematopoietic stem cells and gene editing. *Int J Mol Sci.* (2021) 22:6275. doi: 10.3390/ijms22126275

5. Bolanos-Meade J, Cooke K, Gamper C, Ali S, Ambinder R, Borrello I, et al. Effect of increased dose of total body irradiation on graft failure associated with HLA-haploidentical transplantation in patients with severe haemoglobinopathies: a prospective clinical trial. *Lancet Haematol.* (2019) 6:e183–93. doi: 10.1016/S2352-3026(19)30031-6

6. Eapen M, Brazauskas R, Walters M, Bernaudin F, Bo-Subait K, Fitzhugh C, et al. Effect of donor type and conditioning regimen intensity on allogeneic transplantation outcomes in patients with sickle cell disease: a retrospective multicentre, cohort study. *Lancet Haematol.* (2019) 6:e585–96. doi: 10.1016/S2352-3026(19)30154-1

7. Wilber A, Nienhuis A, Persons D. Transcriptional regulation of fetal to adult hemoglobin switching: new therapeutic opportunities. *Blood.* (2011) 117:3945–53. doi: 10.1182/blood-2010-11-316893

8. Liang S, Moghimi B, Yang T, Strouboulis J, Bungert J. Locus control region mediated regulation of adult beta-globin gene expression. *J Cell Biochem.* (2008) 105:9–16. doi: 10.1002/jcb.21820

9. Sankaran V, Menne T, Xu J, Akie T, Lettre G, Van Handel B, et al. Human fetal hemoglobin expression is regulated by the developmental stage-specific repressor BCL11A. *Science.* (2008) 322:1839–42. doi: 10.1126/science.1165409

10. Martyn G, Wienert B, Yang L, Shah M, Norton L, Burdach J, et al. Natural regulatory mutations elevate the fetal globin gene via disruption of BCL11A or ZBTB7A binding. *Nat Genet.* (2018) 50:498–503. doi: 10.1038/s41588-018-0085-0

11. Steinberg M. Fetal hemoglobin in sickle hemoglobinopathies: high hbf genotypes and phenotypes. *J Clin Med.* (2020) 9:3782. doi: 10.3390/jcm9113782

12. Lamsfus-Calle A, Daniel-Moreno A, Antony J, Epting T, Heumos L, Baskaran P, et al. Comparative targeting analysis of KLF1, BCL11A, and HBG1/2 in CD34(+) HSPCs by CRISPR/Cas9 for the induction of fetal hemoglobin. *Sci Rep.* (2020) 10:10133. doi: 10.1038/s41598-020-66309-x

13. Kanter J, Walters M, Krishnamurti L, Mapara M, Kwiatkowski J, Rifkin-Zenenberg S, et al. Biologic and clinical efficacy of lentiglobin for sickle cell disease. *N Engl J Med.* (2022) 386:617–28. doi: 10.1056/NEJMoa2117175

14. Magrin E, Semeraro M, Hebert N, Joseph L, Magnani A, Chalumeau A, et al. Long-term outcomes of lentiviral gene therapy for the beta-hemoglobinopathies: the HGB-205 trial. *Nat Med.* (2022) 28:81–8. doi: 10.1038/s41591-021-01650-w

15. Locatelli F, Thompson A, Kwiatkowski J, Porter J, Thrasher A, Hongeng S, et al. Betibeglogene autotemcel gene therapy for non-beta(0)/beta(0) genotype beta-thalassemia. *N Engl J Med.* (2022) 386:415–27. doi: 10.1056/NEJMoa2113206

16. Schneiderman J, Thompson A, Walters M, Kwiatkowski J, Kulozik A, Sauer M, et al. Interim results from the phase 3 hgb-207 (northstar-2) and hgb-212 (northstar-3) studies of betibeglogene autotemcel gene therapy (lentiglobin) for the treatment of transfusion-dependent β-thalassemia. *Biol Blood Marrow Transplant.* (2020) 26:S87–8. doi: 10.1016/j.bbmt.2019.12.588

17. European Medicines Agency. *Zynteglo*. (2019). Available online at: https://www.ema.europa.eu/en/medicines/human/EPAR/zynteglo#overview-section (accessed September 1, 2022).

18. U.S. Food and Drug Administration. *FDA Approves First Cell-Based Gene Therapy to Treat Adult and Pediatric Patients With Beta-Thalassemia Who Require Regular Blood Transfusions*. (2022). Available online at: https://www.fda.gov/news-events/press-announcements/fda-approves-first-cell-based-gene-therapy-treat-adult-and-pediatric-patients-beta-thalassemia-who (accessed September 1, 2022).

19. Jones R, DeBaun M. Leukemia after gene therapy for sickle cell disease: insertional mutagenesis, busulfan, both, or neither. *Blood.* (2021) 138:942–7. doi: 10.1182/blood.2021011488

20. Adekile A. The genetic and clinical significance of fetal hemoglobin expression in sickle cell disease. *Med Princ Pract.* (2021) 30:201–11. doi: 10.1159/000511342

21. Grimley M, Asnani M, Shrestha A, Felker S, Lutzko C, Arumugam P, et al. Early results from a phase 1/2 study of aru-1801 gene therapy for sickle cell disease (SCD): manufacturing process enhancements improve efficacy of a modified gamma globin lentivirus vector and reduced intensity conditioning transplant. *Blood.* (2020) 136(Suppl. 1):20–1. doi: 10.1182/blood-2020-140963

22. Esrick E, Lehmann L, Biffi A, Achebe M, Brendel C, Ciuculescu M, et al. Post-transcriptional genetic silencing of BCL11A to treat sickle cell disease. *N Engl J Med.* (2021) 384:205–15. doi: 10.1056/NEJMoa2029392

23. Coquerelle S, Ghardallou M, Rais S, Taupin P, Touzot F, Boquet L, et al. Innovative curative treatment of beta thalassemia: cost-efficacy analysis of gene therapy versus allogenic hematopoietic stem-cell transplantation. *Hum Gene Ther.* (2019) 30:753–61. doi: 10.1089/hum.2018.178

24. Cheng Y, Kang X, Chan P, Ye Z, Chan C, Jin D. Aberrant splicing events caused by insertion of genes of interest into expression vectors. *Int J Biol Sci.* (2022) 18:4914–31. doi: 10.7150/ijbs.72408

25. Cavazzana M, Bushman F, Miccio A, Andre-Schmutz I, Six E. Gene therapy targeting haematopoietic stem cells for inherited diseases: progress and challenges. *Nat Rev Drug Discov.* (2019) 18:447–62. doi: 10.1038/s41573-019-0020-9

26. Cornu T, Mussolino C, Cathomen T. Refining strategies to translate genome editing to the clinic. *Nat Med.* (2017) 23:415–23. doi: 10.1038/nm.4313

27. Carroll D. Genome engineering with zinc-finger nucleases. *Genetics.* (2011) 188:773–82. doi: 10.1534/genetics.111.131433

28. Mussolino C, Cathomen T. TALE nucleases: tailored genome engineering made easy. *Curr Opin Biotechnol.* (2012) 23:644–50. doi: 10.1016/j.copbio.2012.01.013

29. Joung J, Sander J. TALENs: a widely applicable technology for targeted genome editing. *Nat Rev Mol Cell Biol.* (2013) 14:49–55. doi: 10.1038/nrm3486

30. Ran F, Hsu P, Wright J, Agarwala V, Scott D, Zhang F. Genome engineering using the CRISPR-Cas9 system. *Nat Protoc.* (2013) 8:2281–308. doi: 10.1038/nprot.2013.143

31. Carusillo A, Mussolino C. DNA damage: from threat to treatment. *Cells.* (2020) 9:1665. doi: 10.3390/cells9071665

32. Lattanzi A, Camarena J, Lahiri P, Segal H, Srifa W, Vakulskas C, et al. Development of beta-globin gene correction in human hematopoietic stem cells as a potential durable treatment for sickle cell disease. *Sci Transl Med.* (2021) 13:eabf2444. doi: 10.1126/scitranslmed.abf2444

33. DeWitt M, Magis W, Bray N, Wang T, Berman J, Urbinati F, et al. Selection-free genome editing of the sickle mutation in human adult hematopoietic stem/progenitor cells. *Sci Transl Med.* (2016) 8:360ra134. doi: 10.1126/scitranslmed.aaf9336

34. Salisbury-Ruf C, Larochelle A. Advances and obstacles in homology-mediated gene editing of hematopoietic stem cells. *J Clin Med.* (2021) 10:513. doi: 10.3390/jcm10030513

35. Mali P, Aach J, Stranges P, Esvelt K, Moosburner M, Kosuri S, et al. CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering. *Nat Biotechnol.* (2013) 31:833–8. doi: 10.1038/nbt.2675

36. Ran F, Hsu P, Lin C, Gootenberg J, Konermann S, Trevino A, et al. Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. *Cell.* (2013) 154:1380–9. doi: 10.1016/j.cell.2013.08.021

37. Shen B, Zhang W, Zhang J, Zhou J, Wang J, Chen L, et al. Efficient genome modification by CRISPR-Cas9 nickase with minimal off-target effects. *Nat Methods.* (2014) 11:399–402. doi: 10.1038/nmeth.2857

38. Komor A, Kim Y, Packer M, Zuris J, Liu D. Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. *Nature.* (2016) 533:420–4. doi: 10.1038/nature17946

39. Gaudelli N, Komor A, Rees H, Packer M, Badran A, Bryson D, et al. Programmable base editing of A*T to G*C in genomic DNA without DNA cleavage. *Nature.* (2017) 551:464–71. doi: 10.1038/nature24644

40. Anzalone A, Randolph P, Davis J, Sousa A, Koblan L, Levy J, et al. Search-and-replace genome editing without double-strand breaks or donor DNA. *Nature.* (2019) 576:149–57. doi: 10.1038/s41586-019-1711-4

41. Frangoul H, Altshuler D, Cappellini M, Chen Y, Domm J, Eustace B, et al. CRISPR-Cas9 gene editing for sickle cell disease and beta-thalassemia. *N Engl J Med.* (2021) 384:252–60. doi: 10.1056/NEJMoa2031054

42. Grupp S, Bloberger N, Campbell C, Carroll C, Hankins J, Ho T, et al. CTX001 for sickle cell disease: safety and efficacy results from the ongoing climb SCD-121 study of autologous CRISPR-Cas9-modified CD34+ hematopoietic stem and progenitor cells. *HemaSphere.* (2021) 5:365.

43. Alavi A, Krishnamurti L, Abedi M, Galeon I, Reiner D, Smith S, et al. Preliminary safety and efficacy results from precizn-1: an ongoing phase 1/2 study

on zinc finger nuclease-modified autologous CD34+ HSPCS for sickle cell disease (SCD). *Blood.* (2021) 138(Suppl. 1):2930. doi: 10.1182/blood-2021-151650

44. Heath J, de Dreuzy E, Sanchez M, Haskett S, Wang T, Sousa P, et al. editors. PS1518: Genome editing of HBG1/2 promoter leads to robust HbF induction *in vivo*, while editing of BCL11A erythroid enhancer results in defects. *HemaSphere.* (2019) 3:699–700. doi: 10.1097/01.HS9.0000564332.87522.af

45. Gaudelli N. *Applied base editing to treat beta hemoglobinopathies.* (2021). Available online at: https://beamtx.com/science/posters-and-presentations/ (accessed September 1, 2022).

46. Chu S, Ortega M, Feliciano P, Winton V, Xu C, Haupt D, et al. Conversion of HbS to Hb G-makassar by adenine base editing is compatible with normal hemoglobin function. *Blood.* (2021) 138(Suppl. 1):951. doi: 10.1182/blood-2021-150922

47. Morgan R, Gray D, Lomova A, Kohn D. Hematopoietic stem cell gene therapy: progress and lessons learned. *Cell Stem Cell.* (2017) 21:574–90. doi: 10.1016/j.stem.2017.10.010

48. Zonari E, Desantis G, Petrillo C, Boccalatte F, Lidonnici M, Kajaste-Rudnitski A, et al. Efficient *ex vivo* engineering and expansion of highly purified human hematopoietic stem and progenitor cell populations for gene therapy. *Stem Cell Rep.* (2017) 8:977–90. doi: 10.1016/j.stemcr.2017.02.010

49. Wilkinson A, Igarashi K, Nakauchi H. Haematopoietic stem cell self-renewal *in vivo* and *ex vivo*. *Nat Rev Genet.* (2020) 21:541–54. doi: 10.1038/s41576-020-0241-0

50. Boutin J, Cappellen D, Rosier J, Amintas S, Dabernat S, Bedel A, et al. ON-target adverse events of CRISPR-Cas9 nuclease: more chaotic than expected. *CRISPR J.* (2022) 5:19–30. doi: 10.1089/crispr.2021.0120

51. Amendola M, Brusson M, Miccio A. CRISPRthripsis: the risk of CRISPR/Cas9-induced chromothripsis in gene therapy. *Stem Cells Transl Med.* (2022) 11:1003–9. doi: 10.1093/stcltm/szac064

52. Kosicki M, Tomberg K, Bradley A. Repair of double-strand breaks induced by CRISPR-Cas9 leads to large deletions and complex rearrangements. *Nat Biotechnol.* (2018) 36:765–71. doi: 10.1038/nbt.4192

53. Adikusuma F, Piltz S, Corbett M, Turvey M, McColl S, Helbig K, et al. Large deletions induced by Cas9 cleavage. *Nature.* (2018) 560:E8–9. doi: 10.1038/s41586-018-0380-z

54. Turchiano G, Andrieux G, Klermund J, Blattner G, Pennucci V, El Gaz M, et al. Quantitative evaluation of chromosomal rearrangements in gene-edited human stem cells by CAST-Seq. *Cell Stem Cell* (2021) 28:1136–47e5. doi: 10.1016/j.stem.2021.02.002

55. Cullot G, Boutin J, Toutain J, Prat F, Pennamen P, Rooryck C, et al. CRISPR-Cas9 genome editing induces megabase-scale chromosomal truncations. *Nat Commun.* (2019) 10:1136. doi: 10.1038/s41467-019-09006-2

56. Leibowitz M, Papathanasiou S, Doerfler P, Blaine L, Sun L, Yao Y, et al. Chromothripsis as an on-target consequence of CRISPR-Cas9 genome editing. *Nat Genet.* (2021) 53:895–905. doi: 10.1038/s41588-021-00838-7

57. Nahmad A, Reuveni E, Goldschmidt E, Tenne T, Liberman M, Horovitz-Fried M, et al. Frequent aneuploidy in primary human T cells after CRISPR-Cas9 cleavage. *Nat Biotechnol.* (2022) 40:1807–13. doi: 10.1038/s41587-022-01377-0

58. Boutin J, Rosier J, Cappellen D, Prat F, Toutain J, Pennamen P, et al. CRISPR-Cas9 globin editing can induce megabase-scale copy-neutral losses of heterozygosity in hematopoietic cells. *Nat Commun.* (2021) 12:4922. doi: 10.1038/s41467-021-25190-6

59. Samuelson C, Radtke S, Zhu H, Llewellyn M, Fields E, Cook S, et al. Multiplex CRISPR/Cas9 genome editing in hematopoietic stem cells for fetal hemoglobin reinduction generates chromosomal translocations. *Mol Ther Methods Clin Dev.* (2021) 23:507–23. doi: 10.1016/j.omtm.2021.10.008

60. Tsai S, Nguyen N, Malagon-Lopez J, Topkar V, Aryee M, Joung J. CIRCLE-seq: a highly sensitive in vitro screen for genome-wide CRISPR-Cas9 nuclease off-targets. *Nat Methods.* (2017) 14:607–14. doi: 10.1038/nmeth.4278

61. Petri K, Kim D, Sasaki K, Canver M, Wang X, Shah H, et al. Global-scale CRISPR gene editor specificity profiling by ONE-seq identifies population-specific, variant off-target effects. *Biorxiv.* [Preprint]. (2021). doi: 10.1101/2021.04.05.438458

62. Jones S Jr., Hawkins J, Johnson N, Jung C, Hu K, Rybarski J, et al. Massively parallel kinetic profiling of natural and engineered CRISPR nucleases. *Nat Biotechnol.* (2021) 39:84–93. doi: 10.1038/s41587-020-0646-5

63. Tsai S, Zheng Z, Nguyen N, Liebers M, Topkar V, Thapar V, et al. GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. *Nat Biotechnol.* (2015) 33:187–97. doi: 10.1038/nbt.3117

64. Wienert B, Wyman S, Richardson C, Yeh C, Akcakaya P, Porritt M, et al. Unbiased detection of CRISPR off-targets *in vivo* using DISCOVER-Seq. *Science.* (2019) 364:286–9. doi: 10.1126/science.aav9023

65. Teachey D, Pui C. Comparative features and outcomes between paediatric T-cell and B-cell acute lymphoblastic leukaemia. *Lancet Oncol.* (2019) 20:e142–54. doi: 10.1016/S1470-2045(19)30031-2

66. Kayser S, Levis M. Clinical implications of molecular markers in acute myeloid leukemia. *Eur J Haematol.* (2019) 102:20–35. doi: 10.1111/ejh.13172

67. Frock R, Hu J, Meyers R, Ho Y, Kii E, Alt F. Genome-wide detection of DNA double-stranded breaks induced by engineered nucleases. *Nat Biotechnol.* (2015) 33:179–86. doi: 10.1038/nbt.3101

68. Bothmer A, Gareau K, Abdulkerim H, Buquicchio F, Cohen L, Viswanathan R, et al. Detection and modulation of DNA translocations during multi-gene genome editing in T cells. *CRISPR J.* (2020) 3:177–87. doi: 10.1089/crispr.2019.0074

69. Long J, Hoban M, Cooper A, Kaufman M, Kuo C, Campo-Fernandez B, et al. Characterization of gene alterations following editing of the beta-globin gene locus in hematopoietic stem/progenitor cells. *Mol Ther.* (2018) 26:468–79. doi: 10.1016/j.ymthe.2017.11.001

70. Hoban M, Cost G, Mendel M, Romero Z, Kaufman M, Joglekar A, et al. Correction of the sickle cell disease mutation in human hematopoietic stem/progenitor cells. *Blood.* (2015) 125:2597–604. doi: 10.1182/blood-2014-12-615948

71. Hoban M, Lumaquin D, Kuo C, Romero Z, Long J, Ho M, et al. CRISPR/Cas9-mediated correction of the sickle mutation in human CD34+ cells. *Mol Ther.* (2016) 24:1561–9. doi: 10.1038/mt.2016.148

72. Metais J, Doerfler P, Mayuranathan T, Bauer D, Fowler S, Hsieh M, et al. Genome editing of HBG1 and HBG2 to induce fetal hemoglobin. *Blood Adv.* (2019) 3:3379–92. doi: 10.1182/bloodadvances.2019000820

73. Li C, Psatha N, Sova P, Gil S, Wang H, Kim J, et al. Reactivation of gamma-globin in adult beta-YAC mice after ex vivo and *in vivo* hematopoietic stem cell genome editing. *Blood.* (2018) 131:2915–28. doi: 10.1182/blood-2018-03-838540

74. Weber L, Frati G, Felix T, Hardouin G, Casini A, Wollenschlaeger C, et al. Editing a gamma-globin repressor binding site restores fetal hemoglobin synthesis and corrects the sickle cell disease phenotype. *Sci Adv.* (2020) 6:eaay9392. doi: 10.1126/sciadv.aay9392

75. Ravi N, Wienert B, Wyman S, Bell H, George A, Mahalingam G, et al. Identification of novel HPFH-like mutations by CRISPR base editing that elevate the expression of fetal hemoglobin. *Elife.* (2022) 11:e65421. doi: 10.7554/eLife.65421

76. Antoniou P, Hardouin G, Martinucci P, Frati G, Felix T, Chalumeau A, et al. Base-editing-mediated dissection of a gamma-globin cis-regulatory element for the therapeutic reactivation of fetal hemoglobin expression. *Nat Commun.* (2022) 13:6618. doi: 10.1038/s41467-022-34493-1

77. Chu S, Packer M, Rees H, Lam D, Yu Y, Marshall J, et al. Rationally designed base editors for precise editing of the sickle cell disease mutation. *CRISPR J.* (2021) 4:169–77. doi: 10.1089/crispr.2020.0144

78. Zuo E, Sun Y, Wei W, Yuan T, Ying W, Sun H, et al. Cytosine base editor generates substantial off-target single-nucleotide variants in mouse embryos. *Science.* (2019) 364:289–92. doi: 10.1126/science.aav9973

79. Grunewald J, Zhou R, Garcia S, Iyer S, Lareau C, Aryee M, et al. Transcriptome-wide off-target RNA editing induced by CRISPR-guided DNA base editors. *Nature.* (2019) 569:433–7. doi: 10.1038/s41586-019-1161-z

80. Cavazzana M, Mavilio F. Gene therapy for hemoglobinopathies. *Hum Gene Ther.* (2018) 29:1106–13. doi: 10.1089/hum.2018.122

81. Cavazzana M, Ribeil J, Lagresle-Peyrou C, Andre-Schmutz I. Gene therapy with hematopoietic stem cells: the diseased bone marrow's point of view. *Stem Cells Dev.* (2017) 26:71–6. doi: 10.1089/scd.2016.0230

82. Gaudelli N. *Non-genotoxic antibody-based conditioning paired with multiplex base edited HSCs for the potential treatment of sickle cell disease.* (2022). Available online at: https://beamtx.com/science/posters-and-presentations/ (accessed September 1, 2022).

