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BRIEF REPORT

Constitutive hypercoagulability in pediatric sickle cell disease patients with hemoglobin SS genotype

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Abstract

Background: Constitutive inflammation and hemostatic activation have been identified as key contributors to the pathophysiology of sickle cell disease (SCD), leading to clinical consequences such as vaso-occlusive crises and stroke. Patients with hemoglobin SS (HbSS) and hemoglobin SC (HbSC) genotypes are reported to have different symptoms, as do patients in steady-state and crisis situations. Differences among these groups remain unclear in pediatric patients.

Objectives: To compare hemostatic activity in HbSS and HbSC pediatric patients during steady state, in crisis, and in clinical follow-up and compare HbSS and HbSC patients with normal healthy children.

Methods: Whole-blood coagulation assay thromboelastography (TEG) was used to assess hemostatic activity. In parallel, flow cytometry was used to assess procoagulant surface expression of platelets and red blood cells.

Results: TEG results indicated no significant differences in clotting onset (R time), clot maximum amplitude, or maximum rate of thrombus generation among steady-state, crisis, and follow-up subgroups of HbSS and HbSC patients. TEG parameters did not differ significantly between HbSC patients and healthy children, while HbSS patients showed significantly shorter R time and greater maximum amplitude and maximum rate of thrombus generation, all indicative of a constitutive hypercoagulable state. Flow cytometry results did not detect increased platelet integrin $\alpha_{IIb}\beta_3$ activation or red blood cell procoagulant surface expression in SCD patients compared with unaffected children.

Conclusion: Our results indicate that pediatric SCD patients with the HbSS genotype have constitutively activated hemostasis relative to HbSC patients and healthy children. It remains to be determined how treatments that improve clinical outcomes in SCD patients affect this constitutively hypercoagulable state.

KEYWORDS
hypercoagulability, thromboelastography, sickle cell disease

Essentials

- Sickle cell disease patients are at risk for vaso-occlusive crises and stroke.
- We assessed hemostatic activity in SCD children (HbSS and HbSC) via thromboelastography.
- Children were evaluated at baseline and in crisis.
- We determined that HbSS patients are in a constitutive hypercoagulable state.

1 | INTRODUCTION

Sickle cell disease (SCD) is the most common serious inherited blood disorder worldwide, arising from missense variants in both copies of *HBB*, encoding the beta chain of hemoglobin (Hb) [1]. Under hypoxic conditions, Hb variants polymerize and cause red blood cells (RBCs) to form a sickle shape. Sickling of RBCs in SCD patients is accompanied by inflammation, oxidative stress, increased neutrophil adhesiveness, platelet activation, and coagulopathy [1]. The most common SCD genotypes are HbSS (homozygous for Hb variant S) and HbSC (possessing variants HbS and HbC). HbSS patients are reported to have generally more severe and frequent SCD complications, including anemia, infections, vaso-occlusive crises, and thrombotic events [2], as well as other susceptibilities, including increased risk/severity of kidney disease [3]. HbSC patients have a greater risk of specific complications such as severe proliferative retinopathy [4]. Many of the differences in the susceptibility and progression of SCD comorbidities in HbSS and HbSC patients remain poorly understood, highlighting the need to distinguish these groups in studies of SCD pathobiology and therapeutic response [5].

A hypercoagulable state in SCD patients has been directly associated with clinical complications [6], including an estimated incidence of venous thromboembolism of 25% [7]. SCD hypercoagulability has been examined using a range of laboratory approaches, including analysis of platelet-free plasma samples for thrombin generation potential via calibrated automated thrombography (CAT) [8], levels of procoagulant phospholipids and microparticles via functional assays [9], and presence of inflammatory cytokines [10]. These and other tests have indicated that SCD patients have elevated hemostatic biomarkers, including thrombin, thrombin–antithrombin complexes, prothrombin fragment F1.2, and D-dimers [11,12], along with decreased anticoagulant factors [13]. These observations indicate SCD hypercoagulability can be linked to several potential causes, including abnormalities in coagulation factor levels and other aspects of thrombin generation capacity/activation, as well as cellular factors such as adherence of RBCs to the endothelium and platelet activation [1].