83. Antoniani C, Romano O, Miccio A. Concise review: epigenetic regulation of hematopoiesis: biological insights and therapeutic applications. *Stem Cells Transl Med.* (2017) 6:2106–14. doi: 10.1002/sctm.17-0192

84. Boontanrart M, Schroder M, Stehli G, Banovic M, Wyman S, Lew R, et al. ATF4 regulates MYB to increase gamma-globin in response to loss of beta-globin. *Cell Rep.* (2020) 32:107993. doi: 10.1016/j.celrep.2020.107993

85. Ameri K, Harris A. Activating transcription factor 4. *Int J Biochem Cell Biol.* (2008) 40:14–21. doi: 10.1016/j.biocel.2007.01.020

86. Greig K, Carotta S, Nutt S. Critical roles for c-myb in hematopoietic progenitor cells. *Semin Immunol.* (2008) 20:247–56. doi: 10.1016/j.smim.2008.05.003

87. Van der Ploeg L, Flavell R. DNA methylation in the human γδβ-globin locus in erythroid and nonerythroid tissues. *Cell.* (1980) 19:947–58. doi: 10.1016/0092-8674(80)90086-0

88. Busslinger M, Hurst J, Flavell R. DNA methylation and the regulation of globin gene expression. *Cell.* (1983) 34:197–206. doi: 10.1016/0092-8674(83)90150-2

89. Holshouser S, Cafiero R, Robinson M, Kirkpatrick J, Casero R Jr, Hyacinth H, et al. Epigenetic reexpression of hemoglobin f using reversible lsd1 inhibitors: potential therapies for sickle cell disease. *ACS Omega.* (2020) 5:14750–8. doi: 10.1021/acsomega.0c01585

90. Starlard-Davenport A, Fitzgerald A, Pace B. Exploring epigenetic and microRNA approaches for gamma-globin gene regulation. *Exp Biol Med.* (2021) 246:2347–57. doi: 10.1177/15353702211028195

91. Hilton I, D'Ippolito A, Vockley C, Thakore P, Crawford G, Reddy T, et al. Epigenome editing by a CRISPR-Cas9-based acetyltransferase activates genes from promoters and enhancers. *Nat Biotechnol.* (2015) 33:510–7. doi: 10.1038/nbt.3199

92. Mlambo T, Nitsch S, Hildenbeutel M, Romito M, Muller M, Bossen C, et al. Designer epigenome modifiers enable robust and sustained gene silencing in clinically relevant human cells. *Nucleic Acids Res.* (2018) 46:4456–68. doi: 10.1093/nar/gky171

93. Huerne K, Palmour N, Wu A, Beck S, Berner A, Siebert R, et al. Auditing the editor: a review of key translational issues in epigenetic editing. *CRISPR J.* (2022) 5:203–12. doi: 10.1089/crispr.2021.0094

94. Mussolino C, Strouboulis J. Recent approaches for manipulating globin gene expression in treating hemoglobinopathies. *Front Genome Ed.* (2021) 3:618111. doi: 10.3389/fgeed.2021.618111

frontiers | Frontiers in Medicine

TYPE Review
PUBLISHED 23 February 2023
DOI 10.3389/fmed.2023.1036939

OPEN ACCESS

EDITED BY
Robert W. Maitta,
Case Western Reserve University,
United States

REVIEWED BY
Aiming Pang,
Chinese Academy of Medical Sciences and Peking Union Medical College,
China
Erden Atilla,
Fred Hutchinson Cancer Research Center,
United States

*CORRESPONDENCE
Sandeep Kumar Yadav
✉ skyadav@mdanderson.org
Aloukick Kumar Singh
✉ aloukick.singh@cmcvellore.ac.in

†These authors have contributed equally to this work

SPECIALTY SECTION
This article was submitted to Hematology, a section of the journal Frontiers in Medicine

RECEIVED 05 September 2022
ACCEPTED 24 January 2023
PUBLISHED 23 February 2023

CITATION
Bhalla N, Bhargav A, Yadav SK and Singh AK (2023) Allogeneic hematopoietic stem cell transplantation to cure sickle cell disease: A review.
Front. Med. 10:1036939.
doi: 10.3389/fmed.2023.1036939

Allogeneic hematopoietic stem cell transplantation to cure sickle cell disease: A review

Nishka Bhalla[1†], Anjali Bhargav[1†], Sandeep Kumar Yadav[2*] and Aloukick Kumar Singh[1*]

[1]Centre for Stem Cell Research, Christian Medical College, Vellore, Tamilnadu, India, [2]University of Texas MD Anderson Cancer Center, Houston, TX, United States

Sickle cell disease (SCD) had first been mentioned in the literature a century ago. Advancement in the molecular basis of the pathophysiology of the disease opens the door for various therapeutic options. Though life-extending treatments are available for treating patients with SCD, allogeneic hematopoietic stem cell transplantation (HSCT) is the only option as of yet. A major obstacle before HSCT to cure patients with SCD is the availability of donors. Matched sibling donors are available only for a small percentage of patients. To expand the donor pool, different contrasting approaches of allogeneic HSCT like T-cell replete and deplete have been tested. None of those tested approaches have been without the risk of GvHD and graft rejection. Other limitations such as transplantation-related infections and organ dysfunction caused by the harsh conditioning regimen need to be addressed on a priority basis. In this review, we will discuss available allogeneic HSCT approaches to cure SCD, as well as recent advancements to make the approach safer. The center of interest is using megadose T-cell-depleted bone marrow in conjugation with donor-derived CD8 veto T cells to achieve engraftment and tolerance across MHC barriers, under reduced intensity conditioning (RIC). This approach is in phase I/II clinical trial at the MD Anderson Cancer Centre and is open to patients with hemoglobinopathies.

KEYWORDS

sickle cell disease, hematopoietic stem cell transplantation, reduced intensity conditioning, veto cell, bone marrow transplantation

1. Introduction

Sickle cell disease (SCD) is an autosomal recessive inherited genetic disorder caused by a single-nucleotide mutation in the β-globin gene, which leads to the substitution of glutamic acid by valine at position 6. Hemoglobin with this mutation is referred to as hemoglobin S (HbS). HbS polymerizes to form fibers, which change the shape of RBCs into a sickle shape, causing blockage of the circulation and resulting in a sickle cell crisis, hence the name sickle cell disease. HbS polymerization also leads to intravascular hemolysis of red blood cells (RBCs) and subsequent release of hemoglobin and other components into the plasma. This abnormality in hemoglobin may trigger several acute and chronic clinical manifestations such as vaso-occlusive crisis, splenic sequestration crisis, acute chest syndrome, and stroke (1–5). The disease was first described by Dr. James Herrick in 1910 in a 20-year-old patient suffering from severe anemia and malaria (6). During the patient's blood smear examination, Dr. Herrick observed unusually shaped red blood cells that led him to conclude that the possible reason for the disease status is some unknown changes in red blood corpuscles. In 1949, the pioneering work by Linus Pauling showed that sickle cell disease is caused by abnormal hemoglobin (7).

Sickle cell disease is quite prevalent worldwide with the diagnosis of 300,000 new cases every year, and this number could rise to 400,000 by 2050 (8, 9). In the United States, one among 600 African Americans have been reported having SCD, and the disease affects 100,000 Americans (10–12). In sub-Saharan Africa, the mortality rate among newborns to 5-year-olds is 75% (13). However, the picture of the life expectancy of individuals born with SCD in the developed world is quite different, and the majority of SCD-affected subjects attain adulthood age. Nevertheless, the average life expectancy in well-resourced countries is much lower than that of a healthy individual (14–18). The aforementioned prevalence figures describe the magnitude of the problem worldwide; however, SCD has been recognized as a global public health problem by the WHO as early as 2006.

In the last few decades, tremendous advancement has been gained in understanding the pathogenesis of SCD; however, attempts toward the development of a treatment strategy were comparatively slow. Currently, allogeneic hematopoietic stem cell transplantation is the treatment of choice for patients with severe SCD. The HSCT is successful in treating patients with SCD when there is the availability of human leukocyte antigen (HLA)-matched sibling donors; however, its utility is extremely limited in HSCT across MHC barriers. Thus, the scarcity of suitable donors is an existing problem with HSCT. Allogeneic HSCT (allo-HSCT) often has to be used in such cases where a patient is in need of HSCT and the suitably matched donor is lacking. However, this approach is found to be attributed to graft failure after allo-HSCT and the occurrence of GvHD (19–22). The occurrence of GvHD is not the only limitation associated with allo-HSCT, it may be associated with conditioning-related toxicity and graft rejection. Moreover, new therapeutic approaches have been invented recently for the treatment of SCD and other hemoglobinopathies as well. The invention of new diagnostic tools like next-generation sequencing (NGS) together with gene therapy as a therapeutic option revolutionized the treatment options for hemoglobinopathies including SCD (23, 24). The NGS allowed the identification of specific genetic alternation responsible for hemoglobinopathy and gene therapy enables the treatment of genetic disorders through the transplantation of gene-corrected autologous HSCT (25). The autologous gene-corrected hematopoietic stem cells can home to their niches and start hematopoiesis without the risk of GvHD and rejection. Therefore, these corrected hematopoietic stem cells can maintain themselves for a lifetime and leads to the production of corrected RBCs and other cells as well. Gene therapy mainly involves two approaches: lentiviral vector correction and gene editing. In this review, we will mainly focus on the recent advancements with safer allogeneic HSCT approaches to cure SCD.

2. Historical perspective of SCD

Since the discovery of SCD in 1910 by Dr. Herrick, a lot of efforts have been made to elucidate a clear picture of the pathogenesis of the disease (Figure 1). Of noteworthy that with the given symptoms, he was not sure that this was a blood-related disorder or manifestation of another disease. However, up to now, several cases of SCD were identified and described, supporting the belief that it was completely a new disease (26). Three years later in 1927, Hahn and colleagues suggested that anoxia is the principal cause of RBC sickling by demonstrating that shape changes could be induced by saturating a cell suspension with carbon dioxide (27). This concept was again verified by Scriver and Waugh in a case report where they showed that the number of sickle cells in the blood could be changed by making partial changes in oxygen pressure; that is a reversible reaction, and that sickling takes place only when the oxygen pressure falls below pressure of 45 mm Hg (28). Furthermore, in 1948, Watson and colleagues reported the importance of fetal hemoglobin (HbF) in the extension of the sickling period among newborns when compared with mothers with sicklemia (29). These pioneering reports led Linus Pauling to hypothesize that disease might originate from abnormal hemoglobulin and he validated the hypothesis in 1949 by differences in the migration pattern of sickle and normal hemoglobin by gel electrophoresis (7). In the same year, the autosomal recessive inheritance pattern of the disease was elucidated by Dr. Neel (30). Thereupon in 1958, Ingram and colleagues for the first time provided the genetic evidence of the disease that there is a difference in single amino acid between mutant sickle hemoglobin and normal hemoglobin (HbA) (31). Thereafter, it could be demonstrated in 2004 that abnormal HbS leads to polymerization in deoxygenation conditions (32). These studies provided the platform for further investigation of SCD on a molecular basis.

3. Pathophysiology of SCD

The term SCD comprises a group of disorders among which disease pathology results due to the inheritance of the mutated sickle cell gene either homozygously or as a double heterozygote in combination with another gene. Thus, the disease pathology is mainly influenced by hemoglobin genes individually. However, among the majority of the population, the most frequent genotype manifesting disease is homozygous sickle cell (33). Over the 12 decades, four major pathological processes, namely HbS polymerization, vaso-occlusion, hemolysis-mediated endothelial dysfunction, and sterile inflammation, have emerged and are characterized (34).

Hemoglobin S polymerization under low oxygen is the primary event that leads to the complex pathophysiology and severe manifestations of SCD (Figure 2). Under the deoxygenated condition, the change of glutamic acid with valine triggers interactions with other hemoglobin molecules that cause aggregation of hemoglobin molecules into the large polymers. The polymerization of deoxygenated HbS causes structural and functional changes in RBCs. These distorted or damaged rigid sickle-shaped RBCs lost their flexibility and become more adhesive in nature. The process of sickling is not a continuous process; the sickling and unsickling occur at regular intervals and one of the major effects of the sickling and unsickling cycle is that it decreases the life span of RBCs (35, 36). The clinical outcome is an acute systemic painful vaso-occlusive crisis (VOC) due to the occlusion of blood vessels in every compartment of the body and chronic hemolytic anemia (37). The periodic recurrence of painful VOC is an essential feature of SCD.

All these events lead to the induction of a pro-inflammatory response that involves neutrophils, platelets, and vascular endothelium (34). It has been well established that nitric oxide (NO) is essential for normal vascular function (Figure 2). However, in SCD, there is a continuous release of hemoglobin from distorted cells from hemolysis depletes hemopexin and haptoglobin, a consequence of which is the reduction in the bio-availability of nitric oxide (NO), and vascular endothelial dysfunction that causes organ damage in SCD (38). The NO prevents endothelial damage by inhibiting, neutrophils, platelets, and the expression of adhesion molecules like VCAM-1 and ICAM-1 at the transcriptional level (39). Among patients with SCD, the levels of neutrophils, monocytes, and platelets remain above that of normal

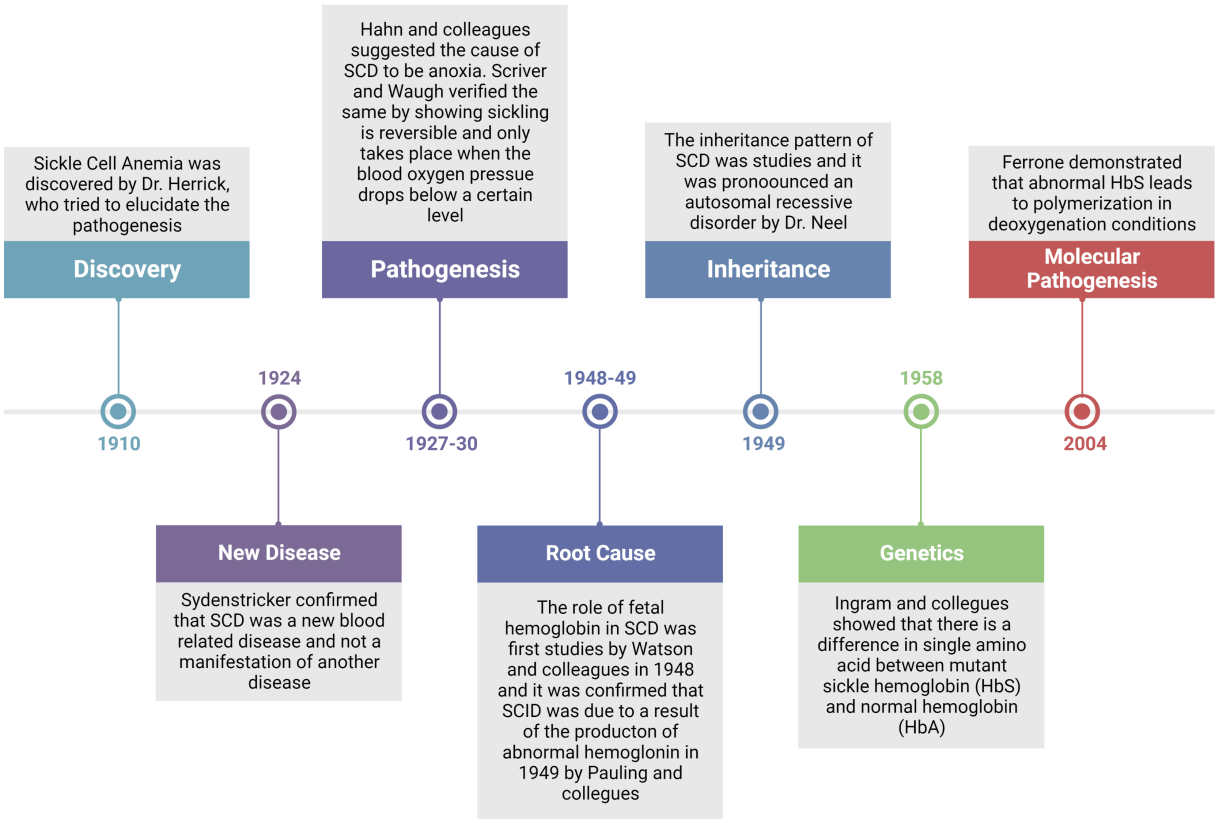

FIGURE 1
Timeline review of major historical events since the discovery of the disease and up to the established molecular pathogenesis of the disease.

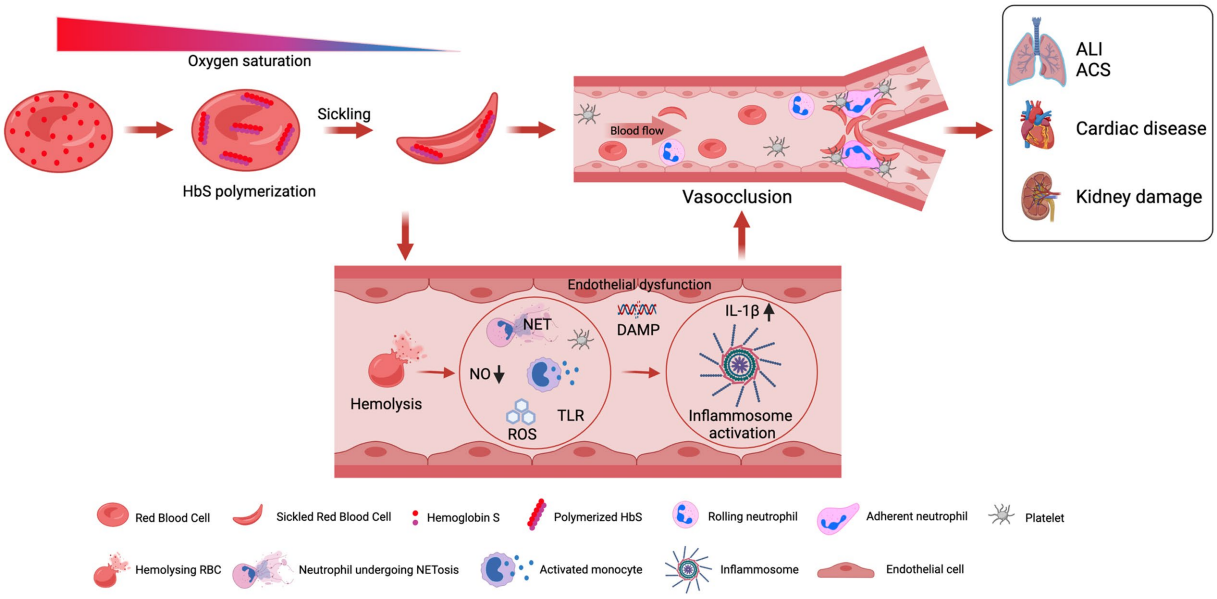

FIGURE 2
A graphical illustration of pathophysiologies associated with sickle cell disease.

healthy subjects, which further get more increased during acute events (40).

To this end, various studies reported the correlation between neutrophilia and the severity of SCD (41, 42). A recent study reported that neutrophil interactions with RBCs and endothelium play a central role in VOC by increasing the expression of E and P selectins identified as current therapeutic targets to deal with VOC (43). In addition, the *in vivo* studies in SCD mice also showed that the interaction of neutrophils

with adherent leukocytes can induce VOC (44). There are a series of reports that suggests the potential role of neutrophilia in the severity of SCD by triggering alternation in neutrophil surface marker expression, adhesion, migration, and intracellular oxidative stress (42). However, the underlying mechanism remains elusive. Furthermore, vitamin E levels remain diminished in patients with SCD, and neutrophils acquire a pro-inflammatory phenotype due to the loss of antioxidants. Furthermore, the administration of granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) in order to correct neutropenia worsens the disease status, suggesting the role of neutrophils in SCD pathogenesis (45). Thus, it was evident from various studies that anomalous interactions between endothelial cells, platelets, erythrocytes, and leukocytes comprise the pathophysiology of SCD during acute pain crises.

4. Therapeutic strategies for the treatment of SCD

The discovery of these pathophysiological targets has led the insights for the initiation of clinical trials by targeting platelets, adhesion molecules, and coagulation for the prevention of VOC-induced pain in SCD (46–49). To this end, in the transgenic SCD model, it has been demonstrated that anti-P-selectin can efficiently inhibit the adhesion of both sickle RBC and leukocytes to endothelial cells, suggesting a critical for P-selectin in cell adhesion (50). Very recently a humanized monoclonal antibody crizanlizumab (Adakveo) has been reported to block the P-selectin interaction with circulating leukocytes and the therapy was observed to be significantly effective in lowering the rate of sickle-related pain crises. This study also showed that the reduction in the annual crisis was more significant in patients those patients (50%) who were treated only with crizanlizumab (5 mg/kg) than those who received both the crizanlizumab and hydroxyurea combination therapy (32.1%) (51). Subsequently, this drug was got approved by the FDA in 2019. A study conducted in the SCD Berkeley mice model reported that a synthetic pan-selectin inhibitor, rivipansel (GMI-1070), can effectively inhibit E-selectin-mediated adhesion as well as RBC-leukocyte interactions, leading to improved microcirculatory blood flow and improved survival (52). Very recently, the clinical trial results were published and showed that Rivipansel administered early in VOC results in clinically meaningful benefits for adults and children with SCD, shortening IV opioid use and hospital stay (53).

These efforts by the researchers led to the development of a therapeutic agent targeting P-selectin for the treatment of VOC, which subsequently got approved by the FDA in 2019. The effective anti-adherence agents can act as a blocker to cell–cell interactions that can protect from aggressive erythrocyte aggregation and adherence to vascular endothelium.

One of the newer therapeutic approaches is the induction of fetal hemoglobin (HbF, a2γ2). The HbF is the principal hemoglobin present during the gestation period. Approximately 60%–80% of total hemoglobin in newborns constitutes HbF, it is completely replaced by adult hemoglobin (hemoglobin A, α2β2) at the age of 6–12 months, and in an adult, person HbF constitutes less than 1% of the total hemoglobin. Genome-wide association studies (GWAS) identified BCL11A as a key regulator of hemoglobin switching from HbF to HbA and as a key therapeutic target for HbF induction (54). Pharmacological interventions like the use of Hydroxyurea (HU) resulted in the evaluation of HBF levels in patients with SCD leading to the amelioration of pain crisis (55, 56). Throughout the period, various drugs were tested for induction of HbF, while the most widely used for SCD is HU, and we discussed the mechanism of action of a few of them here. The HU induces HbF by mainly three pathways: (1) epigenetic modification: HU triggers reduced methylation of CpG island, which leads to increased expression of γ-globin. (2) Signal transduction pathway: activation of soluble guanylate cyclase (sGC) leads to the upregulation of the γ-globulin gene at the transcription level in erythroid cell lines and primary erythroblasts (57). (3) Post-transcriptional regulation: HU controls HbF levels by regulating the expression of micro RNAs involved in modulating HbF synthesis. The HU intervention leads to the upregulation of reactive oxygen species (ROS) like NO in patients with SCD, which in turn activates soluble guanylate cyclase (sGC), stimulates cyclic monophosphate (cGMP) and protein kinase G (PKG) pathway for the γ-globulin expression (58). However, various signaling pathways may contribute to the HU-induced NO-cGMP pathway (59). Furthermore, HbF level can also be induced by the combination therapy of HU along with salubrinal, identified as a key regulator of γ-globulin gene expression (60). The drugs like azacytidine and decitabine are used as replacement therapy for HU for HbF induction, mainly for the treatment of HU-resistant patients with SCD; however, later are found potentially more effective in the induction of HbF (61, 62). Other drugs, namely trichostatin-A and valproic acid also observed to play a key role in the induction of HbF (63, 64). Despite the availability of new therapeutic drugs to ameliorate the VOC, none of them proved to be as effective as HU, which can exert an anti-sickling effect through induction of HbF (65). Though, HU has been potentially observed to cure various issues related to SCD e.g. RBC hydration, normalization of neutrophil count, reduction in leukocyte adhesion, and expression of pro-inflammatory molecules, a significant number of patients do not benefit from HU due to suboptimal HbF responses and side effects. Nevertheless, all these efforts well established the HbF as a clinical approach to treating patients with SCD, and the findings led the scientists to consider other approaches like genetics to induce HbF (66). To this end, the identification of the transcriptional regulator of HbF, BCL11A, MYB, and KLF1 completely opened new windows for the treatment of SCD, i.e., intervention through genetic and genomic approaches (67, 68). In 2017, Ribeil and colleagues treated patients with SCD with lentiviral vector-mediated addition of the anti-sickling β globin gene (T87Q) into autologous hematopoietic stem cells (HSCs). The study showed promising results; 15 months after treatment, the level of the anti-sickling β-globin remained high (50%) (69). However, the treatment of choice is HSCT using immunologically matched siblings as donors (70).

Considering the pathophysiology of SCD, different targets can be selected for the introduction of therapeutic interventions. Currently, the major attention has focused on therapeutic approaches for modifying the patient's genotype. Other targets include the prevention of HbS polymerization, VOC, and inflammation (Figure 3). The HbF induction can overcome the problem of HbS polymerization; however, there are also other approaches to overcome the problem. One approach recently approved by FDA is the increased oxygen affinity of the hemoglobin molecule by Oxbryta (Voxelotor)™ (71). Furthermore, we will discuss in detail about the recent advancements in allogeneic HSCT-based approaches for the treatment of sickle cell disease.

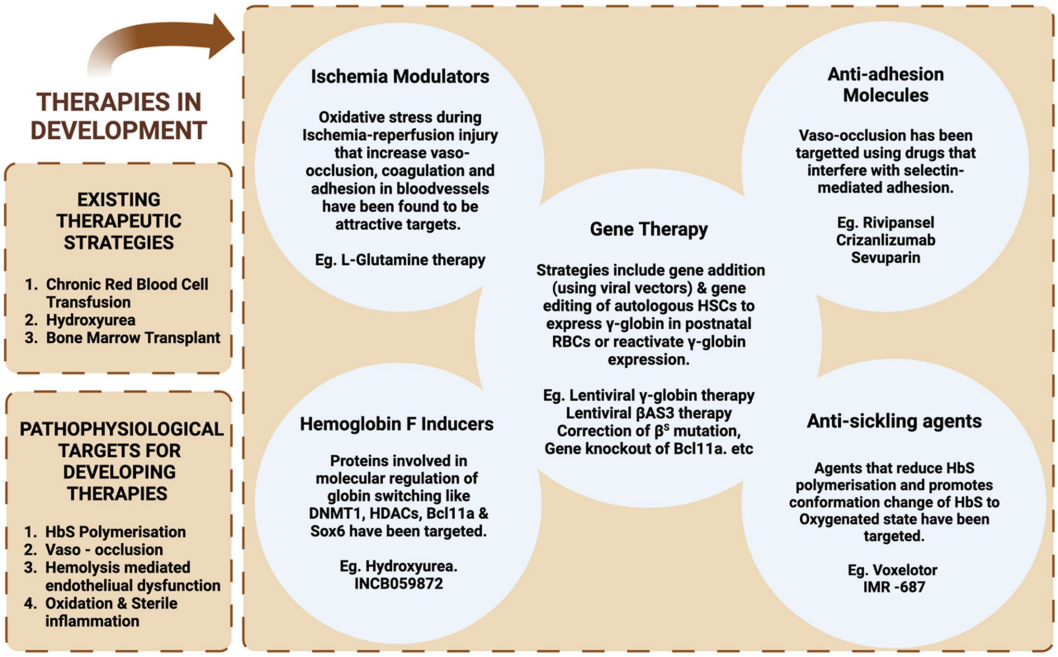

FIGURE 3
A diagrammatic representation of various therapeutic strategies: current and under development for the treatment of SCD.

5. Allogeneic HSCT-based therapeutic strategies to treat SCD

5.1. Matched sibling HSCT under myeloablative conditioning

The first HSCT for SCD was performed in 1984 when an 8-year-old girl with acute myeloid leukemia (AML) and an underlying SCD was subjected to HLA-matched HSCT to cure AML. The patient got cured of leukemia, and at the same time, a reversal in sickle status was observed (72, 73). This pioneer report by Johnson et al. encouraged researchers to go forward with HSCT as a therapeutic option for SCD (73). In 1988, after 4 years of this initial report, Vermylen et al. reported the outcome of matched related donor (MRD) HSCT using myeloablative conditioning with busulfan and cyclophosphamide in five children with SCD. Here, four out of five showed rapid and stable engraftment while one rejected the graft and underwent a second HSCT after 62 days of the first one. Thereafter, among all the children, the outcome of HSCT was uneventful, without any episodes of vaso-occlusion (VOC) and hemolysis with a hemoglobin pattern of donor type (74). Subsequently, in 1996, Walters and colleagues reported the outcome of HLA-matched HSCT in a group of 22 subjects (<16 years), preconditioned with cyclophosphamide, busulfan, and alemtuzumab (ATG). Stable engraftment was observed in 16 patients and stable mixed chimerism was observed in one patient (1/16). Twenty patients survived with a median follow-up of 23.9 months. A total of four patients rejected the graft including one patient with accompanied bone marrow aplasia. The central nervous system hemorrhage or GvHD was the possible cause of death of two patients. The overall survival and event-free survival at 4 years were 91% and 73%, respectively (75).

After 10 years of his first report, Vermylen et al. published the outcome of HLA-matched HSCT under myeloablative conditioning (busulfan, cyclophosphamide, and ATG) in 50 patients. Overall survival, event-free survival, and disease-free survival at 11 years were observed at 93, 82, and 85%, respectively. The study also advocated the advantage of early intervention for a better outcome of HSCT in treating SCD (76). Immediately after this report, Walters et al., in 2000, also reported the outcome of MRD-HSCT with myeloablative conditioning in 50 children with SCD. The patients received matched sibling bone marrow allografts between September 1991 and March 1999, with ages ranging from 3.3 to 15.9 (median 9.9) years. Three patients died due to intracranial hemorrhage or GvHD. Five patients rejected the graft out of 47 surviving patients, five patients rejected the graft with the presence of recurrent SCD, four had stable mixed chimerism, and among 38, full donor chimerism was observed. The authors also reported ovarian dysfunction in five of the seven evaluated female participants (77).

Another report published in 2007 (78) confirmed the findings of Vermylen et al. and Walters et al. The study reported overall survival and event-free survival of 93.1% and 86.1%, respectively. In this study, for the first 12 patients, busulfan and cyclophosphamide combination were used as a myeloablative conditioning regimen. Furthermore, ATG was also included in the regimen to prevent unstable mixed chimerism and rejection with a notably encouraging reduction in rejection rate (22.6% vs. 2.9%). Acute and chronic GvHD was observed in 20% and 12.6% of patients, respectively. The gonadal dysfunction was again observed as a common transplantation-related manifestation (78).

An international survey related to HLA identical sibling HSCT included 1,000 recipients of HLA-identical sibling transplants performed between 1986 and 2013 and reported to the European Society for Blood and Marrow Transplantation, Eurocord, and the Center for International Blood and Marrow Transplant Research. The majority of patients received a myeloablative conditioning regimen (87%) and only 13% of patients received reduced intensity conditioning. The 5-year event-free survival was approximately 91.4% and overall survival was 92.9%. Graft failure was observed in 23 patients and 7% of patients died and the most common cause of transplantation-related death was infection (70).

A very recent study from Spain reported the outcome of myeloablative sibling HSCT in 45 patients. Most patients received a conditioning regimen based on busulfan and cyclophosphamide (69%) and the remaining received treosulfan, thiotepa, and fludarabine. Event-free survival and overall survival at 3 years post-HSCT were 89.4% and 92.1%, respectively. Grade III–IV and chronic GvHD were evident in 6.8% and 5.4% of patients, respectively (79). HSCT studies on patients with SCD involving matched sibling donors are summarized in Supplementary Table 1.

5.2. Matched sibling HSCT under non-myeloablative conditioning

Encouraging clinical success was observed with matched sibling myeloablative HSCT; however, associated toxicities with preconditioning regimens like organ damage and gonadal dysfunction make them less attractive to cure SCD especially when patients are already suffering from organ damage due to their SCD status. Moreover, transplantation-related mortality and morbidity can outweigh the morbidity and mortality of SCD. To address these issues, collective effort from clinicians and researchers led to the development of reduced-intensity conditioning (RIC) regimens. HSCT under RIC proved to be advantageous for patients with SCD with underlying organ dysfunction/damage in a safer way which was not possible with myeloablative conditioning regimens. The initial attempts to cure SCD using HSCT under RIC were disappointing. In a study where a non-myeloablative-HSCT approach (fludarabine +200 cGy total body irradiation; two patients also received ATG) was used to treat SCD (six patients) and thalassemia (one patient) followed by a combination of immunosuppressants. Initially, 6 out of 7 patients who underwent HSCT exhibited donor chimerism and patients showed marked correction in hematological parameters. However, once the post-transplantation was immunosuppression tapered, loss of the donor graft, autologous hematopoietic recovery, and disease recurrence was observed (80). A similar outcome was reported in another study where RIC-based HSCT was used to cure a group of 13 patients with non-malignant diseases. Of the four patients with hemoglobinopathies, stable engraftment was observed in only one patient (81). After these initial unsuccessful attempts toward the development of a safer RIC-HSCT approach, the first successful approach was reported by Hsieh et al. in 2009. A total of 10 patients with SCD underwent non-myeloablative conditioning (300 cGy TBI+ alemtuzumab). Sirolimus (rapamycin) was used as GvHD prophylaxis. At a median follow-up of 30 months, stable donor-derived chimerism was observed in nine patients, which was enough to reverse the sickle cell disease status. Interestingly, no evidence of either acute or chronic GvHD was observed (82). In 2014, Hsieh et al. published the outcome of RIC-based sibling HSCT in 30 patients using the same approach. Of 30 patients, 29 patients survived during a median follow-up of 3.4 years, and stable donor chimerism was observed in 26 patients without any evidence of GvHD (83). It also became evident from these studies that alemtuzumab can prevent graft rejection and GvHD by deleting anti-donor host and anti-host donor T cells. Considering the utility of alemtuzumab in RIC-based HSCT for sickle cell disease, other groups also used alemtuzumab-based RIC regimen with reasonably good transplantation outcomes. Bhatia et al. used fludarabine-, busulfan-, and alemtuzumab-based RIC regimen in 18 patients with SCD. A total of 15 and 3 patients received matched sibling bone marrow and cord blood, respectively. All patients showed a higher percentage of donor chimerism. However, grade II-IV GvHD was evident in 17% of patients and chronic GvHD in 11% of patients as well (84). A similar outcome of RIC-based HSCT was reported by King et al. in 2015 (85). In the extended patient number (52 patients: 43 patients with SCD and 8 patients with thalassemia), they demonstrated the utility of RIC-based matched sibling donor HSCT (alemtuzumab, fludarabine, and melphalan). During a three-year follow-up, overall survival and event-free survival were 94.2% and 92.3%, respectively. Acute GvHD and chronic GvHD incidences were observed in 23% and 13% of patients, respectively. Three deaths due to the GvHD were recorded.

Later in 2016 Sharaf et al., confirmed the findings of Hsieh et al., by using RIC-based matched sibling donor HSCT in 13 adult patients with SCD. Stable donor chimerism observed in 12 patients was sufficient to reverse the SCD status without any occurrence of GvHD (86). These findings suggest that 300 cGy TBI is sufficient to create the required niche in the host bone marrow to establish stable donor chimerism. Moreover, alemtuzumab's lymph-toxic effect persists for weeks that deletes both host and donor T-cell clones potentially responsible for graft rejection and GvHD.

5.3. Matched unrelated and haploidentical donors for HSCT

Though the outcomes of non-myeloablative matched sibling HSCT were encouraging, practically it is impossible to find MSD for every patient with SCD in need of HSCT (75, 77, 82, 87). Thus, the paucity of MSD greatly reduced the HSCT utility to cure SCD. Furthermore, the HSCT experience with SCD also suggests that a reversal in disease patterns could be gained even without the complete replacement of the patient's stem cells (22, 76, 77, 88, 89). The aforementioned reports potentially suggested that the matched unrelated donor (MUD) or haploidentical donor-based HSCT could be implemented in patients with SCD. In another study, Strocchio et al. (90) reported the outcome of Treosulfan-based myeloablative conditioning in 15 patients with SCD including 6 receiving Matched Unrelated Donor (MUD) HSCT. Here, one out of six patients with MUD-HSCT showed graft failure. Disease-free survival at 5 years among those who received MUD-HSCT was 83%, comparatively lower than those who received MSD-HSCT (96%). However, there was no incidence of GvHD among groups (90). Contrary to this report, Shenoy et al. with a larger cohort of pediatric patients with SCD underwent MUD-HSCT($n = 29$) using a conditioning regimen of alemtuzumab, fludarabine and melphalan reported only 76% and 69% event-free survival rates at 1 and 2 years, respectively. Notably, there was a higher incidence of GvHD; grade II–IV 28% and chronic 62% (20). Very recently, Gluckman et al. published a report of a retrospective survey on 70 MUD-HSCT in patients with SCD carried out in the European Society for Blood and Marrow Transplantation Centers from 2005 to 2017. The overall survival of transplanted patients was 90% and event-free survival was 76% with the incidence of 25% and 23% of acute and chronic GvHD, respectively (91). These studies witnessed that the MUD-HSCT can expand the available donor pool for patients with SCD but to a limited extent. To this end, Tozatto-Maio et al. in 2020, by estimating the HLA haplotypes of 185 patients and by conducting a search for matched donors in international registries, reported that approximately 47% of patients with SCD had a matched donor (92). Considering the scarcity of donors for patients with SCD, haploidentical HSCTs (haplo-HSCT) were included to increase the donor pool possibly

for every patient. The very first report on the use of haploidentical HSCT to cure SCD was published in 2004. A 14-year-old boy received the non-myeloablative conditioning regimen of TBI and fludarabine. Cyclosporine and mycophenolate mofetil were used as GVHD prophylaxis. The graft was rejected with a complete absence of donor cells during the 5-month study (93). Principally, two main approaches were used to overcome the HLA barriers: (1) haplo-HSCT with T-cell-depleted grafts; (2) T-cell replete (TCR) grafts or un-manipulated grafts. Both approaches were increasingly tested and developed to overcome the underlying bidirectional issues of GvHD and graft rejection (94). HSCT studies on patients with SCD involving unmatched donors are summarized in Supplementary Table 2.

5.3.1. Haplo-HSCT using T-cell replete graft

The T-cell replete (TCR) haplo-HSCT is based on *in vivo* strategies to overcome the bidirectional alloreactivity and to induce tolerance across MHC barriers. The use of post-transplantation cyclophosphamide completely revolutionized the field by selective deletion of alloreactive T cells. HSCs are protected from the adverse effect of cyclophosphamide due to the expression of the drug-metabolizing enzyme aldehyde dehydrogenase which is not expressed by T cells (94–97). Moreover, cyclophosphamide-induced preferential expansion of regulatory T cells may also contribute to GvHD prevention (95).

In 2008, Brodsky et al. published the outcome of haplo-HSCT in one patient with SCD. The patient received the conditioning regimen of cyclophosphamide, TBI, and fludarabine, and the GvHD prophylaxis regimen of tacrolimus, mycophenolate mofetil along with post-transplantation cyclophosphamide. The patients with SCD exhibited donor-type chimerism and during a follow-up of the 1-year patient remained in remission without any evidence of GvHD (98). In 2012, Bolanos-Meade and colleagues explored the use of haplo-HSCT and post-transplantation cyclophosphamide in an extended number of patients ($n = 17$). Of the 17 patients, 14 patients transplanted with allografts from haploidentical-related donors were preconditioned with ATG, fludarabine, TBI, and cyclophosphamide. Mycophenolate mofetil and post-transplantation cyclophosphamide (days +3 and +4) were used as GvHD prophylaxis. During a median follow-up of 711 days, donor cell engraftment was observed in only 57% of patients with SCD, while the remaining 43% rejected the graft. Autologous hematopoiesis recovery was observed in all patients who rejected the graft. GvHD was not observed in any of the transplanted patients and overall survival was 100% (19). In this study, all the engrafted patients showed correction in SCD status. Moreover, the study also highlighted the need for improvement in the current TCR haplo-HSCT and post-transplantation cyclophosphamide approach.

To address the issues of GvHD and graft failure, Fitzhugh et al. introduced a modified non-myeloablative conditioning regimen with ATG, 400 cGy TBI, and escalating doses of post-transplantation cyclophosphamide (0, 50, and 100 mg/kg) for haplo-HSCT. A total of 23 patients (21 patients with SCD and 2 patients with thalassemia) were included in the study. The patients were distributed in three cohorts based on escalating doses of cyclophosphamide. The patients of cohort 1 ($n = 3$) did not receive post-transplantation cyclophosphamide, whereas those in cohort 2 ($n = 8$) and cohort 3 ($n = 12$) received 50 and 100 mg/kg cyclophosphamide, respectively. The results showed that 10 out of 12 patients from cohort 3 developed donor chimerism, whereas only five out of eight and one out of three patients showed engraftment from cohorts 1 and 2, respectively. After 1 year of post-transplantation, 50% of patients from cohort 3 remained disease free which was the highest among the groups. Moreover, the incidence of GvHD was observed in all cohorts of patients (no GvHD in cohort 1, grade I acute GvHD in one patient from each cohort 2 and 3) (99). In this study, the conditioning protocol modifications weighing to minimize the occurrence of GvHD was possibly the major cause of rejections resulted. However, in cohort 3, stable engraftment was observed in six (50%) of transplanted subjects who received 100 mg/kg post-transplantation cyclophosphamide. Though the outcomes were not very promising, this study set a platform for the future use of post-transplantation cyclophosphamide.

Saraf et al. reported the outcome of haplo-HSCT in 10 patients. The first two patients received the conditioning regimen of alemtuzumab, 300 cGy TBI, and cyclophosphamide of 50 mg/kg on days +3 and 4. Both the patients rejected the graft, so they adopted John Hopkins university's conditioning protocol. For the remaining eight patients, they used mobilized blood as a source of stem cells and a conditioning regimen containing TBI 300 cGy, ATG, cyclophosphamide, and fludarabine. Post-transplantation cyclophosphamide (days +3 and 4; 50 mg/kg) along with mycophenolate mofetil and sirolimus was used as GvHD prophylaxis. The engraftment was observed in all eight patients; secondary graft failure was observed in one patient at day 90 who showed autologous hematopoietic recovery. Two patients developed acute GvHD and one chronic patient developed chronic GvHD and later died due to unexplained reasons. With a median follow-up of 16 months, the remaining seven patients were alive. The study highlighted the safety of the modified John Hopkins approach (86). Although higher engraftment and reduced mortality were observed using the modified John Hopkins approach, this approach was not devoid of transplantation-related toxicities and viral reactivation.

At the same time, another multicentric study published a report of 16 patients with SCD who underwent haplo-HSCT under non-myeloablative conditioning and post-transplantation cyclophosphamide (John Hopkins approach) (100). For the first three patients conditioning regimen was ATG, fludarabine, cyclophosphamide, and 200 cGy TBI; GvHD prophylaxis consisted of post-transplantation cyclophosphamide, mycophenolate mofetil, and sirolimus. Two out of three patients showed primary graft failure. To overcome the problem of graft rejection, thiotepa was included in the conditioning regimen and then the remaining 13 and 2 patients who previously rejected the graft underwent haplo-HSCT with thiotepa augmented conditioning regimen. During a median follow-up of 13.3 months, 14 of 15 patients had donor engraftment > 95% with 100% overall survival. Grades III–IV GVHD occurred in only one patient and chronic GvHD was also observed in only one patient. This study suggested that the use of post-transplantation cyclophosphamide and thiotepa in a conditioning regimen (John Hopkins approach) improves donor engraftment without the higher incidence of fatal GvHD. However, again there was an incidence of viral reactivation and transplantation-related toxicities among transplanted patients.

A second study by Bolanos-Meade et al. on 12 patients with SCD reported the outcome of haplo-HSCT with a modified regimen (400 cGy TBI, ATG, fludarabine, cyclophosphamide, and post-transplantation cyclophosphamide) (101). Graft failure was observed in one patient (8%), and overall survival was 100%. Three patients (25%) experienced acute GVHD, and one patient developed chronic GVHD.

Kharya et al. (102) reported the outcome of haplo-HSCT in 25 patients using mobilized blood graft instead of bone marrow. All the patients received the conditioning regimen used by de la Fuente et al., in 2019 (100). Two courses of immune suppression were used to prevent

GvHD, pre-transplant (fludarabine, cyclophosphamide, and dexamethasone), and post-transplant (cyclophosphamide, sirolimus, and mycophenolate mofetil). Engraftment was observed in all the patients. Unfortunately, 12% (3) died due to transplantation-related mortality. Overall survival was 88% with the incidence of acute and chronic GvHD in 20% and 12% of patients, respectively.

5.3.2. Haplo-HSCT using T-cell-depleted graft

Graft-derived T cells are considered key players in the occurrence of GvHD. *Ex vivo* depletion of T cells from the graft is one of the effective approaches for the prevention of GvHD (103). One of the major advantages of this approach is that it restricts the prolonged use of immunosuppressive drugs, which may make the host more prone to infection and can cause multi-organ toxicity (103). The approach involves either extensive depletion of T cells from hematopoietic grafts or a positive selection of CD34+ cells. Later it can cause a delay in immune reconstitution due to the positive selection of CD34 graft lacking B and NK cells that do not happen during T-cell depletion.

Initial attempts to deplete T cells from hematopoietic grafts were carried out in the late 1980s *via* agglutination with soybean lectin and resetting the residual T cells with sheep RBC (104–106), and the approach was further advanced to use the monoclonal antibodies directed against T cells (106, 107). Initially, the approach was observed as potentially useful in the prevention of GvHD with increased cases of relapse among patients with leukemia (108). Moreover, graft failure was another issue associated with the TCD approach (109). These findings suggested that donor T cells have a promising role in graft survival; they can prevent graft rejection by counteracting against residual host immune cells left after preconditioning. Despite this, encouraging results have been observed by using TCD hematopoietic graft.