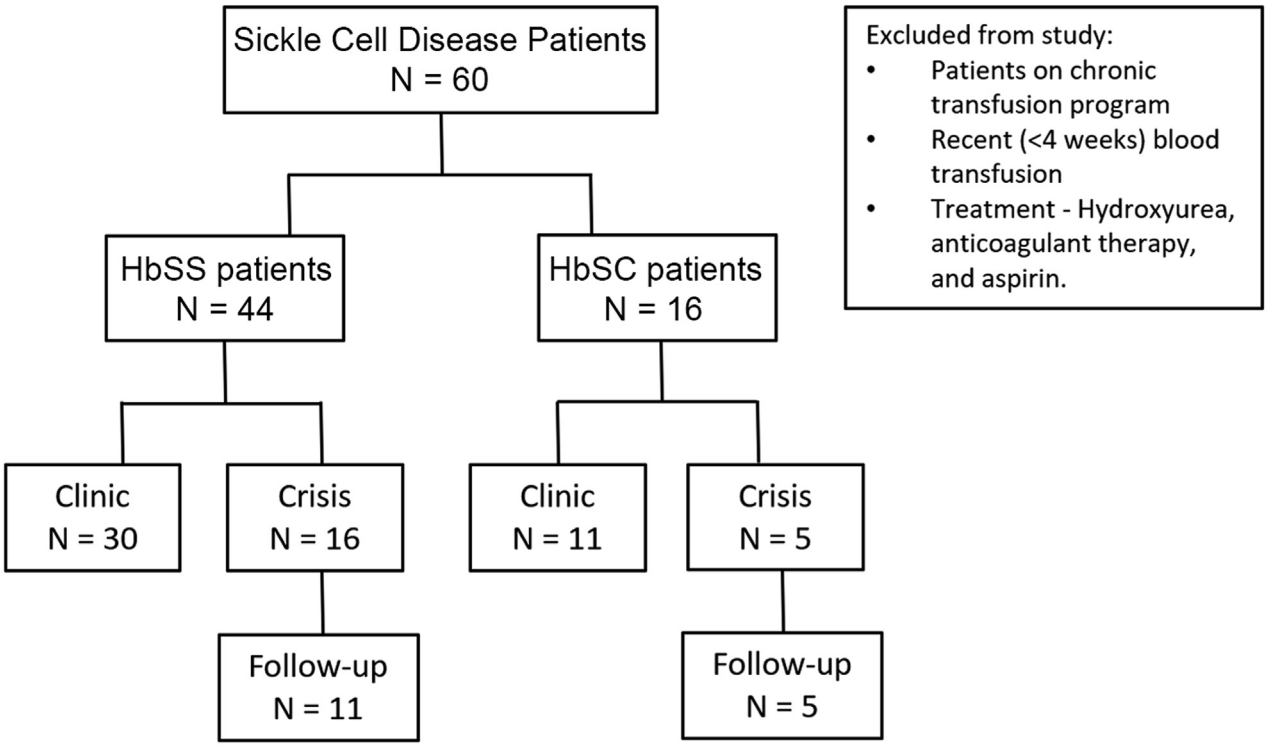
The potential complexity of hypercoagulability in SCD patients has prompted the application of global assays of hemostasis, such as thromboelastography (TEG), which allows *ex vivo* monitoring of coagulation in whole blood [14,15]. As with CAT, a variety of clinical and laboratory variables can influence TEG results [16], and in several studies employing these assays, we have minimized these factors by having tests performed by a single operator under consistent conditions [17–22]. TEG has been employed (along with CAT) to assess chronic hypercoagulability in adult SCD patients of HbSS and HbSB°–thalassemia genotypes, where TEG results were consistent with hypercoagulability relative to normal healthy controls (NC) [14]. Similar results were reported from a pilot study that compared clotting activation in children with SCD and sickle cell trait, as well as NC [11]. In a cross-sectional study of pediatric SCD patients, Yee et al. [23] examined 35 HbSS, 7 HbSC, and 4 HbSB° patients under baseline conditions (ie, steady state) and in the setting of acute illness (ie, in crisis), stating that relative to 20 NC samples, those from steady-state HbSS and HbSC patients showed decreased reaction time (R, minutes) and increased maximum amplitude (MA, mm) and coagulation index (CI) values, indicative of hypercoagulability. They also reported that 5 HbSS patients with current/recent crises showed increased TEG MA and CI values compared with the steady-state HbSS patient group.

In this prospective study, we used TEG to examine hemostatic activity in blood samples from a large pediatric (age range, 1.5-18.5 years) SCD cohort of 60 patients: 44 HbSS and 16 HbSC plus an NC group of 16 individuals in the same age range. SCD patients were evaluated in the context of one or more of the following: a regular clinic visit (steady state), during a crisis event, and during clinical follow-up. In the process of intravasular blood coagulation, platelets initiate and promote thrombin generation by binding to vascular surfaces, releasing granule contents and exposing procoagulant phosphatidylserine (PS) on their surface (detected via annexin V binding), while erythrocytes (RBCs) can also present PS and provide a procoagulant surface [24]. Thus, in parallel with TEG assays, patient samples were examined by whole-blood flow cytometry to assess potential procoagulant surface markers of RBCs and platelets to potentially gain insights into the cellular basis of SCD patient hypercoagulability.