Dallas et al. (110) reported the outcome of TCD-haplo-HSCT in eight SCD children with a median age of 9.0 ± 5.0. The CD34+ cells were selected by CliniMACS (Miltenyi Biotech) and *in vivo* CD3 depletion was accompanied by muromonab. During a median follow-up of 7.4 ± 2.4 years, overall survival and disease-free survival were observed at 75% and 38%, respectively. The incidence of acute and chronic GvHD was 50 and 38%, respectively. The major aim of the study was to expand the donor pool availability with a reduced rate of GvHD and sustainable long-term outcomes of HSCT.

Foell et al. (21) published the report of haplo-HSCT in nine patients who failed hydroxyurea treatment, by using a Treosulfan-based conditioning regimen using T-and B-cell-depleted haploidentical grafts from first-degree related relatives who were fully haplotype mismatched. The myeloablative conditioning regimen consisted of ATG, fludarabine, and treosulfan. Considering the higher chances of graft rejection, immunosuppression was continued up to 120 days post-HSCT by using cyclosporine and mycophenolate mofetil. Stable donor cell engraftment was observed in all patients during a median follow-up of 26 months. The study aimed to test whether treosulfan is effective in creating sufficient niches for HSCT with reduced toxicities and GvHD. Despite the myeloablative nature of conditioning, it was tolerable among the patients, and only mild grade 1–2 toxicities were observed. However, viral reactivation emerged as a common problem. However, this terosulfan-based novel conditioning regimen opened a new door for HSCT to induce engraftment with reduced transplantation toxicities. The same year, Gilman et al. (111) reported the outcome of haplo-HSCT by using a RIC regimen and CD34+ selected or T-cell-depleted graft in 10 patients. The conditioning regimen consisted of melphalan, thiotepa, fludarabine, and ATG. Methotrexate was used as GvHD prophylaxis. During a median follow-up of 49 months, 9 out of 10 survived with stable donor engraftment and without sickle cell disease complications. Acute and chronic GvHD was observed in two and one patients, respectively. The viral reactivation again emerged as an issue that needed to be addressed.

Very recently, Gaziev and colleagues showed the results of haplo-HSCT by using T-cell receptor αβ+/CD19+ depleted grafts (112). The conditioning regimen consisted of busulfan, thiotepa, cyclophosphamide, and ATG preceded by fludarabine, hydroxyurea, and azathioprine. A total of 14 patients, 11 with thalassemia and 3 with SCD, were included in the study. The resultant data were compared with the outcome of haplo-HSCT among a group of 40 patients by using CD34+ selected from peripheral blood and bone marrow ($n = 32$), CD34+ selected from peripheral blood, and CD3+/CD19+ from depleted bone marrow grafts ($n = 8$). The 5-year probability of overall survival and disease-free survival were observed at 84% and 69%, respectively. The incidence of graft failure was 14%. The results showed that a higher incidence of graft failure was observed in the CD34 group (45%) when compared with the TCR group (14%). The incidence of acute and chronic GvHD was comparable among the groups (28% vs. 29% and 10% vs. 21%, respectively). Viral reactivation was also common in both groups. The study showed that TCR αβ+/CD19+ depleted grafts were associated with reduced incidence of GvHD as well as a delay in immune reconstitution associated with mortality and morbidity remained a challenge to be addressed (112).

Similarly, the occurrence of GvHD and graft failure was observed in a very recent study that reported the outcome of 25 patients with SCD who underwent haplo-HSCT using a myeloablative conditioning regimen (ATG, fludarabine, and treosulfan). The graft was T and B cell depleted. Secondary graft failure was observed in two patients who died due to transplantation-related complications. Another patient died due to viral reactivation reducing the overall survival to 88%. The incidence of acute GvHD was 32% (7 out of 25), and 18% (4 out of 25) developed mild to moderate GvHD (113). The patients in the MSD-SCT group had an OS of 100%, and no graft failure occurred. The incidence of grade I-II acute GVHD was 23%, and mild to moderate chronic GvHD occurred in two patients (15%).

5.3.3. Veto cells-based TCD approach

Both the approaches either TCR or TCD showed encouraging success in the treatment of SCD by increasing donor availability, improving engraftment, and minimizing the risk of GvHD. However, both tested approaches with different conditioning regimens failed to establish the ideal protocol for HSCT to cure SCD without graft failure, the incidence of GvHD, and viral reactivation. To this end, a preclinical study by Singh and colleagues in a well-defined SCD mice model showed that durable engraftment of allogeneic hematopoietic stem cells could be achieved by using donor-derived CD8+ veto T cells with a complete reversal in disease pattern (Figure 4). The authors reported that graft rejection of T-cell-depleted non-myeloablative HSCT can be overcome in fully mismatched SCD recipient mice by using a mega dose of bone marrow, anti-third party veto cells, and short-term dose of rapamycin without the occurrence of GvHD. The current approach is under phase I/II clinical trial to cure hematological malignancies (NCT03622788). The approach is based on the generation of antiviral donor-derived CD8+ veto T cells. Extensive research by Prof. Yair Reisner's group provided sufficient information that anti-third party CD8+ veto can induce tolerance and engraftment of haplo-HSCs by specifically deleting only alloreactive T cells directed against veto cells

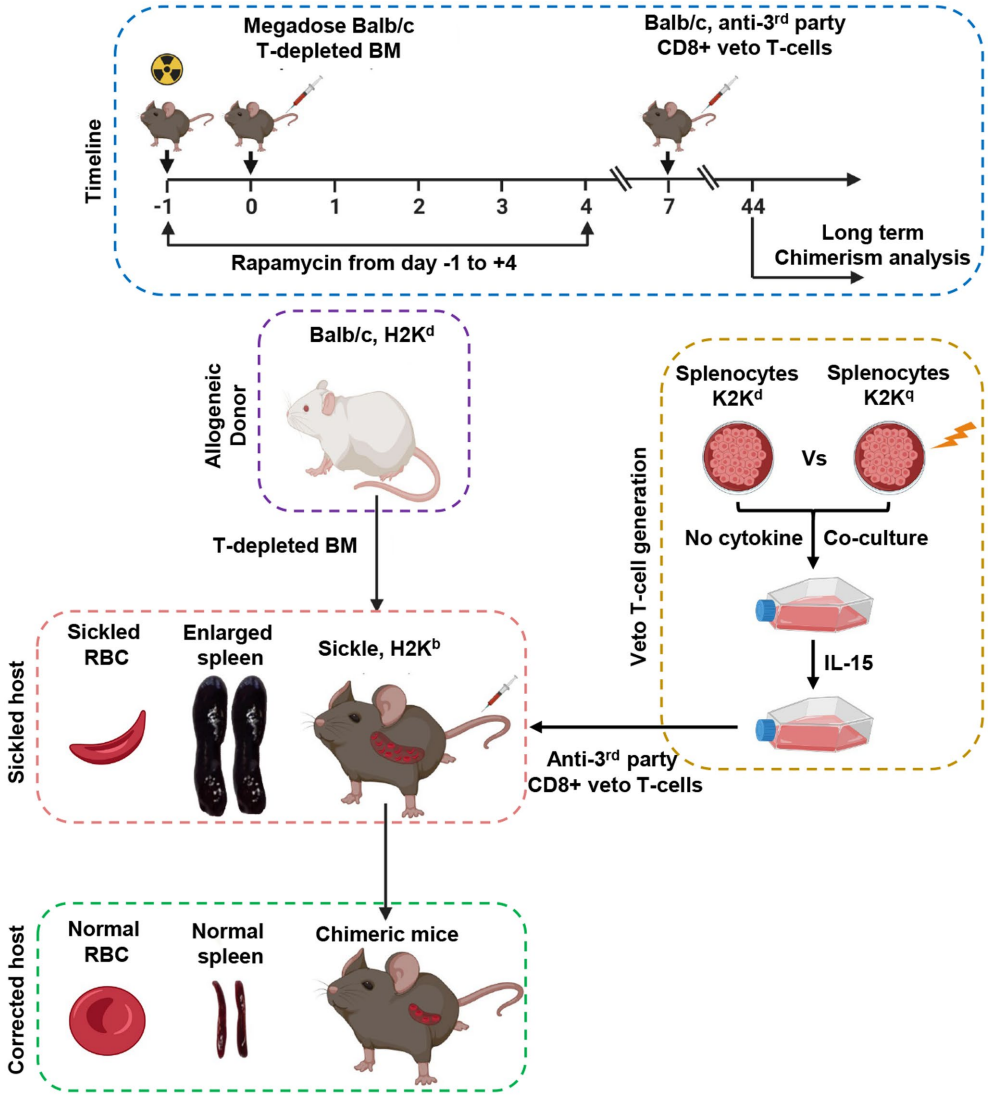

FIGURE 4
Schematic representation of the allogeneic hematopoietic stem transplantation with anti-third party veto cells under RIC to correct sickle cell disease in a murine model.

thus sparing non-alloreactive immune cells to fight against infection (114, 115). The veto cells directed against viral antigens do not cause GvHD and would also protect against viral reactivation a common problem in transplantation settings. The clinical trial results are awaited, their success will open a new door to treating hematological or non-hematological disease by HSCT without the risk of graft failure, GvHD, and viral reactivation under reduced intensity conditioning by using megadose of peripheral blood stem cells and donor derive CD8+ veto T cells.

6. Conclusion

Tremendous success has been achieved by MSD and haplo-HSCT to cure SCD. However, in every approach, the incidence of GvHD, graft failure, and viral reactivation are existing issues. To this end, Veto CD8 T-cell-based haplo-HSCT approach seems promising in overcoming HLA barriers as well as viral reactivation. Only preclinical data are currently available right now. A clinical trial testing the safety and efficacy of central memory CD8 veto cells in recipients of non-myeloablative T-cell-depleted HLA-haplotype-matched transplants in patients with hematological cancers is in progress. Furthermore, different gene therapy-based approaches are also underway in clinical trials showing promising results. However, all of these approaches are in their early phase still a long road to go and allo-HSCT is the only treatment of choice for the treatment of SCD.

Author contributions

AS and SKY conceptualized, supervised, and co-wrote the manuscript. NB and AB facilitated a compilation of relevant literature, manuscript writing, figure preparation, and participated in discussions.

All authors contributed to the article and approved the submitted version.

Acknowledgments

The authors gratefully acknowledge BioRender.com for creating figures.

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.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2023.1036939/full#supplementary-material

References

1. Glassberg, J. Evidence-based management of sickle cell disease in the emergency department. *Emerg Med Pract*. (2011) 13:1–20. PMID: 22164362

2. Esham, KS, Rodday, AM, Smith, HP, Noubary, F, Weidner, RA, Buchsbaum, RJ, et al. Assessment of health-related quality of life among adults hospitalized with sickle cell disease vaso-occlusive crisis. *Blood Adv*. (2020) 4:19–27. doi: 10.1182/bloodadvances.2019000128

3. El Hoss, S, Cochet, S, Marin, M, Lapoumeroulie, C, Dussiot, M, Bouazza, N, et al. Insights into determinants of spleen injury in sickle cell anemia. *Blood Adv*. (2019) 3:2328–36. doi: 10.1182/bloodadvances.2019000106

4. Badawy, SM, and Payne, AB. Association between clinical outcomes and metformin use in adults with sickle cell disease and diabetes mellitus. *Blood Adv*. (2019) 3:3297–306. doi: 10.1182/bloodadvances.2019000838

5. Reeves, SL, Jary, HK, Gondhi, JP, Kleyn, M, and Dombkowski, KJ. Health outcomes and services in children with sickle cell trait, sickle cell anemia, and normal hemoglobin. *Blood Adv*. (2019) 3:1574–80. doi: 10.1182/bloodadvances.2018028043

6. Herrick, JB. Peculiar elongated and sickle-shaped red blood corpuscles in a CASE of severe anemia. *Arch Intern Med*. (1910) VI:517–21.

7. Pauling, L, Itano, HA, Singer, SJ, and Wells, IC. Sickle cell anemia a molecular disease. *Science*. (1949) 110:543–8. doi: 10.1126/science.110.2865.543

8. Piel, FB, Hay, SI, Gupta, S, Weatherall, DJ, and Williams, TN. Global burden of sickle cell anaemia in children under five, 2010-2050: modelling based on demographics, excess mortality, and interventions. *PLoS Med*. (2013) 10:e1001484. doi: 10.1371/journal.pmed.1001484

9. Piel, FB, Steinberg, MH, and Rees, DC. Sickle cell disease. *N Engl J Med*. (2017) 376:1561–73. doi: 10.1056/NEJMra1510865

10. Herrick, JB. Peculiar elongated and sickle-shaped red blood corpuscles in a case of severe anemia. *Yale J Biol Med*. (1910) 74:179–84.

11. Weatherall, DJ, and Clegg, JB. Inherited haemoglobin disorders: an increasing global health problem. *Bull World Health Organ*. (2001) 79:704–12. PMID: 11545326

12. Hassell, KL. Sickle cell disease: a continued call to action. *Am J Prev Med*. (2016) 51:S1–2. doi: 10.1016/j.amepre.2015.11.002

13. McGann, PT, Hernandez, AG, and Ware, RE. Sickle cell anemia in sub-Saharan Africa: advancing the clinical paradigm through partnerships and research. *Blood*. (2017) 129:155–61. doi: 10.1182/blood-2016-09-702324

14. Telfer, P, Coen, P, Chakravorty, S, Wilkey, O, Evans, J, Newell, H, et al. Clinical outcomes in children with sickle cell disease living in England: a neonatal cohort in East London. *Haematologica*. (2007) 92:905–12. doi: 10.3324/haematol.10937

15. Quinn, CT, Rogers, ZR, McCavit, TL, and Buchanan, GR. Improved survival of children and adolescents with sickle cell disease. *Blood*. (2010) 115:3447–52. doi: 10.1182/blood-2009-07-233700

16. Elmariah, H, Garrett, ME, De Castro, LM, Jonassaint, JC, Ataga, KI, Eckman, JR, et al. Factors associated with survival in a contemporary adult sickle cell disease cohort. *Am J Hematol*. (2014) 89:530–5. doi: 10.1002/ajh.23683

17. Gardner, K, Douiri, A, Drasar, E, Allman, M, Mwirigi, A, Awogbade, M, et al. Survival in adults with sickle cell disease in a high-income setting. *Blood*. (2016) 128:1436–8. doi: 10.1182/blood-2016-05-716910

18. Serjeant, GR, Chin, N, Asnani, MR, Serjeant, BE, Mason, KP, Hambleton, IR, et al. Causes of death and early life determinants of survival in homozygous sickle cell disease: the Jamaican cohort study from birth. *PLoS One*. (2018) 13:e0192710. doi: 10.1371/journal.pone.0192710

19. Bolanos-Meade, J, Fuchs, EJ, Luznik, L, Lanzkron, SM, Gamper, CJ, Jones, RJ, et al. HLA-haploidentical bone marrow transplantation with posttransplant cyclophosphamide expands the donor pool for patients with sickle cell disease. *Blood*. (2012) 120:4285–91. doi: 10.1182/blood-2012-07-438408

20. Shenoy, S, Eapen, M, Panepinto, JA, Logan, BR, Wu, J, Abraham, A, et al. A trial of unrelated donor marrow transplantation for children with severe sickle cell disease. *Blood*. (2016) 128:2561–7. doi: 10.1182/blood-2016-05-715870

21. Foell, J, Pfirstinger, B, Rehe, K, Wolff, D, Holler, E, and Corbacioglu, S. Haploidentical stem cell transplantation with CD3(+)-/CD19(+)-depleted peripheral stem cells for patients with advanced stage sickle cell disease and no alternative donor: results of a pilot study. *Bone Marrow Transplant*. (2017) 52:938–40. doi: 10.1038/bmt.2017.49

22. Fitzhugh, CD, Cordes, S, Taylor, T, Coles, W, Roskom, K, Link, M, et al. At least 20% donor myeloid chimerism is necessary to reverse the sickle phenotype after allogeneic HSCT. *Blood*. (2017) 130:1946–8. doi: 10.1182/blood-2017-03-772392

23. Kaestner, L, and Bianchi, P. Trends in the development of diagnostic tools for red blood cell-related diseases and anemias. *Front Physiol*. (2020) 11:387. doi: 10.3389/fphys.2020.00387

24. Bueren, JA, Quintana-Bustamante, O, Almarza, E, Navarro, S, Rio, P, Segovia, JC, et al. Advances in the gene therapy of monogenic blood cell diseases. *Clin Genet*. (2020) 97:89–102. doi: 10.1111/cge.13593

25. Ferrari, S, Vavassori, V, Canarutto, D, Jacob, A, Castiello, MC, Javed, AO, et al. Gene editing of hematopoietic stem cells: hopes and hurdles toward clinical translation. *Front Genome Ed*. (2021) 3:618378. doi: 10.3389/fgeed.2021.618378

26. Sydenstricker, VP. Further observations on sickle cell anemia. *J Am Med Assoc*. (1924) 83:12–7. doi: 10.1001/jama.1924.02660010016004

27. Hahn, EV, and Gillespie, EB. Sickle cell anemia: report of a CASE greatly improved by splenectomy. Experimental Study of sickle cell formation. *Arch Intern Med*. (1927) 39:233–54. doi: 10.1001/archinte.1927.00130020072006

28. Scriver, JB, and Waugh, TR. Studies on a case of sickle-cell Anaemia. *Can Med Assoc J*. (1930) 23:375–80. PMID: 20317973

29. Watson, J. The significance of the paucity of sickle cells in newborn negro infants. *Am J Med Sci*. (1948) 215:419–23. doi: 10.1097/00000441-194804000-00008

30. Neel, JV. The inheritance of sickle cell anemia. *Science*. (1949) 110:64–6. doi: 10.1126/science.110.2846.64

31. Ingram, VMAbnormal human haemoglobins. I. The comparison of normal human and sickle-cell haemoglobins by fingerprinting. *Biochim Biophys Acta*. (1958) 28:539–45. doi: 10.1016/0006-3002(58)90516-X

32. Ferrone, FA. Polymerization and sickle cell disease: a molecular view. *Microcirculation*. (2004) 11:115–28. doi: 10.1080/10739680490278312

33. Serjeant, GR. The natural history of sickle cell disease. *Cold Spring Harb Perspect Med*. (2013) 3:a011783. doi: 10.1101/cshperspect.a011783

34. Sundd, P, Gladwin, MT, and Novelli, EM. Pathophysiology of sickle cell disease. *Annu Rev Pathol*. (2019) 14:263–92. doi: 10.1146/annurev-pathmechdis-012418-012838

35. Bunn, HF. Pathogenesis and treatment of sickle cell disease. *N Engl J Med*. (1997) 337:762–9. doi: 10.1056/NEJM199709113371107

36. Hebbel, RP. Reconstructing sickle cell disease: a data-based analysis of the hyperhemolysis paradigm for pulmonary hypertension from the perspective of evidence-based medicine. *Am J Hematol*. (2011) 86:123–54. doi: 10.1002/ajh.21952

37. Manwani, D, and Frenette, PS. Vaso-occlusion in sickle cell disease: pathophysiology and novel targeted therapies. *Blood*. (2013) 122:3892–8. doi: 10.1182/blood-2013-05-498311

38. Salinas Cisneros, G, and Thein, SL. Recent advances in the treatment of sickle cell disease. *Front Physiol*. (2020) 11:435. doi: 10.3389/fphys.2020.00435

39. Palmer, RM, Ashton, DS, and Moncada, S. Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature*. (1988) 333:664–6. doi: 10.1038/333664a0

40. Villagra, J, Shiva, S, Hunter, LA, Machado, RF, Gladwin, MT, and Kato, GJ. Platelet activation in patients with sickle disease, hemolysis-associated pulmonary hypertension, and nitric oxide scavenging by cell-free hemoglobin. *Blood*. (2007) 110:2166–72. doi: 10.1182/blood-2006-12-061697

41. Ohene-Frempong, K, Weiner, SJ, Sleeper, LA, Miller, ST, Embury, S, Moohr, JW, et al. Cerebrovascular accidents in sickle cell disease: rates and risk factors. *Blood*. (1998) 91:288–94. PMID: 9414296

42. Miller, ST, Sleeper, LA, Pegelow, CH, Enos, LE, Wang, WC, Weiner, SJ, et al. Prediction of adverse outcomes in children with sickle cell disease. *N Engl J Med*. (2000) 342:83–9. doi: 10.1056/NEJM200001133420203

43. Zhang, D, Xu, C, Manwani, D, and Frenette, PS. Neutrophils, platelets, and inflammatory pathways at the nexus of sickle cell disease pathophysiology. *Blood*. (2016) 127:801–9. doi: 10.1182/blood-2015-09-618538

44. Turhan, A, Weiss, LA, Mohandas, N, Coller, BS, and Frenette, PS. Primary role for adherent leukocytes in sickle cell vascular occlusion: a new paradigm. *Proc Natl Acad Sci U S A*. (2002) 99:3047–51. doi: 10.1073/pnas.052522799

45. Wali, Y, Beshlawi, I, Fawaz, N, Alkhayat, A, Zalabany, M, Elshinawy, M, et al. Coexistence of sickle cell disease and severe congenital neutropenia: first impressions can be deceiving. *Eur J Haematol*. (2012) 89:245–9. doi: 10.1111/j.1600-0609.2012.01827.x

46. Telen, MJ. Beyond hydroxyurea: new and old drugs in the pipeline for sickle cell disease. *Blood*. (2016) 127:810–9. doi: 10.1182/blood-2015-09-618553

47. Telen, MJ, Batchvarova, M, Shan, S, Bovee-Geurts, PH, Zennadi, R, Leitgeb, A, et al. Sevuparin binds to multiple adhesive ligands and reduces sickle red blood cell-induced vaso-occlusion. *Br J Haematol*. (2016) 175:935–48. doi: 10.1111/bjh.14303

48. Telen, MJ, Malik, P, and Vercellotti, GM. Therapeutic strategies for sickle cell disease: towards a multi-agent approach. *Nat Rev Drug Discov*. (2019) 18:139–58. doi: 10.1038/s41573-018-0003-2

49. Nasimuzzaman, M, and Malik, P. Role of the coagulation system in the pathogenesis of sickle cell disease. *Blood Adv*. (2019) 3:3170–80. doi: 10.1182/bloodadvances.2019000193

50. Gutsaeva, DR, Parkerson, JB, Yerigenahally, SD, Kurz, JC, Schaub, RG, Ikuta, T, et al. Inhibition of cell adhesion by anti-P-selectin aptamer: a new potential therapeutic agent for sickle cell disease. *Blood*. (2011) 117:727–35. doi: 10.1182/blood-2010-05-285718

51. Ataga, KI, Kutlar, A, Kanter, J, Liles, D, Cancado, R, Friedrisch, J, et al. Crizanlizumab for the prevention of pain crises in sickle cell disease. *N Engl J Med*. (2017) 376:429–39. doi: 10.1056/NEJMoa1611770

52. Chang, J, Patton, JT, Sarkar, A, Ernst, B, Magnani, JL, and Frenette, PS. GMI-1070, a novel pan-selectin antagonist, reverses acute vascular occlusions in sickle cell mice. *Blood*. (2010) 116:1779–86. doi: 10.1182/blood-2009-12-260513

53. Ali, MA, Ahmad, A, Chaudry, H, Aiman, W, Aamir, S, Anwar, MY, et al. Efficacy and safety of recently approved drugs for sickle cell disease: a review of clinical trials. *Exp Hematol*. (2020) 92:11–18.e1. doi: 10.1016/j.exphem.2020.08.008

54. Basak, A, and Sankaran, VG. Regulation of the fetal hemoglobin silencing factor BCL11A. *Ann N Y Acad Sci*. (2016) 1368:25–30. doi: 10.1111/nyas.13024

55. Charache, S, Dover, GJ, Moore, RD, Eckert, S, Ballas, SK, Koshy, M, et al. Hydroxyurea: effects on hemoglobin F production in patients with sickle cell anemia. *Blood*. (1992) 79:2555–65. doi: 10.1182/blood.V79.10.2555.2555

56. Charache, S, Terrin, ML, Moore, RD, Dover, GJ, Barton, FB, Eckert, SV, et al. Effect of hydroxyurea on the frequency of painful crises in sickle cell anemia. Investigators of the Multicenter Study of Hydroxyurea in Sickle Cell Anemia. *N Engl J Med*. (1995) 332:1317–22. PMID: 7715639

57. Nahavandi, M, Tavakkoli, F, Wyche, MQ, Perlin, E, Winter, WP, and Castro, O. Nitric oxide and cyclic GMP levels in sickle cell patients receiving hydroxyurea. *Br J Haematol*. (2002) 119:855–7. doi: 10.1046/j.1365-2141.2002.03919.x

58. Conran, N, Oresco-Santos, C, Acosta, HC, Fattori, A, Saad, ST, and Costa, FF. Increased soluble guanylate cyclase activity in the red blood cells of sickle cell patients. *Br J Haematol*. (2004) 124:547–54. doi: 10.1111/j.1365-2141.2004.04810.x

59. Kuroyanagi, Y, Kaneko, Y, Muta, K, Park, BS, Moi, P, Ausenda, S, et al. cAMP differentially regulates gamma-globin gene expression in erythroleukemic cells and primary erythroblasts through c-Myb expression. *Biochem Biophys Res Commun*. (2006) 344:1038–47. doi: 10.1016/j.bbrc.2006.03.203

60. Hahn, CK, and Lowrey, CH. Eukaryotic initiation factor 2alpha phosphorylation mediates fetal hemoglobin induction through a post-transcriptional mechanism. *Blood*. (2013) 122:477–85. doi: 10.1182/blood-2013-03-491043

61. Fard, AD, Hosseini, SA, Shahjahani, M, Salari, F, and Jaseb, K. Evaluation of novel fetal hemoglobin inducer drugs in treatment of beta-Hemoglobinopathy disorders. *Int J Hematol Oncol Stem Cell Res*. (2013) 7:47–54. PMID: 24505535

62. Fathallah, H, and Atweh, GF. Induction of fetal hemoglobin in the treatment of sickle cell disease. *Hematology Am Soc Hematol Educ Program*. (2006) 2006:58–62. doi: 10.1182/asheducation-2006.1.58

63. Pace, BS, Qian, XH, Sangerman, J, Ofori-Acquah, SF, Baliga, BS, Han, J, et al. p38 MAP kinase activation mediates gamma-globin gene induction in erythroid progenitors. *Exp Hematol*. (2003) 31:1089–96. doi: 10.1016/S0301-472X(03)00235-2

64. Marianna, P, Kollia, P, Akel, S, Papassotiriou, Y, Stamoulakatou, A, and Loukopoulos, D. Valproic acid, trichostatin and their combination with hemin preferentially enhance gamma-globin gene expression in human erythroid liquid cultures. *Haematologica*. (2001) 86:700–5. PMID: 11454524

65. Ware, RE, and Aygun, B. Advances in the use of hydroxyurea. *Hematology Am Soc Hematol Educ Program*. (2009) 2009:62–9. doi: 10.1182/asheducation-2009.1.62

66. Nevitt, SJ, Jones, AP, and Howard, J. Hydroxyurea (hydroxycarbamide) for sickle cell disease. *Cochrane Database Syst Rev*. (2017) 2017:CD002202. doi: 10.1002/14651858.CD002202.pub2

67. Vinjamur, DS, Bauer, DE, and Orkin, SH. Recent progress in understanding and manipulating haemoglobin switching for the haemoglobinopathies. *Br J Haematol*. (2018) 180:630–43. doi: 10.1111/bjh.15038

68. Sankaran, VG, Lettre, G, Orkin, SH, and Hirschhorn, JN. Modifier genes in Mendelian disorders: the example of hemoglobin disorders. *Ann N Y Acad Sci*. (2010) 1214:47–56. doi: 10.1111/j.1749-6632.2010.05821.x

69. Ribeil, JA, Hacein-Bey-Abina, S, Payen, E, Magnani, A, Semeraro, M, Magrin, E, et al. Gene therapy in a patient with sickle cell disease. *N Engl J Med*. (2017) 376:848–55. doi: 10.1056/NEJMoa1609677

70. Gluckman, E, Cappelli, B, Bernaudin, F, Labopin, M, Volt, F, Carreras, J, et al. Sickle cell disease: an international survey of results of HLA-identical sibling hematopoietic stem cell transplantation. *Blood*. (2017) 129:1548–56. doi: 10.1182/blood-2016-10-745711

71. Vichinsky, E, Hoppe, CC, Ataga, KI, Ware, RE, Nduba, V, El-Beshlawy, A, et al. A phase 3 randomized trial of Voxelotor in sickle cell disease. *N Engl J Med*. (2019) 381:509–19. doi: 10.1056/NEJMoa1903212

72. Johnson, FL, Look, AT, Gockerman, J, Ruggiero, MR, Dalla-Pozza, L, and Billings, FT 3rd. Bone-marrow transplantation in a patient with sickle-cell anemia. *N Engl J Med*. (1984) 311:780–3. doi: 10.1056/NEJM198409203111207

73. Johnson, FL. Bone marrow transplantation in the treatment of sickle cell anemia. *Am J Pediatr Hematol Oncol*. (1985) 7:254–7. doi: 10.1097/00043426-198507030-00008

74. Vermylen, C, Fernandez Robles, E, Ninane, J, and Cornu, G. Bone marrow transplantation in five children with sickle cell anaemia. *Lancet*. (1988) 1:1427–8. PMID: 2898584

75. Walters, MC, Patience, M, Leisenring, W, Eckman, JR, Buchanan, GR, Rogers, ZR, et al. Barriers to bone marrow transplantation for sickle cell anemia. *Biol Blood Marrow Transplant*. (1996) 2:100–4. PMID: 9118298

76. Vermylen, C, Cornu, G, Ferster, A, Brichard, B, Ninane, J, Ferrant, A, et al. Haematopoietic stem cell transplantation for sickle cell anaemia: the first 50 patients transplanted in Belgium. *Bone Marrow Transplant*. (1998) 22:1–6. doi: 10.1038/sj.bmt.1701291

77. Walters, MC, Storb, R, Patience, M, Leisenring, W, Taylor, T, Sanders, JE, et al. Impact of bone marrow transplantation for symptomatic sickle cell disease: an interim report. Multicenter investigation of bone marrow transplantation for sickle cell disease. *Blood*. (2000) 95:1918–24. doi: 10.1182/blood.V95.6.1918

78. Bernaudin, F, Socie, G, Kuentz, M, Chevret, S, Duval, M, Bertrand, Y, et al. Long-term results of related myeloablative stem-cell transplantation to cure sickle cell disease. *Blood*. (2007) 110:2749–56. doi: 10.1182/blood-2007-03-079665

79. Benitez-Carabante, MI, Belendez, C, Gonzalez-Vicent, M, Alonso, L, Uria-Oficialdegui, ML, Torrent, M, et al. Grupo Espanol de Trasplante de Medula Osea en Ninos, matched sibling donor stem cell transplantation for sickle cell disease: results from the Spanish group for bone marrow transplantation in children. *Eur J Haematol*. (2021) 106:408–16. doi: 10.1111/ejh.13566

80. Iannone, R, Casella, JF, Fuchs, EJ, Chen, AR, Jones, RJ, Woolfrey, A, et al. Results of minimally toxic nonmyeloablative transplantation in patients with sickle cell anemia and beta-thalassemia. *Biol Blood Marrow Transplant*. (2003) 9:519–28. doi: 10.1016/S1083-8791(03)00192-7

81. Jacobsohn, DA, Duerst, R, Tse, W, and Kletzel, M. Reduced intensity haemopoietic stem-cell transplantation for treatment of non-malignant diseases in children. *Lancet*. (2004) 364:156–62. doi: 10.1016/S0140-6736(04)16628-2

82. Hsieh, MM, Kang, EM, Fitzhugh, CD, Link, MB, Bolan, CD, Kurlander, R, et al. Allogeneic hematopoietic stem-cell transplantation for sickle cell disease. *N Engl J Med*. (2009) 361:2309–17. doi: 10.1056/NEJMoa0904971

83. Hsieh, MM, Fitzhugh, CD, Weitzel, RP, Link, ME, Coles, WA, Zhao, X, et al. Nonmyeloablative HLA-matched sibling allogeneic hematopoietic stem cell transplantation for severe sickle cell phenotype. *JAMA*. (2014) 312:48–56. doi: 10.1001/jama.2014.7192

84. Bhatia, M, Jin, Z, Baker, C, Geyer, MB, Radhakrishnan, K, Morris, E, et al. Reduced toxicity, myeloablative conditioning with BU, fludarabine, alemtuzumab and SCT from sibling donors in children with sickle cell disease. *Bone Marrow Transplant*. (2014) 49:913–20. doi: 10.1038/bmt.2014.84

85. King, AA, Kamani, N, Bunin, N, Sahdev, I, Brochstein, J, Hayashi, RJ, et al. Successful matched sibling donor marrow transplantation following reduced intensity conditioning in children with hemoglobinopathies. *Am J Hematol*. (2015) 90:1093–8. doi: 10.1002/ajh.24183

86. Saraf, SL, Oh, AL, Patel, PR, Jalundhwala, Y, Sweiss, K, Koshy, M, et al. Nonmyeloablative stem cell transplantation with Alemtuzumab/low-dose irradiation to cure and improve the quality of life of adults with sickle cell disease. *Biol Blood Marrow Transplant*. (2016) 22:441–8. doi: 10.1016/j.bbmt.2015.08.036

87. Walters, MC. Update of hematopoietic cell transplantation for sickle cell disease. *Curr Opin Hematol*. (2015) 22:227–33. doi: 10.1097/MOH.0000000000000136

88. Craddock, C. Nonmyeloablative stem cell transplants. *Curr Opin Hematol*. (1999) 6:383. doi: 10.1097/00062752-199911000-00005

89. Krishnamurti, L, Blazar, BR, and Wagner, JE. Bone marrow transplantation without myeloablation for sickle cell disease. *N Engl J Med*. (2001) 344:68. doi: 10.1056/NEJM200101043440119

90. Strocchio, L, Zecca, M, Comoli, P, Mina, T, Giorgiani, G, Giraldi, E, et al. Treosulfan-based conditioning regimen for allogeneic haematopoietic stem cell transplantation in children with sickle cell disease. *Br J Haematol*. (2015) 169:726–36. doi: 10.1111/bjh.13352

91. Gluckman, E, Fuente, J, Cappelli, B, Scigliuolo, GM, Volt, F, Tozatto-Maio, K, et al. Inborn errors working parties of the, the role of HLA matching in unrelated donor hematopoietic stem cell transplantation for sickle cell disease in Europe. *Bone Marrow Transplant*. (2020) 55:1946–54. doi: 10.1038/s41409-020-0847-z

92. Tozatto-Maio, K, Torres, MA, Degaide, NHS, Cardoso, JF, Volt, F, Pinto, ACS, et al. HLA-matched unrelated donors for patients with sickle cell disease: results of international donor searches. *Biol Blood Marrow Transplant*. (2020) 26:2034–9. doi: 10.1016/j.bbmt.2020.07.015

93. Raj, A, Bertolone, SJ, Mangold, S, and Edmonds, HL Jr. Assessment of cerebral tissue oxygenation in patients with sickle cell disease: effect of transfusion therapy. *J Pediatr Hematol Oncol*. (2004) 26:279–83. doi: 10.1097/00043426-200405000-00004

94. Patel, DA, Akinsete, AM, Connelly, JA, and Kassim, AA. T-cell deplete versus T-cell replete haploidentical hematopoietic stem cell transplantation for sickle cell disease: where are we? *Expert Rev Hematol*. (2019) 12:733–52. doi: 10.1080/17474086.2019.1642103

95. Kanakry, CG, Ganguly, S, Zahurak, M, Bolanos-Meade, J, Thoburn, C, Perkins, B, et al. Aldehyde dehydrogenase expression drives human regulatory T cell resistance to posttransplantation cyclophosphamide. *Sci Transl Med*. (2013) 5:211ra157. doi: 10.1126/scitranslmed.3006960

96. Kanakry, CG, Coffey, DG, Towlerton, AM, Vulic, A, Storer, BE, Chou, J, et al. Origin and evolution of the T cell repertoire after posttransplantation cyclophosphamide. *JCI Insight*. (2016) 1:e86252. doi: 10.1172/jci.insight.86252

97. Luznik, L, Jalla, S, Engstrom, LW, Iannone, R, and Fuchs, EJ. Durable engraftment of major histocompatibility complex-incompatible cells after nonmyeloablative conditioning with fludarabine, low-dose total body irradiation, and posttransplantation cyclophosphamide. *Blood*. (2001) 98:3456–64. doi: 10.1182/blood.V98.12.3456

98. Brodsky, RA, Luznik, L, Bolanos-Meade, J, Leffell, MS, Jones, RJ, and Fuchs, EJ. Reduced intensity HLA-haploidentical BMT with post transplantation cyclophosphamide in nonmalignant hematologic diseases. *Bone Marrow Transplant*. (2008) 42:523–7. doi: 10.1038/bmt.2008.203

99. Fitzhugh, CD, Hsieh, MM, Taylor, T, Coles, W, Roskom, K, Wilson, D, et al. Cyclophosphamide improves engraftment in patients with SCD and severe organ damage who undergo haploidentical PBSCT. *Blood Adv*. (2017) 1:652–61. doi: 10.1182/bloodadvances.2016002972

100. de la Fuente, J, Dhedin, N, Koyama, T, Bernaudin, F, Kuentz, M, Karnik, L, et al. Haploidentical bone Marrow transplantation with post-transplantation cyclophosphamide plus Thiotepa improves donor engraftment in patients with sickle cell anemia: results of an international learning collaborative. *Biol Blood Marrow Transplant*. (2019) 25:1197–209. doi: 10.1016/j.bbmt.2018.11.027

101. Bolanos-Meade, J, Cooke, KR, Gamper, CJ, Ali, SA, Ambinder, RF, Borrello, IM, et al. Effect of increased dose of total body irradiation on graft failure associated with HLA-haploidentical transplantation in patients with severe haemoglobinopathies: a prospective clinical trial. *Lancet Haematol*. (2019) 6:e183–93. doi: 10.1016/S2352-3026(19)30031-6

102. Kharya, G, Bakane, A, Agarwal, S, and Rauthan, A. Pre-transplant myeloid and immune suppression, upfront plerixafor mobilization and post-transplant cyclophosphamide: novel strategy for haploidentical transplant in sickle cell disease. *Bone Marrow Transplant*. (2021) 56:492–504. doi: 10.1038/s41409-020-01054-3

103. Aversa, F. Setting the standard in T-cell-depleted haploidentical transplantation and beyond. *Best Pract Res Clin Haematol*. (2011) 24:325–9. doi: 10.1016/j.beha.2011.04.008

104. Reisner, Y. Differential agglutination by soybean agglutinin of human leukemia and neuroblastoma cell lines: potential application to autologous bone marrow transplantation. *Proc Natl Acad Sci U S A*. (1983) 80:6657–61. doi: 10.1073/pnas.80.21.6657

105. Reisner, Y, Kapoor, N, Kirkpatrick, D, Pollack, MS, Dupont, B, Good, RA, et al. Transplantation for acute leukaemia with HLA-A and B nonidentical parental marrow cells fractionated with soybean agglutinin and sheep red blood cells. *Lancet*. (1981) 2:327–31. PMID: 6115110

106. Frame, JN, Collins, NH, Cartagena, T, Waldmann, H, O'Reilly, RJ, Dupont, B, et al. T cell depletion of human bone marrow. Comparison of Campath-1 plus complement, anti-T cell ricin a chain immunotoxin, and soybean agglutinin alone or in combination with sheep erythrocytes or immunomagnetic beads. *Transplantation*. (1989) 47:984–8. doi: 10.1097/00007890-198906000-00013

107. Champlin, RE, Passweg, JR, Zhang, MJ, Rowlings, PA, Pelz, CJ, Atkinson, KA, et al. T-cell depletion of bone marrow transplants for leukemia from donors other than HLA-identical siblings: advantage of T-cell antibodies with narrow specificities. *Blood*. (2000) 95:3996–4003. doi: 10.1182/blood.V95.12.3996

108. Goldman, JM, Gale, RP, Horowitz, MM, Biggs, JC, Champlin, RE, Gluckman, E, et al. Bone marrow transplantation for chronic myelogenous leukemia in chronic phase. Increased risk for relapse associated with T-cell depletion. *Ann Intern Med*. (1988) 108:806–14. doi: 10.7326/0003-4819-108-6-806

109. Kernan, NA, Flomenberg, N, Dupont, B, and O'Reilly, RJ. Graft rejection in recipients of T-cell-depleted HLA-nonidentical marrow transplants for leukemia. Identification of host-derived antidonor allocytotoxic T lymphocytes. *Transplantation*. (1987) 43:842–7. doi: 10.1097/00007890-198743060-00014

110. Dallas, MH, Triplett, B, Shook, DR, Hartford, C, Srinivasan, A, Laver, J, et al. Long-term outcome and evaluation of organ function in pediatric patients undergoing haploidentical and matched related hematopoietic cell transplantation for sickle cell disease. *Biol Blood Marrow Transplant*. (2013) 19:820–30. doi: 10.1016/j.bbmt.2013.02.010

111. Gilman, AL, Eckrich, MJ, Epstein, S, Barnhart, C, Cannon, M, Fukes, T, et al. Alternative donor hematopoietic stem cell transplantation for sickle cell disease. *Blood Advances*. (2017) 1:1215–23. doi: 10.1182/bloodadvances.2017005462

112. Gaziev, J, Isgro, A, Sodani, P, Paciaroni, K, De Angelis, G, Marziali, M, et al. Haploidentical HSCT for hemoglobinopathies: improved outcomes with TCRalphabeta(+)/CD19(+)-depleted grafts. *Blood Adv*. (2018) 2:263–70. doi: 10.1182/bloodadvances.2017012005

113. Foell, J, Kleinschmidt, K, Jakob, M, Troeger, A, and Corbacioglu, S. Alternative donor: alphass/CD19 T-cell-depleted haploidentical hematopoietic stem cell transplantation for sickle cell disease. *Hematol Oncol Stem Cell Ther*. (2020) 13:98–105. doi: 10.1016/j.hemonc.2019.12.006

114. Singh, AK, Schetzen, E, Yadav, SK, Lustig, EB, Liu, WH, Yadav, RK, et al. Correction of murine sickle cell disease by allogeneic haematopoietic cell transplantation with anti-3rd party veto cells. *Bone Marrow Transplant*. (2021) 56:1818–27. doi: 10.1038/s41409-021-01237-6

115. Ophir, E, Or-Geva, N, Gurevich, I, Tal, O, Eidelstein, Y, Shezen, E, et al. Murine anti-third-party central-memory CD8(+) T cells promote hematopoietic chimerism under mild conditioning: lymph-node sequestration and deletion of anti-donor T cells. *Blood*. (2013) 121:1220–8. doi: 10.1182/blood-2012-07-441493

frontiers | Frontiers in Medicine

TYPE Original Research
PUBLISHED 18 April 2023
DOI 10.3389/fmed.2023.1101501

OPEN ACCESS

EDITED BY
Magali J. Fontaine,
University of Maryland,
United States

REVIEWED BY
Wassim El Nemer,
French Blood Establishment (EFS),
France
Roberto Motterlini,
Institut National de la Santé et de la Recherche Médicale (INSERM),
France
Diana Imhof,
University of Bonn,
Germany

*CORRESPONDENCE
John D. Belcher
✉ belcher@umn.edu

†PRESENT ADDRESSES
Domi Stickens,
Arrakis Therapeutics,
Waltham, MA, United States
Maneet Singh,
Wave Life Sciences,
Cambridge, MA,
United States
Margaret Biddle,
Entact Bio,
Watertown, MA,
United States

SPECIALTY SECTION
This article was submitted to Hematology, a section of the journal Frontiers in Medicine

RECEIVED 17 November 2022
ACCEPTED 27 March 2023
PUBLISHED 18 April 2023

CITATION
Belcher JD, Nataraja S, Abdulla F, Zhang P, Chen C, Nguyen J, Ruan C, Singh M, Demes S, Olson L, Stickens D, Stanwix J, Clarke E, Huang Y, Biddle M and Vercellotti GM (2023) The BACH1 inhibitor ASP8731 inhibits inflammation and vaso-occlusion and induces fetal hemoglobin in sickle cell disease. *Front. Med.* 10:1101501. doi: 10.3389/fmed.2023.1101501

The BACH1 inhibitor ASP8731 inhibits inflammation and vaso-occlusion and induces fetal hemoglobin in sickle cell disease

John D. Belcher[1]*, Selvaraj Nataraja[2], Fuad Abdulla[1], Ping Zhang[1], Chunsheng Chen[1], Julia Nguyen[1], Conglin Ruan[1], Maneet Singh[2†], Shilpa Demes[3], Lyndsay Olson[2], Domi Stickens[2†], Jeff Stanwix[2], Emer Clarke[4], Yongzhao Huang[4], Margaret Biddle[2†] and Gregory M. Vercellotti[1]

[1]Department of Medicine, Division of Hematology, Oncology and Transplantation, University of Minnesota, Minneapolis, MN, United States, [2]Mitobridge Inc., Cambridge, MA, United States, [3]Astellas Pharma Global Development Inc., Northbrook, IL, United States, [4]ReachBio, Seattle, WA, United States

In sickle cell disease (SCD), heme released during intravascular hemolysis promotes oxidative stress, inflammation, and vaso-occlusion. Conversely, free heme can also activate expression of antioxidant and globin genes. Heme binds to the transcription factor BACH1, which represses NRF2-mediated gene transcription. ASP8731, is a selective small molecule inhibitor of BACH1. We investigated the ability of ASP8731 to modulate pathways involved in SCD pathophysiology. In HepG2 liver cells, ASP8731 increased *HMOX1* and *FTH1* mRNA. In pulmonary endothelial cells, ASP8731 decreased *VCAM1* mRNA in response to TNF-α and blocked a decrease in glutathione in response to hemin. Townes-SS mice were gavaged once per day for 4weeks with ASP8731, hydroxyurea (HU) or vehicle. Both ASP8731 and HU inhibited heme-mediated microvascular stasis and in combination, ASP8731 significantly reduced microvascular stasis compared to HU alone. In Townes-SS mice, ASP8731 and HU markedly increased heme oxygenase-1 and decreased hepatic ICAM-1, NF-kB phospho-p65 protein expression in the liver, and white blood cell counts. In addition, ASP8731 increased gamma-globin expression and HbF+ cells (F-cells) as compared to vehicle-treated mice. In human erythroid differentiated CD34+ cells, ASP8731 increased *HGB* mRNA and increased the percentage of F-cells 2-fold in manner similar to HU. ASP8731 and HU when given together induced more HbF+ cells compared to either drug alone. In CD34+ cells from one donor that was non-responsive to HU, ASP8731 induced HbF+ cells ~2-fold. ASP8731 and HU also increased *HBG* and *HBA*, but not *HBB* mRNA in erythroid differentiated CD34+ cells derived from SCD patients. These data indicate that BACH1 may offer a new therapeutic target to treat SCD.

KEYWORDS

BACH1, Nrf2, sickle cell disease, hemoglobin F, gamma globin, HMOX1, vaso-occlusion, NF-kappa B

Introduction

Sickle cell disease (SCD) is caused by a point mutation in the β-globin chain of hemoglobin, resulting in a valine substitution for glutamate at position 6 resulting in the mutation of hemoglobin A (HbA, α_2/β_2) to hemoglobin S (HbS, $\alpha_2/\beta^S{}_2$). When deoxygenated during transit through the venous circulation, HbS polymerizes, which leads to the formation of red blood cells (RBCs) that are dehydrated, stiffer, more adhesive, and abnormally shaped (1). Repeated bouts of HbS polymerization and depolymerization as RBCs circulate through the venous and arterial vasculature shortens their lifespan and promotes intravascular and extravascular hemolysis that releases toxic free heme. Free heme promotes oxidative stress, inflammation, and vaso-occlusion (2, 3).

Despite its toxicity, free heme also activates antioxidant and globin gene expression (4–9). Heme binds to BTB and CNC homolog 1 (BACH1), which functions as a transcriptional repressor of nuclear factor erythroid 2-related factor 2 (NRF2) (10). The release of heme-BACH1 from antioxidant response elements (ARE) permits the binding of NRF2 to ARE and the cell-specific transcription of antioxidant genes such as *HMOX1* (heme oxygenase 1, HO-1), *GR* (glutathione reductase), and *NQO1* (NAD(P)H dehydrogenase [quinone] 1). Increasing HO-1 expression has been shown to reduce inflammation, adhesion molecules, and stasis in a mouse model of SCD (11, 12). Additionally, polymorphisms in the *HMOX1* promoter that correspond to increased HO-1 levels have been correlated with reduced incidents of acute chest syndrome in pediatric SCD patients and overall reductions in vaso-occlusive pain crises and hospitalization rates (13, 14).

Previous studies demonstrated increases in nuclear Nrf2 expression and downstream expression of Nrf2-responsive genes including *Hmox1*, *Nqo1*, and *Hbg* (gamma globin) after administration of dimethyl fumarate (DMF) to murine SCD models (15, 16). Importantly, heme-induced microvascular stasis was inhibited in an HO-1 dependent manner (15). In addition, DMF increased the expression of other NRF2-responsive genes including proteins involved in hemoglobin, heme, and iron clearance as well as a decrease in markers of inflammation such as nuclear factor kappa B (NF-κB) phospho-p65, toll-like receptor 4 (TLR4), adhesion molecules, and pro-inflammatory cytokines (3, 15, 16). A decrease in hepatic lesions and increased circulating hemoglobin F (HbF)-containing RBCs (F-cells) was also observed in these studies (3, 15, 16). Conversely, a loss of NRF2 function exacerbates SCD pathophysiology and inhibits HbF expression (17, 18).

ASP8731 (previously known as ML-0207) was identified as a selective small molecule BACH1 inhibitor that relieves BACH1 repression of NRF2 pathways in human and murine cells (19). We investigated the capability of ASP8731 to increase antioxidant and anti-inflammatory gene expression in cell culture, decrease microvascular stasis (vaso-occlusion) and white blood cell (WBC) counts, and induce gamma globin and F-cells in a preclinical murine model of SCD and human CD34 cells during erythroid differentiation.

Materials and methods

Mice

Animal experiments were approved by the Institutional Animal Care and Use Committee at the University of Minnesota. These studies used male and female knock-in Townes-sickle (HbSS) mice ($h\alpha/h\alpha$, $h\gamma^A/h\gamma^A$, $h\beta^S/h\beta^S$) on a 129/B6 mixed genetic background (20). All mice were genotyped, housed in specific pathogen-free rooms to limit infections, and kept on a 12-h (h) light/dark cycle at 21°C. All animals were monitored daily for health problems, food and water levels, and cage conditions. All animals were included in each analysis and there were no adverse events that required changes to the protocol. Mice were 12–16 weeks of age.

ASP8731 and hydroxyurea administration to mice

ASP8731 is a selective small molecule BACH1 inhibitor, which activates antioxidant NRF2-responsive genes (19). We examined the cytoprotective effects of ASP8731 on human cells and in Townes HbSS mice. Mice were gavaged (10 ml/kg) once daily for 14 days consecutively or 6 days per week for 4 weeks with the indicated dose of ASP8731 or hydroxyurea (HU) suspended in 0.05% w/v Tween 80 + 0.45% hydroxypropyl methylcellulose (HPMC).

Tissue collection

At the end of each experiment, mice were euthanized in a CO_2 atmosphere and blood was collected from the inferior vena cava into EDTA tubes and processed at 4°C. Livers were excised, flash frozen in liquid N_2, and stored at -85°C until use.

HepG2 cell culture and gene expression

Human hepatoma cells (HepG2, ATCC HB-8065) were grown in Minimum Essential Media (EMEM Corning #MT-10-010-CV) supplemented with 1X MEM Non-essential Amino Acids Solution (Gibco 100X Solution #11140–50), 1 mM sodium pyruvate (Gibco 100 mM solution, #MT-25-000-CI) and 10% heat-inactivated fetal bovine serum (Gibco #16140–071). Cells were spilt 2–3 times weekly at 1:4 or 1:6. HepG2 cells were plated at a density of 250 k cells in 12-well plates coated with collagen (ENZO Life Sciences, Cat#ALX-522-440-0050). Approximately 24 h post-seeding, cells were treated with a dose titration of ASP8731 or DMF (Fisher Scientific Cat#50–144-5120) as a positive control *via* media change at three biological replicates per treatment. Compounds were dissolved in 0.1% DMSO, untreated wells had the same concentration of DMSO to account for any effect of the vehicle. At 24 h post-treatment, cells were washed in PBS and frozen (-80°C) before RNA isolation and analysis as described below. Further, in independent experiment, cells were checked for treatment toxicity by MTT assay (no toxicity observed- data not shown).

PAEC cell culture and gene expression

Human primary pulmonary arterial endothelial cells (PAEC, Lonza CC-2530) were grown in EGM-2 media (Lonza CC-3162). Cells were sub-cultured when they reached 70–85% confluence. Cells were seeded at 5 K viable cells/cm^2. HPAEC were plated at 200 K/cells per well on 12-well plates (TrueLine #TR5001) in growth

media and cultured at 37°C in 5% CO_2. Approximately 24 h post-seeding, cells were treated with dose titration of ASP8731 in 0.1% DMSO *via* media change, control cells had the same amount of DMSO ($n = 3$ replicates per treatment). At 24 h post-treatment with ASP8731, TNF-α (10 ng/ml, Invitrogen) or hemin (50 μM, Sigma-Aldrich) was added to the media and the cells were incubated an additional 4 h (TNF-α) or 30 min (hemin). Treatment of 50 uM hemin for 30 min was determined to be the minimal dose that elicits a decrease in GSH, and is within the physiological levels reported in SCD and thalassemia patients. After treatment was complete, media was aspirated off, cells were washed with PBS and aspirated dry. For the TNF-α experiments, RNA was isolated and *VCAM1* mRNA was measured by Nanostring gene expression analysis as described below. For the hemin experiments, cellular glutathione (GSH) was measured using a GSH-Glo™ glutathione assay kit (Promega) following the manufacturer's protocol. Cell viability was observed by MTT assay as described by the manufacturer (Sigma-Aldrich, Cat# CT02).

CD34 cell isolation, expansion, and differentiation

Six normal human bone marrow (BM) samples from 2 African Americans, 3 Hispanics, and 1 Caucasian were obtained from three commercial sources (Lonza, NorCal Biologics, and iSpecimen). SCD subject whole blood (~50 ml/subject) was obtained from Sanguine Biosciences ($n = 3$ subjects). The whole blood was diluted 2-fold with PBS containing 2% FBS and then layered on ficoll for peripheral blood mononuclear blood cell (PBMC) isolation using density gradient centrifugation. The whole blood was centrifuged at 1200 rpm for 25 min and PBMC were harvested and counted. PBMC and BM derived CD34+ cells were enriched using Miltenyi's CD34+ microbeads (positive selection method). BM derived $CD34^+$ cells were stored in liquid nitrogen (−152°C) until required for HbF induction. On the day of the experiment, CD34+ cells were thawed in Iscove's modified Dulbecco's medium (IMDM, Hyclone) containing 10% heat-inactivated fetal bovine serum (FBS) and then cells were pelleted by centrifugation. The cell pellets were re-suspended in X-Vivo medium (Lonza) and viability was assessed with trypan blue. $CD34^+$ cells from SCD whole blood were cultured soon after isolation. $CD34^+$ cells were expanded in X-Vivo media containing rhIL-3 (10 ng/ml), rhSCF (100 ng/mL) and rhFlt-3 ligand (100 ng/mL). Approximately 10,000–12,500 cells in 250 μL volume were plated per well in round-bottom 96-well plates and culture for 7 days in a humidified incubator at 37°C/5% CO_2. After 7 days, the cells were washed twice with X-Vivo media and resuspended in freshly prepared erythroid differentiation media containing X-Vivo-15, rhIL-3 (10 ng/mL), rhSCF (100 ng/mL), and Epo (3 U/mL). BACH1 inhibitor ASP8731 (1, 3, 10 μM) and/or HU (3, 10 μM) positive control for HbF induction were added either alone or in combination to the media. Standard cultures (solvent and compound free) and solvent control cultures (containing DMSO) were also initiated. Each test condition was set up in triplicate. Following culture in differentiation media for 7 days, the induction of HbF was assessed by flow cytometry. In addition, cells were collected on days 3 or 7 of differentiation for gene expression analysis. RNA from erythroid differentiated CD34 cells was isolated and analyzed for gene expression as described below.

mRNA analysis

RNA was isolated using Machery-Nagel isolation kit (NucleoSpin RNA, Cat#740955.250) and quantified using a NanoDrop spectrophotometer (ThermoFisher Scientific). Isolated RNA was analyzed on a NanoString SPRINT profiler and nSolver version 4.0 software. A custom Tag-24 CodeSet and exploratory gene expression biomarker probes, were hybridized with a total mRNA input of 200 ng. To hybridize the probes to the target genes of interest, a thermocycler block was set to 67C (with the lid set to 72C) and samples were incubated overnight for a total of 16 h. The samples were cooled to 4C at the end of 16 h and remained at 4C until samples were pooled and added to wells of a SPRINT cartridge. Positive and negative controls were assessed for run QC, ensuring linearity and ample assay limit of detection, respectively. Raw data from the reference genes in study samples were assessed for stability across the experiment utilizing the GeNorm protocol for assessment of expression. Reference genes that exhibited differential expression ie oscillated randomly or excessively above/below the experiment geometric mean were excluded from content normalization calculations. The limit of detection for each sample was determined as the average of negative controls in each sample plus two times the standard deviation of the negative controls within the sample; the experimental limit of detection was calculated as the geometric mean of all individual limit of detection calculations across the experiment. CLTC, POLR2A, RPL27, and TBP were used as reference genes for normalization. Data was graphed as fold-change compared to DMSO treatment. Probe Sequences are listed in Supplementary Table 1.

Measurement of microvascular stasis (vaso-occlusion)

After 4 weeks treatment with ASP8731 or HU, HbSS mice were anesthetized with ketamine and xylazine and dorsal skinfold chambers were implanted (21). Flowing subcutaneous venules (20–23 venules/mouse) in the chamber window were selected and mapped using intravital microscopy as previously described (21). After selection of flowing venules, mice were infused with a bolus infusion *via* tail vein with hemin (3.2 μmol/kg). One hour after hemin infusion, the venules were visually re-examined for stasis (no flow). The static venules in each mouse were counted and percent stasis at 1 h was calculated by dividing the number of static venules by the total (static + flowing) number of venules.

Western blots

Hepatic microsomes and nuclear extracts were isolated from frozen organs as previously described (12). Immunoblots of cellular subfractions (30–50 μg protein) were run on 4–20% SDS PAGE gels (Bio-Rad #3450033), transferred to PVDF membranes, and immunostained with primary antibodies to NF-κB phospho-p65 (Ser536, Cell Signaling #3031), total p65 (Cell Signaling #3034), ICAM-1 (Abcam #ab124759), HO-1 (Enzo #ADI-111), BACH1 (US Biological Life Sciences #220980), or GAPDH (Sigma-Aldrich #G9545). Primary antibodies were detected with suitable secondary antibodies conjugated to alkaline phosphatase and visualized with

ECF substrate (GE Healthcare) and a Typhoon FLA 9500 imager (GE Healthcare).

White blood cell (WBC) counts

EDTA blood was diluted 1:20 or 1:40 in 3% glacial acetic acid to lyse RBCs. WBCs were counted manually using a hemocytometer and a light microscope.

HbF by FACS

Differentiated CD34+ cells collected at day 7 of erythroid differentiation were washed with PBS. For flow cytometry analysis, non-specific binding of antibodies was blocked with 10% FBS with human IgG (20 μg/mL) at 4°C for 20 min. Following blocking, the surface markers of the cells were stained with an antibody cocktail containing CD235a-PE/Cy7, and CD71-APC at 4°C for 30 min. The cells were washed with PBS, re-suspended in 1x Cytofix/Cytoperm solution (BD Biosciences), and fixed for 20 min at 4°C. Following fixation, cells were washed with Perm/Wash Buffer (BD Biosciences) and stained with anti-HbF-PE for 30 min at room temperature. For isotype control, IgG-PE was used in place of HbF-PE. Finally, the cells were washed twice with Perm/Wash Buffer, resuspended in PBS containing 2% FBS, and then analyzed by flow cytometry using a Beckman Coulter CytoFlex cytometer. Both percentages of various cell populations as well as events (cells) per μL were collected. The HbF^+ cells were found within the $CD71^+$ bright cell population. HU was used as a positive control throughout the experiment.

Statistical analysis

Comparisons of multiple treatment groups were made using one-way ANOVA with Tukey's or Dunnett's multiple comparison test (GraphPad Prism version 9.4). Normality of data was assessed using the Shapiro–Wilk test. Non-normally distributed data was analyzed for differences between treatment groups using the Kruskal-Wallis test with Dunn's multiple comparisons test.

Results

Effects of ASP8731 on antioxidant and anti-inflammatory genes *in vitro*

Since BACH1 represses NRF2-responsive pathways in cells, we investigated the ability of ASP8731 to modulate antioxidant and anti-inflammatory genes in cell culture. HEPG2 cells were treated with increasing concentrations of the BACH1 inhibitor ASP8731 (0.1–50 μM) or DMF (1–250 μM), a positive control for NRF2 gene transcription, for 24 h. ASP8731 potently induced HO-1 (*HMOX1*) and ferritin heavy chain (*FTH*) mRNA in HEPG2 cells, even more potently than DMF (Figures 1A,B). Human primary pulmonary arterial endothelial cells (PAEC) were incubated with ASP8731 (0, 1, 3, 10 μM) or DMSO vehicle for 24 h and then TNF-α (10 ng/ml), was added for an additional 4 h followed by RNA isolation and measurement of *VCAM1* mRNA. *VCAM1* mRNA was maximally increased in TNF-α-treated cells in the absence of BACH1 inhibitor ASP8731 and decreased as the concentration of ASP8731 increased (Figure 1C). PAEC were incubated with DMSO vehicle or ASP8731 (0, 1, or 3 μM) for 24 h and then cells were exposed to heme-mediated oxidative stress. Hemin (50 μM) was added for an additional 30 min followed by measurement of glutathione (GSH) levels as a marker of oxidative stress. GSH levels were decreased in hemin-treated cells in the absence of BACH1 inhibitor ASP8731 and increased as the concentration of ASP8731 increased (Figure 1D). The effects of treatments on HepG2 and PAEC viability were measured by MTT assay. ASP8731 at the doses tested did not affect the viability of HepG2 cells or HPAEC cells in the presence of DMSO or hemin.

Microvascular stasis (vaso-occlusion) in HbSS mice

Since activation of NRF2-responsive genes has been shown to be beneficial in SCD mice, BACH1 inhibition was also evaluated. Townes HbSS mice were gavaged once daily, 6 days per week for 4 weeks with vehicle (VEH), ASP8731 (1, 3, 25 mg/kg body weight), hydroxyurea (HU, 100 mg/kg), or ASP8731 (25 mg/kg) + HU (100 mg/kg). On the last day of treatment, dorsal skinfold chambers were implanted on the mice. Flowing venules (20–23 venules/mouse) in the chamber window were selected and mapped using intravital microscopy. Mice were then infused with hemin (3.2 μmol/kg body weight) *via* the tail vein. One hour after hemin infusion, the venules were visually re-examined for stasis (no flow). The static venules in each mouse were counted and percent stasis at 1 h was calculated by dividing the number of static venules by the total (static + flowing) number of venules (Figure 2A). ASP8731 inhibited hemin-induced stasis in a dose responsive manner with the highest stasis occurring in HbSS mice pretreated with vehicle (33%) and decreasing to 26, 21, and 7% stasis in mice treated with 1, 3, and 25 mg/kg ASP8731, respectively. Treatment with HU (100 mg/kg) decreased stasis to 13%, with the lowest stasis (3.5%) seen in mice treated with ASP8731 (25 mg/kg) + HU (100 mg/kg).

White blood cell counts in HbSS mice

After stasis measurements, blood samples were collected 4 h after the infusion of hemin. White blood cells (WBC) were counted manually in whole blood. WBC counts decreased in a manner similar to stasis (Figure 2B). ASP8731 significantly inhibited WBC counts in a dose-responsive manner with the highest WBC counts occurring in HbSS mice pretreated with vehicle (28×10^3/μL) and decreasing to 22×10^3/μL, 18×10^3/μL, and 13×10^3/μL in mice treated with 1, 3, and 25 mg/kg ASP8731, respectively. Treatment with HU (100 mg/kg) decreased the WBC count to 11×10^3/μL. Treatment with ASP8731 (25 mg/kg) + HU (100 mg/kg) decreased the WBC count to 12×10^3/μL.

Red blood cell indices in HbSS mice

There were no significant differences in any of the red blood cell indices, including red blood cell counts, hemoglobin levels, hematocrits, and reticulocyte counts in any of the treatment groups as compared to

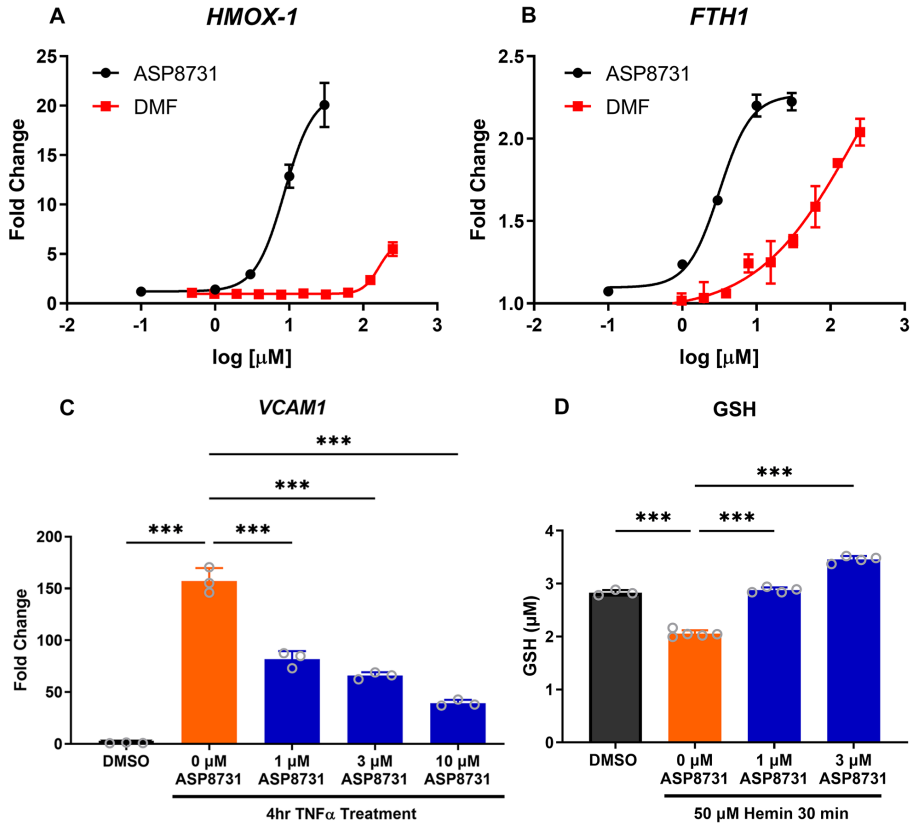

FIGURE 1
BACH1 inhibitor ASP8731 modulated multiple pathways *in vitro* that are involved in SCD pathophysiology. ASP8731, dimethyl fumarate (DMF), or DMSO vehicle were incubated with HepG2 cells for 24h and **(A)** *HMOX1* (HO-1) and **(B)** *FTH* (ferritin heavy chain) mRNA were measured. **(C)** Human primary pulmonary arterial endothelial cells (PAEC) were incubated with ASP8731 (1, 3, 10μM) or DMSO vehicle for 24h and then TNF-α (10ng/ml), was added for an additional 4h followed by RNA isolation and measurement of *VCAM1* mRNA. **(D)** PAEC were incubated with DMSO vehicle or ASP8731 (0, 1, or 3μM) for 24h and then hemin (50μM) was added for an additional 30min followed by measurement of glutathione (GTH) levels. Values are means ± SEM. ***p <0.001, one-way ANOVA with Tukey's multiple comparison test.

vehicle treated HbSS mice (Supplementary Figure 1). In addition, the spleen weights as a percent of body weight were also not significantly different between treatment groups suggesting that BACH1 inhibition did not significantly inhibit hemolysis in HbSS Townes mice.

Hepatic HO-1, NF-κB, ICAM-1, and BACH1 expression in HbSS mice

In a separate cohort of HbSS mice, we examined the effects of ASP8731 and HU on HO-1, NF-κB phospho-p65 and ICAM-1 expression in the liver. HbSS mice ($n = 3$ mice/treatment, 2 males and 1 female) were gavaged once daily for 14 consecutive days with vehicle (VEH), ASP8731, or HU at the indicated doses. On the last day of treatment, mice were infused with hemin (3.2 μmol/kg) *via* the tail vein. Livers were collected 4 h after the infusion of hemin. Hepatic HO-1, NF-κB phospho-p65, and ICAM-1 proteins were analyzed on Western blots (Figures 3A–C) Relative expression of the bands on the Western blots were quantified using densitometry (Supplementary Figure 2). Microsomal HO-1 was significantly increased in the livers of HbSS mice treated with ASP8731 (25 mg/kg), HU (100 mg/kg), and ASP8731 (25 mg/kg) + HU (100 mg/kg). Nuclear NF-κB phospho-p65, a marker of NF-κB activation, was significantly decreased in the livers of HbSS mice at all treatment doses of ASP8731 and HU. Microsomal ICAM-1 was also significantly decreased in livers at all doses of ASP8731 and HU. Treatment of HbSS mice with ASP8731 increased nuclear BACH1 expression in the liver (Supplementary Figure 3). Similarly, HU also increased nuclear BACH1 protein expression.

Hepatic heme oxygenase activity in HbSS mice

In a separate set of studies, ASP8731 dose responsively increased heme oxygenase (HO) activity in the livers of HbSS mice (Supplementary Figure 4). HU also increased HO activity to a somewhat lesser degree than ASP8731, but the highest HO activity was measured in mice treated with ASP8731 + HU.

Gamma globin and HbF expression in HbSS mice

BACH1 inhibitor ASP87311 was examined for its ability to increase expression of gamma globins and HbF-containing F-cells.

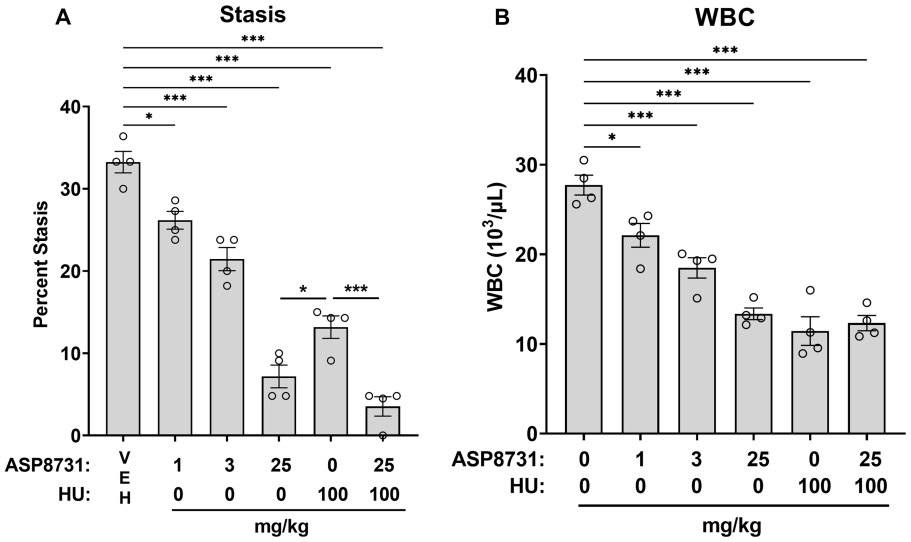

FIGURE 2
Bach1 inhibitor ASP8731 decreases microvascular stasis and WBC counts in Townes HbSS mice. Townes HbSS mice were gavaged once daily, 6days per week for 4weeks with vehicle (VEH), ASP8731, and/or HU at the indicated doses (n=4 mice/group, 2 male and 2 female). On the last day of treatment, mice were implanted with dorsal skinfold chambers and infused with hemin (3.2μmol/kg body weight). Flowing venules (20–24 venules/mouse) in the chamber window were selected and mapped using intravital microscopy prior to hemin infusion. **(A)** Microvascular stasis was measured in the same venules 1h after hemin infusion. **(B)** WBC counts were measured in blood 4h after hemin infusion. Individual values are presented as circles with bars representing the means ± SEM; *p <0.05 and ***p <0.001, one-way ANOVA with Tukey's multiple comparison test.

Gamma and beta-S globin levels were measured by reverse phase HPLC in the red blood cells of Townes HbSS mice after treatment with ASP8731 (3 or 25 mg/kg) or vehicle once daily for 14 days. The ratio of gamma globin to gamma globin + beta-S globin increased from a mean of 5.8% in vehicle treated HbSS mice to 7.8 and 8.3% in HbSS mice treated with 3 and 25 mg/kg ASP8731, respectively (Figure 4A). Similarly, HbF containing F-cells measured by FACS significantly increased from 1.0% of red blood cells in vehicle-treated HbSS mice to 1.8 and 2.1% in HbSS mice treated with 3 and 25 mg/kg ASP8731, respectively (Figure 4B).

HGB mRNA and F-cells in human erythroid differentiated CD34 cells

The effects of BACH1 inhibitor ASP8731 and HU on gamma globin gene (*HBG*) mRNA levels and F-cell production were measured in human BM-derived CD34 cells during erythroid differentiation. HGB mRNA was significantly increased in CD34 cells differentiated in the presence of ASP8731 at 0.3 and 1 μM or in the presence of HU at 3 and 10 μM (Figure 4C) as compared to the DMSO vehicle (VEH). The percent F-cells was significantly increased in CD34 cells differentiated in 1 μM ASP8731 or 10 μM HU (Figure 4D; Supplementary Table 2). Differentiation of cells to CD71+ cells (reticulocyte marker) was not affected by treatment with HU or ASP8731 (Supplementary Table 2). In preliminary time course study, incubation of compounds for different time (days, 3, 7, and 10) did not change the % CD71+ cells (data not shown). F-cells were increased by both ASP8731 and HU individually, but the combination of both compounds increased F-cells higher than either drug alone (Figure 4E). In one healthy CD34 donor's cells that were non-responsive to HU, ASP8731 was able to induce F-cells at 1 and 3 μM (Figure 4F).

Gamma-, alpha-, and beta-globin in erythroid differentiated CD34 cells derived from SCD subjects

The effects of BACH1 inhibitor ASP8731 and HU were examined in erythroid differentiated CD34 cells derived from the peripheral blood of SCD subjects (Figure 5). Levels of (A) gamma globin (*HBG*), (B) alpha globin (*HBA*), (C) beta globin (*HBB*), (D) HO-1 (*HMOX1*), (E) NAD(P)H quinone dehydrogenase 1 (*NQO1*), (F) glutamate-cysteine ligase modifier subunit (*GCLM*), (G) solute carrier family 7 member 11 (*SLC7A11*), and (H) solute carrier family 48 member 1 (*SLC48A1*) mRNA levels were measured using Nanostring. ASP8731 (10 μM) and the combination of ASP8731 (10 μM) + HU (10 μM) significantly increased the mRNA of every gene tested except *HBB* and *HMOX1*. The lack of statistical difference in HMOX1 gene was primarily due to very high level of expression in 1 patient sample, thus fold change at 3, 1, and 0.3 μM ASP8731 and 10 μM HU were 38.16 ± 41.5; 3.96 ± 1.28, 1.55 ± 0.8, and 1.51 ± 0.3, respectively. In contrast HU alone at 10 μM concentration did not significantly increase the mRNA of any of the genes tested. These data suggest that the BACH1 inhibitor ASP8731 is a potent inducer of HbF and antioxidant response genes in SCD patients.

Discussion

BACH1 inhibitor ASP8731 potently modulated multiple pathways that affect SCD pathophysiology. In HepG2 liver cells, ASP8731 increased *HMOX1* and *FTH1* mRNA. In pulmonary endothelial cells, ASP8731 decreased *VCAM1* mRNA in response to TNF-α and prevented a decrease in glutathione in response to hemin. BACH1 has

10.3389/fmed.2023.1101501

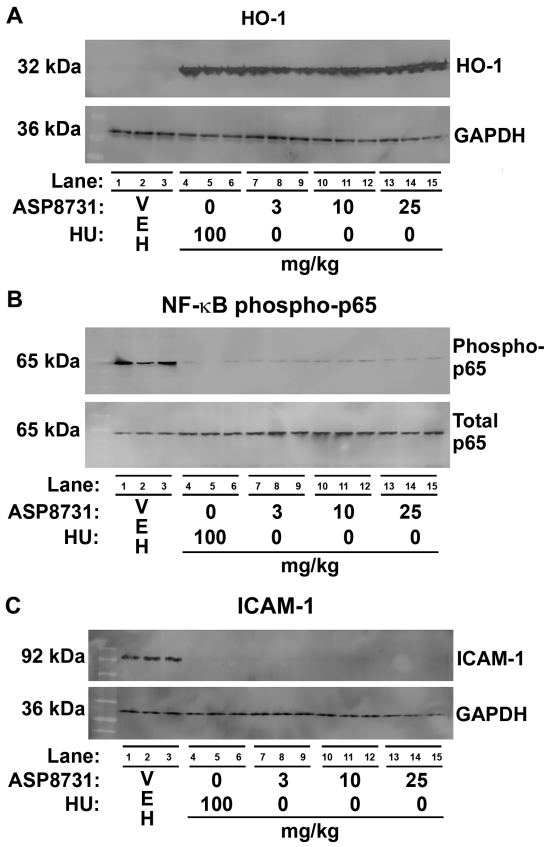

FIGURE 3
Bach1 inhibitor ASP8731 had anti-inflammatory properties in Townes HbSS mice. Townes HbSS mice (n =3 mice/treatment, 2 males and 1 female) were gavaged once daily for 14 consecutive days with vehicle (VEH), ASP8731, or HU at the indicated doses. On the last day of treatment, mice were infused with hemin (3.2μmol/kg). Livers were excised 4h after the infusion of hemin. Proteins in liver were analyzed by Western blots. **(A)** HO-1 and GAPDH. **(B)** NF-κB phospho-and total p65. **(C)** ICAM-1 and GAPDH using liver microsomes **(A,C)** and nuclear extracts **(B)**.

been reported to repress transcription of genes that reduce labile iron, oxidative stress, and ferroptosis (22).

Both ASP8731 and HU markedly inhibited heme-mediated microvascular stasis in a dorsal skin-fold chamber model in HbSS mice while increasing hepatic HO-1 expression. HO-1 induction might have had a large impact on the inhibition of microvascular stasis in HbSS mice in part due to the many anti-inflammatory effects of HO-1 (11, 12). Markers of inflammation including ICAM-1 and NF-kB phospho-p65 expression in the liver and blood WBC counts were significantly decreased by ASP8731 treatment as compared to vehicle controls. Inhibition of HO-1 activity with tin protoporphyrin has been previously shown to abrogate the beneficial effects of NRF2 activation on microvascular stasis (15). The potent anti-inflammatory effects of HO-1 are related in part to the release of carbon monoxide and biliverdin and the post-transcriptional induction of ferritin heavy chain (*FTH1*) by iron generated by HO-1-mediated heme degradation (11, 23, 24).

Paradoxically, treatment of HbSS mice with ASP8731 increased nuclear BACH1 expression in the liver, suggesting a there is a feedback loop that increases nuclear BACH1 protein expression upon inhibition of BACH1. Similarly, HU also increased nuclear BACH1 protein expression. HU is a known nitric oxide (NO) donor (25). The ability of NO alone or in combination with heme to induce Nrf2/HO-1 has been reported (26).

Microvascular stasis was measured in the subcutaneous venules in the skin, but we did not directly measure inflammatory markers in the skin. However, it has previously been shown that inflammation in HbSS mice is occurring in blood vessels throughout the vasculature including the skin, liver, kidneys, and lungs (3, 11, 15, 27). We measured hepatic HO-1 expression and activity in HbSS mice, but not in other organs. We previously showed that genetic overexpression of hepatic HO-1 has systemic effects on microvascular stasis in the distal skin (12). It is likely that ASP8731 had anti-inflammatory effects in all tissues as evidenced by the significant reduction in the WBC counts.

HU induced HO-1 to a similar degree as ASP8731 in HbSS mice. To our knowledge, this is the first time HU has been reported to increase HO-1 expression. Lanaro and colleagues (28) reported increased *HMOX1* mRNA in the mononuclear cells and neutrophils of SCD patients. In that study, SCD patients taking HU had a trend toward higher *HMOX1* mRNA than SCD patients not on HU, but this difference did not appear to be significant. HU increases nitric oxide (NO) production, acting as both a NO donor and a stimulator of NO synthase (25, 29). Bioactivation of NO is a plausible mechanism for *HMOX1* induction by HU as NO can activate KEAP1/NRF2 signaling (30).

Clinical studies have shown that increasing HbF levels with HU inhibits the polymerization of HbS, decreases the severity of many clinical features of SCD, and improves survival (31–33). SCD patients with hereditary persistence of F-cells have a more benign clinical course (34). Given the modest increase in F-cells in HbSS mice treated with ASP8731 (0.8% F-cells in vehicle treated mice compared to 1.8 and 2.1% in mice treated with 3 mg/kg and 25 mg/kg of ASP8731, respectively), F-cell induction is probably not a major factor in the anti-vaso-occlusion protection seen in HbSS mice. Humans at birth express 2 gamma-globin genes (*HBG1* and *HBG2*). *HBG1* codes for A-gamma ($^{A}\gamma$)-globin and *HBG2* codes for G-gamma ($^{G}\gamma$)-globin. The 2 globin chains differ by one amino acid, with ($^{A}\gamma$) having an alanine and ($^{G}\gamma$) having a glycine at position 136. Two gamma globin chains with two alpha (α)-globin chains form HbF (α_2/γ_2), which is normally replaced by HbA shortly after birth. Townes HbSS mice have the human *HBG1* gene, but not the human *HBG2* gene, and thus they can only express $^{A}\gamma$-globin. Murine *HBB* genes in the Townes model were replaced by human *HBG1* and *HBBS* genes and some proximal but not some distal gene-regulatory elements. The HbSS Townes humanized knock-in mouse model recapitulates human globin gene switching (35). HU suppresses erythropoiesis in Townes HbSS mice to create an early persistent F-cell phenotype without re-activating γ-globin transcription (35). Townes mouse HSCs, in contrast to human HSCs, fail to induce HbF to therapeutic levels after genetic disruption of the BCL11A binding site in the *HBG1* promoter (36). This is likely due to missing distal regulatory elements of the globin genes in the Townes model (36). Thus, interpretation of HbF induction in Townes HbSS mice has limitations.

BACH1 and BACH2 support erythropoiesis by regulating heme metabolism in committed erythroid cells, driving cells toward erythroid differentiation and away from myeloid differentiation (37) by repressing C/EBPβ, a transcription factor regulating myelopoiesis

10.3389/fmed.2023.1101501

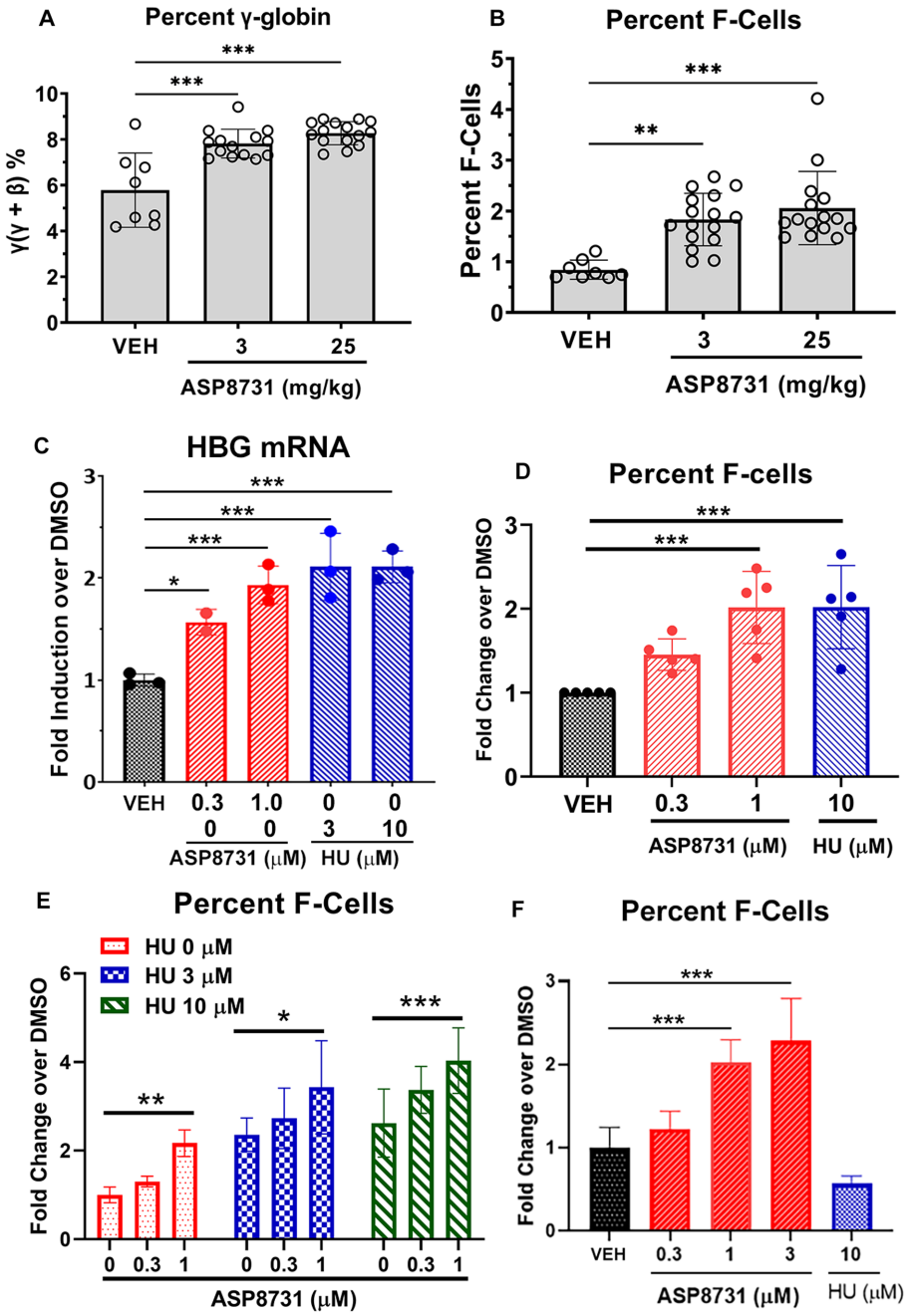

FIGURE 4
BACH1 inhibitor ASP8731 induces gamma globin and F-cells in HbSS mice and human erythroid differentiated CD34 cells. Townes HbSS mice (*n* =8–16 mice/treatment, equal males and females) were gavaged once daily for 14 consecutive days with vehicle (VEH, *n* =8 mice) or ASP8731 at 3mg/kg (*n* =16 mice) or 25mg/kg (*n* =16 mice). Whole blood was collected on the last day of treatment and the red blood cells (RBC) were lysed in water and membrane debris was moved by centrifugation and filtration. **(A)** Gamma (ɣ)-A and beta (β)-S globins in the lysed RBC supernatants were measured by reverse phase HPLC and expressed as percent gamma-globin (ɣ/ɣ +β). **(B)** HbF containing F-cells in whole blood were measured by FACS and expressed as percent F-cells [(HbF+ and Ter119+)/total RBC (Ter119+)]. **(C–F)** Human BM derived CD34+ cells were expanded (days 1–7) and differentiated (days 7–14) to erythroid cells in cell culture. Cells were differentiated in the presence of the indicated concentrations of ASP8731, HU, or DMSO (VEH) on days 7–14. Cells were collected for *HBG* mRNA or percent F-cell FACS analysis on day 14. **(C)** *HBG* mRNA was measured by Nanostring analysis (*n* =3 subjects). **(D–F)** Percent F-cells were measured by FACS. Percent F-cells [(CD71+ and HbF+)/(CD71+)] was calculated and expressed as fold change over DMSO (VEH)-treated cells. **(D)** Values are mean±SD of 5 subjects. **(E)** Values are the mean of 3 subjects measured in triplicate. **(F)** In one subject's CD34 cells, HbF was not increased in response to HU, but these cells were responsive to ASP8731. Mean±SD of triplicate measurements from one donor. **p* <0.05, ***p* <0.01, and ****p* <0.001, one-way ANOVA with Tukey's multiple comparison test.

and inflammation. Heme-BACH1 interaction also plays an important role in globin gene expression. The binding of heme to CP motifs in BACH1 inhibits BACH1 repressor activity. The human globin gene cluster spans ~70 kb and contains 5 globin genes including ϵ, γ_G, γ_A, δ, and β that are controlled by the microlocus control region (μLCR) (38). BACH1 forms heterodimers with small

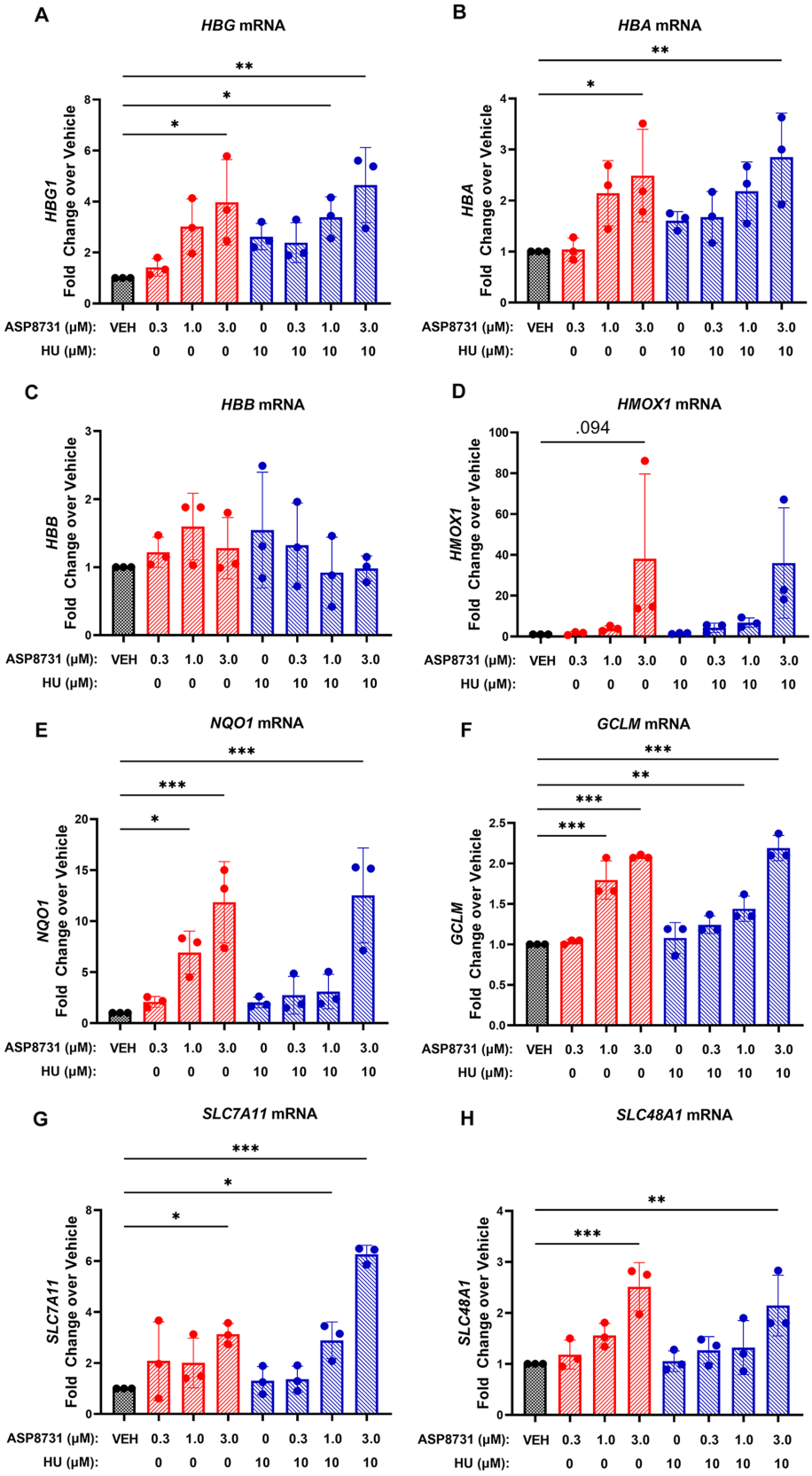

FIGURE 5
BACH1 inhibitor ASP8731 induces gamma-and alpha-globin, but not beta-globin in erythroid differentiated CD34 cells derived from SCD subjects. Peripheral blood CD34+ cells isolated from SCD subjects (n =3) were expanded (days 1–7) and differentiated (days 7–10) to erythroid cells in cell culture. Cells were differentiated in the presence of the indicated concentrations of ASP8731, HU, or DMSO (VEH) on days 7–10. Cells were collected

(Continued)

FIGURE 5 (Continued)
for mRNA analysis by Nanostring on day 10. **(A)** *HBG* mRNA; **(B)** *HBA* mRNA; **(C)** *HBB* mRNA – the probes used for Nanostring for *HBB* do not distinguish between normal and sickle; **(D)** *HMOX1* mRNA; **(E)** *NQO1* mRNA; **(F)** *GCLM* mRNA; **(G)** *SLC7A11* mRNA; and **(H)** *SLC48A11* mRNA. Values are expressed as fold change over DMSO vehicle and bars are mean±SD. *p <0.05, **p <0.01, and ***p <0.001 compared to vehicle, one-way ANOVA followed by Dunnett's multiple comparison test.

Maf proteins that repress globin expression by binding to Maf recognition elements (MARE) in the μLCR (9). Heme increases globin gene expression by binding to BACH1 and blocking the interaction of BACH1 with MARE regions in the μLCR (38, 39). A role for BACH1 in the control of gamma globin expression has been suggested, but not shown directly (40, 41). Our studies indicate that BACH1 inhibition by ASP8731 induces gamma-globin and HbF expression in human erythroid cells.

In BM derived CD34+ cells from normal subjects, BACH1 inhibitor induced *HBG* mRNA and percent F-cells. ASP8731 and heme alleviate BACH1 repression, leading to activation of NRF2-responsive genes. Activation of NRF2 promotes $^{A}\gamma$- and $^{G}\gamma$-globin expression in erythroid cells (42–46), which increase HbF expression in red blood cells of SCD patients. Interestingly, ASP8731 + HU potentiated the induction of F-cells over HU alone. In one donor's CD34 cells that were non-responsive to HU, ASP8731 was able to induce F-cells. The effectiveness of HU to induce HbF is variable in SCD patients and there are concerns over the long-term efficacy of HU (31, 47–49). Induction of F-cells in an HU non-responder by BACH1 inhibition, albeit a single subject, suggests this therapeutic might be an option for patients who are not responsive to HU. During differentiation of CD34+ cells from SCD patients, BACH1 inhibition increased the expression of *HBG*, *HBA* and other NRF2 responsive genes.

In conclusion, BACH1 inhibitors are promising therapeutics for SCD that enhance the cytoprotective oxidative stress responses and thereby reduce inflammation and vaso-occlusion. BACH1 inhibitors have the added benefit of inducing HbF expression and thereby potentially reducing HbS polymerization and hemolysis. The present data support evaluation of BACH1 inhibitors as therapeutics for SCD patients.

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 animal study was reviewed and approved by University of Minnesota's Institutional Animal Care and Use Committee.

Author contributions

JB wrote the manuscript, analyzed data, oversaw the studies in mice, and prepared figures. FA, CC, PZ, JN, and CR conducted mouse experiments and data collection, processing, and analysis. GV analyzed data and edited the manuscript. JS, LO, EC, and YH planned and conducted cell culture experiments, data collection, processing, and analysis, and prepared figures. SD, MS, MB, DS, and SN: conception, study design and data interpretation. All authors contributed to the article and approved the submitted version.

Funding

This study was funded by Astellas Pharma Inc. and investigators received funding to conduct this study from the study sponsor.

Conflict of interest

JB and GV are consultants and receive research funding from Astellas Pharma/Mitobridge. SN, SD, LO, and JS are employees of Astellas Pharma US Inc. MS, DS, and MB were employees of Astellas Pharma US Inc./Mitobridge at the time the study was conducted. EC and YH were employed by company ReachBio.

The remaining 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.

The authors declare that this study received funding from Astellas-Mitobridge. The funder had the following involvement: study design, data collection, analysis, interpretation of data, and the writing of this article.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2023.1101501/full#supplementary-material

References

1. Telen MJ. Beyond hydroxyurea: new and old drugs in the pipeline for sickle cell disease. *Blood*. (2016) 127:810–9. doi: 10.1182/blood-2015-09-618553

2. Charache S, Dover GJ, Moore RD, Eckert S, Ballas SK, Koshy M, et al. Hydroxyurea: effects on hemoglobin F production in patients with sickle cell anemia. *Blood*. (1992) 79:2555–65. doi: 10.1182/blood.V79.10.2555.2555

3. Belcher JD, Chen C, Nguyen J, Milbauer L, Abdulla F, Alayash AI, et al. Heme triggers TLR4 signaling leading to endothelial cell activation and vaso-occlusion in murine sickle cell disease. *Blood*. (2014) 123:377–90. doi: 10.1182/blood-2013-04-495887

4. Ogawa K. Heme metabolism in stress response. *Nihon Eiseigaku Zasshi*. (2002) 56:615–21. doi: 10.1265/jjh.56.615

5. Sun J, Hoshino H, Takaku K, Nakajima O, Muto A, Suzuki H, et al. Hemoprotein Bach1 regulates enhancer availability of heme oxygenase-1 gene. *EMBO J*. (2002) 21:5216–24. doi: 10.1093/emboj/cdf516

6. Sun J, Brand M, Zenke Y, Tashiro S, Groudine M, Igarashi K. Heme regulates the dynamic exchange of Bach1 and NF-E2-related factors in the Maf transcription factor network. *Proc Natl Acad Sci U S A*. (2004) 101:1461–6. doi: 10.1073/pnas.0308083100

7. Dhakshinamoorthy S, Jain AK, Bloom DA, Jaiswal AK. Bach1 competes with Nrf2 leading to negative regulation of the antioxidant response element (ARE)-mediated NAD(P)H:quinone oxidoreductase 1 gene expression and induction in response to antioxidants. *J Biol Chem*. (2005) 280:16891–900. doi: 10.1074/jbc.M500166200

8. Ishikawa M, Numazawa S, Yoshida T. Redox regulation of the transcriptional repressor Bach1. *Free Radic Biol Med*. (2005) 38:1344–52. doi: 10.1016/j.freeradbiomed.2005.01.021

9. Igarashi K, Sun J. The heme-Bach1 pathway in the regulation of oxidative stress response and erythroid differentiation. *Antioxid Redox Signal*. (2006) 8:107–18. doi: 10.1089/ars.2006.8.107

10. Zhang X, Guo J, Wei X, Niu C, Jia M, Li Q, et al. Bach1: function, regulation, and involvement in disease. *Oxidative Med Cell Longev*. (2018) 2018:1347969. doi: 10.1155/2018/1347969

11. Belcher JD, Mahaseth H, Welch TE, Otterbein LE, Hebbel RP, Vercellotti GM. Heme oxygenase-1 is a modulator of inflammation and vaso-occlusion in transgenic sickle mice. *J Clin Invest*. (2006) 116:808–16. doi: 10.1172/JCI26857

12. Belcher JD, Vineyard JV, Bruzzone CM, Chen C, Beckman JD, Nguyen J, et al. Heme oxygenase-1 gene delivery by sleeping beauty inhibits vascular stasis in a murine model of sickle cell disease. *J Mol Med (Berl)*. (2010) 88:665–75. doi: 10.1007/s00109-010-0613-6

13. Bean CJ, Boulet SL, Ellingsen D, Pyle ME, Barron-Casella EA, Casella JF, et al. Heme oxygenase-1 gene promoter polymorphism is associated with reduced incidence of acute chest syndrome among children with sickle cell disease. *Blood*. (2012) 120:3822–8. doi: 10.1182/blood-2011-06-361642

14. Bakr S, Khorshied M, Talha N, Jaffer KY, Soliman N, Eid K, et al. Implication of HMOX1 and CCR5 genotypes on clinical phenotype of Egyptian patients with sickle cell anemia. *Ann Hematol*. (2019) 98:1805–12. doi: 10.1007/s00277-019-03697-9

15. Belcher JD, Chen C, Nguyen J, Zhang P, Abdulla F, Nguyen P, et al. Control of oxidative stress and inflammation in sickle cell disease with the Nrf2 activator dimethyl fumarate. *Antioxid Redox Signal*. (2017) 26:748–62. doi: 10.1089/ars.2015.6571

16. Krishnamoorthy S, Pace B, Gupta D, Sturtevant S, Li B, Makala L, et al. Dimethyl fumarate increases fetal hemoglobin, provides heme detoxification, and corrects anemia in sickle cell disease. JCI. *Insight*. (2017) 2:e96409. doi: 10.1172/jci.insight.96409

17. Zhu X, Xi C, Thomas B, Pace BS. Loss of NRF2 function exacerbates the pathophysiology of sickle cell disease in a transgenic mouse model. *Blood*. (2018) 131:558–62. doi: 10.1182/blood-2017-10-810531

18. Li B, Zhu X, Ward CM, Starlard-Davenport A, Takezaki M, Berry A, et al. MIR-144-mediated NRF2 gene silencing inhibits fetal hemoglobin expression in sickle cell disease. *Exp Hematol*. (2019) 70:85–96.e5. doi: 10.1016/j.exphem.2018.11.002

19. Nataraja S, Singh M, Demes S, Olson L, Stanwix J, Biddle M, et al. ML-0207/ASP8731: a novel BACH1 inhibitor that induces fetal hemoglobin in treatment of sickle cell disease. *Blood*. (2021) 138:854–4. doi: 10.1182/blood-2021-151606

20. Wu LC, Sun CW, Ryan TM, Pawlik KM, Ren J, Townes TM. Correction of sickle cell disease by homologous recombination in embryonic stem cells. *Blood*. (2006) 108:1183–8. doi: 10.1182/blood-2006-02-004812

21. Kalambur VS, Mahaseth H, Bischof JC, Kielbik MC, Welch TE, Vilbäck Å, et al. Microvascular blood flow and stasis in transgenic sickle mice: utility of a dorsal skin fold chamber for intravital microscopy. *Am J Hematol*. (2004) 77:117–25. doi: 10.1002/ajh.20143

22. Nishizawa H, Matsumoto M, Shindo T, Saigusa D, Kato H, Suzuki K, et al. Ferroptosis is controlled by the coordinated transcriptional regulation of glutathione and labile iron metabolism by the transcription factor BACH1. *J Biol Chem*. (2020) 295:69–82. doi: 10.1074/jbc.RA119.009548

23. Belcher JD, Gomperts E, Nguyen J, Chen C, Abdulla F, Kiser ZM, et al. Oral carbon monoxide therapy in murine sickle cell disease: beneficial effects on vaso-occlusion, inflammation and anemia. *PLoS One*. (2018) 13:e0205194. doi: 10.1371/journal.pone.0205194

24. Vercellotti GM, Khan FB, Nguyen J, Chen C, Bruzzone CM, Bechtel H, et al. H-ferritin ferroxidase induces cytoprotective pathways and inhibits microvascular stasis in transgenic sickle mice. *Front Pharmacol*. (2014) 5:79. doi: 10.3389/fphar.2014.00079

25. Gladwin MT, Shelhamer JH, Ognibene FP, Pease-Fye ME, Nichols JS, Link B, et al. Nitric oxide donor properties of hydroxyurea in patients with sickle cell disease. *Br J Haematol*. (2002) 116:436–44. doi: 10.1046/j.1365-2141.2002.03274.x

26. Naughton P, Hoque M, Green CJ, Foresti R, Motterlini R. Interaction of heme with nitroxyl or nitric oxide amplifies heme oxygenase-1 induction: involvement of the transcription factor Nrf2. *Cell Mol Biol (Noisy-le-Grand)*. (2002) 48:885–94.

27. Vercellotti GM, Dalmasso AP, Schaid TR Jr, Nguyen J, Chen C, Ericson ME, et al. Critical role of C5a in sickle cell disease. *Am J Hematol*. (2019) 94:327–37. doi: 10.1002/ajh.25384

28. Lanaro C, Franco-Penteado CF, Albuqueque DM, Saad STO, Conran N, Costa FF. Altered levels of cytokines and inflammatory mediators in plasma and leukocytes of sickle cell anemia patients and effects of hydroxyurea therapy. *J Leukoc Biol*. (2009) 85:235–42. doi: 10.1189/jlb.0708445

29. Subotički T, Ajtić OM, Đikić D, Santibanez JF, Tošić M, Čokić VP. Nitric oxide synthase dependency in hydroxyurea inhibition of Erythroid progenitor growth. *Genes (Basel)*. (2021) 12:1145. doi: 10.3390/genes12081145

30. Um HC, Jang JH, Kim DH, Lee C, Surh YJ. Nitric oxide activates Nrf2 through S-nitrosylation of Keap1 in PC12 cells. *Nitric Oxide*. (2011) 25:161–8. doi: 10.1016/j.niox.2011.06.001

31. Charache S, Terrin ML, Moore RD, Dover GJ, Barton FB, Eckert SV, et al. Effect of hydroxyurea on the frequency of painful crises in sickle cell anemia. Investigators of the multicenter study of hydroxyurea in sickle cell anemia. *N Engl J Med*. (1995) 332:1317–22. doi: 10.1056/NEJM199505183322001

32. Steinberg MH, Barton F, Castro O, Pegelow CH, Ballas SK, Kutlar A, et al. Effect of hydroxyurea on mortality and morbidity in adult sickle cell anemia: risks and benefits up to 9 years of treatment. *JAMA*. (2003) 289:1645–51. doi: 10.1001/jama.289.13.1645

33. Smith WR, Ballas SK, McCarthy WF, Bauserman RL, Swerdlow PS, Steinberg MH, et al. The association between hydroxyurea treatment and pain intensity, analgesic use, and utilization in ambulatory sickle cell anemia patients. *Pain Med*. (2011) 12:697–705. doi: 10.1111/j.1526-4637.2011.01096.x

34. Steinberg MH. Fetal hemoglobin in sickle cell anemia. *Blood*. (2020) 136:2392–400. doi: 10.1182/blood.2020007645

35. Walker AL, Crosby D, Miller V, Weidert F, Ofori-Acquah S. Hydroxyurea decouples persistent F-cell elevation and induction of γ-globin. *Exp Hematol*. (2022) 112-113:15–23.e1. doi: 10.1016/j.exphem.2022.07.005

36. Woodard KJ, Doerfler PA, Mayberry KD, Sharma A, Levine R, Yen J, et al. Limitations of mouse models for sickle cell disease conferred by their human globin transgene configurations. *Dis Model Mech*. (2022) 15:dmm049463. doi: 10.1242/dmm.049463

37. Kato H, Itoh-Nakadai A, Matsumoto M, Ishii Y, Watanabe-Matsui M, Ikeda M, et al. Infection perturbs Bach2-and Bach1-dependent erythroid lineage 'choice' to cause anemia. *Nat Immunol*. (2018) 19:1059–70. doi: 10.1038/s41590-018-0202-3

38. Tahara T, Sun J, Nakanishi K, Yamamoto M, Mori H, Saito T, et al. Heme positively regulates the expression of beta-globin at the locus control region via the transcriptional factor Bach1 in erythroid cells. *J Biol Chem*. (2004) 279:5480–7. doi: 10.1074/jbc.M302733200

39. Tahara T, Sun J, Igarashi K, Taketani S. Heme-dependent up-regulation of the alpha-globin gene expression by transcriptional repressor Bach1 in erythroid cells. *Biochem Biophys Res Commun*. (2004) 324:77–85. doi: 10.1016/j.bbrc.2004.09.022

40. Lee TY, Muniandy L, Teh LK, Abdullah M, George E, Sathar J, et al. Correlation of BACH1 and hemoglobin E/Beta-thalassemia globin expression. *Turk J Haematol*. (2016) 33:15–20. doi: 10.4274/tjh.2014.0197

41. Zakaria NA, Islam MA, Abdullah WZ, Bahar R, Mohamed Yusoff AA, Abdul Wahab R, et al. Epigenetic insights and potential modifiers as therapeutic targets in β-thalassemia. *Biomol Ther*. (2021) 11:755. doi: 10.3390/biom11050755

42. Marini MG, Chan K, Casula L, Kan YW, Cao A, Moi P. hMAF, a small human transcription factor that heterodimerizes specifically with Nrf1 and Nrf2. *J Biol Chem*. (1997) 272:16490–7. doi: 10.1074/jbc.272.26.16490

43. Macari ER, Lowrey CH. Induction of human fetal hemoglobin via the NRF2 antioxidant response signaling pathway. *Blood*. (2011) 117:5987–97. doi: 10.1182/blood-2010-10-314096

44. Pullarkat V, Meng Z, Tahara SM, Johnson CS, Kalra VK. Proteasome inhibition induces both antioxidant and hb f responses in sickle cell disease via the nrf2 pathway. *Hemoglobin*. (2014) 38:188–95. doi: 10.3109/03630269.2014.898651

45. Promsote W, Makala L, Li B, Smith SB, Singh N, Ganapathy V, et al. Monomethyl fumarate induces gamma-globin expression and fetal hemoglobin production in cultured human retinal pigment epithelial (RPE) and erythroid cells, and in intact retina. *Invest Ophthalmol Vis Sci*. (2014) 55:5382–93. doi: 10.1167/iovs.14-14179

46. Zhu X, Xi C, Ward A, Takezaki M, Shi H, Peterson KR, et al. NRF2 mediates γ-globin gene regulation through epigenetic modifications in a β-YAC transgenic mouse model. *Exp Biol Med (Maywood)*. (2020) 245:1308–18. doi: 10.1177/1535370220945305

47. Steinberg MH, McCarthy W, Castro O, Ballas SK, Armstrong FD, Smith W, et al. The risks and benefits of long-term use of hydroxyurea in sickle cell anemia: a 17.5 year follow-up. *Am J Hematol*. (2010) 85:403–8. doi: 10.1002/ajh.21699

48. Steinberg MH, Lu ZH, Barton FB, Terrin ML, Charache S, Dover GJ, et al. Fetal hemoglobin in sickle cell anemia: determinants of response to hydroxyurea. *Mult Study Hydroxyurea Blood*. (1997) 89:1078–88. doi: 10.1182/blood.V89.3.1078

49. Lavelle D, Engel JD, Saunthararajah Y. Fetal hemoglobin induction by epigenetic drugs. *Semin Hematol*. (2018) 55:60–7. doi: 10.1053/j.seminhematol.2018.04.008

Frontiers in Medicine

Translating medical research and innovation into improved patient care

A multidisciplinary journal which advances our medical knowledge. It supports the translation of scientific advances into new therapies and diagnostic tools that will improve patient care.

Discover the latest Research Topics

See more →

Frontiers

Avenue du Tribunal-Fédéral 34
1005 Lausanne, Switzerland
frontiersin.org

Contact us

+41 (0)21 510 17 00
frontiersin.org/about/contact