2 | METHODS

2.1 | Patients

All work involving patients was carried out in accordance with the Declaration of Helsinki and the Research Ethics Board of the Hospital for Sick Children. Informed consent was obtained for all patients involved in this study. The study design and description of the patient cohort and healthy children comparison group are summarized in Figure 1. Exclusion criteria were patients on chronic transfusion program or recent (<4 weeks) blood transfusion, hydroxyurea,



Variable	SCD Clinic N = 41	SCD Crisis N = 21	Normal Healthy Controls N = 16
Age (years), median (range)	9.4 (1.6-18.5)	8.9 (2.7-16.4)	9.8 (1.8-16.6)
Sex (female), n (%)	21 (51)	12 (57)	5 (45)
SS Genotype, n (%)	30 (73)	16 (76)	n/a
SC Genotype, n (%)	11 (27)	5 (24)	n/a
Haemoglobin (g/L), M (SD)	91 (13)	90 (16)	134 (16.4)
Platelets (10^9/L), M (SD)	375 (136.5)	354 (131.6)	291 (83.3)
WBC (10^9/L), M (SD)	12.3 (4.7)	12.8 (5.3)	6.5 (1.5)

FIGURE 1 Description of the sickle cell disease (SCD) patient cohort and genotypic/clinical subgroups, and summary of patient and normal healthy control characteristics. Two HbSS patients who were initially tested at clinic baseline were subsequently also tested in crisis. Hemoglobin (Hb); hemoglobin SC, (HbSC); hemoglobin SS, (HbSS); WBC, white blood cells.

anticoagulant therapy, or aspirin. SCD patients were grouped according to genotype (HbSS and HbSC) and clinical status: patients in steady state attending a regular clinic visit (clinic) and those presenting at the hospital with a vaso-occlusive episode resulting in admission for symptom management (crisis). A subset of patients from the crisis group were tested after resolution of acute symptoms (follow-up). The NC group consisted of individuals without SCD who included siblings of study patients (1 each of HbAS and HbAC genotypes) and unaffected hospital patients who had been followed in clinic without another active disease process for at least 1 year. The NC and SCD patient cohorts are described in Figure 1. For HbSC patients, the mean age was 7.6 years, with 56% female; for HbSS patients, the mean age was 9.1 years, with 57% female. Of note, 2 HbSS patients who were initially tested at clinic baseline were subsequently also tested in crisis. Therefore the total number of HbSS patients was 44 as shown in Figure 1.

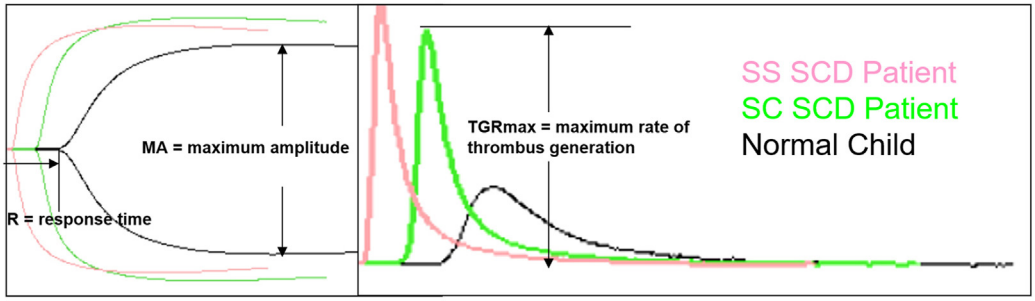
2.2 | Sample collection

Blood was collected into 3.2% buffered citrate tubes for TEG and clinical laboratory analysis and into CTAD tubes containing buffered sodium citrate equivalent to 3.2% sodium citrate and theophylline adenosine dipyridamole.

2.3 | Hemostasis testing

TEG analysis employed a Haemoscope Thrombelastograph Hemostasis Analyzer with data acquired by the TEG Analytical Software version 4 (Haemonetics Corporation). TEG parameters analyzed were 1) R (in minutes), measured from the start of the recording until the clot amplitude reaches 2 mm, an indicator of clotting latency; 2) the MA (in mm) of the TEG tracing, an indicator of clotting strength; and

A



B

Group (n)	R	MA	TGRmax
	min	mm	mm/min
SS Clinic (30)	4.7 (1.6)	69.1 (4.5)	15.8 (3.7)
SS Crisis (16)	4.5 (1.5)	69.7 (5.9)	18.5 (5.7)
SS Follow-up (11)	4.1 (1.5)	70.8 (6.0)	17.2 (4.3)
SC Clinic (11)	6.4 (3.0)	61.7 (9.4)	12.2 (4.4)
SC Crisis (5)	6.1 (1.3)	66.7 (2.4)	12.7 (2.8)
SC Follow-up (5)	7.1 (2.2)	69.5 (2.6)	13.3 (1.7)
Pooled SS (57)	4.5 (1.6)	69.6 (5.2)	16.8 (4.5)
Pooled SC (21)	6.5 (2.4)**	64.8 (7.6)*	12.6 (3.5)**
Healthy children (16)	7.6 (2.1)****	61.8 (3.3)***	10.9 (3.3)****

C

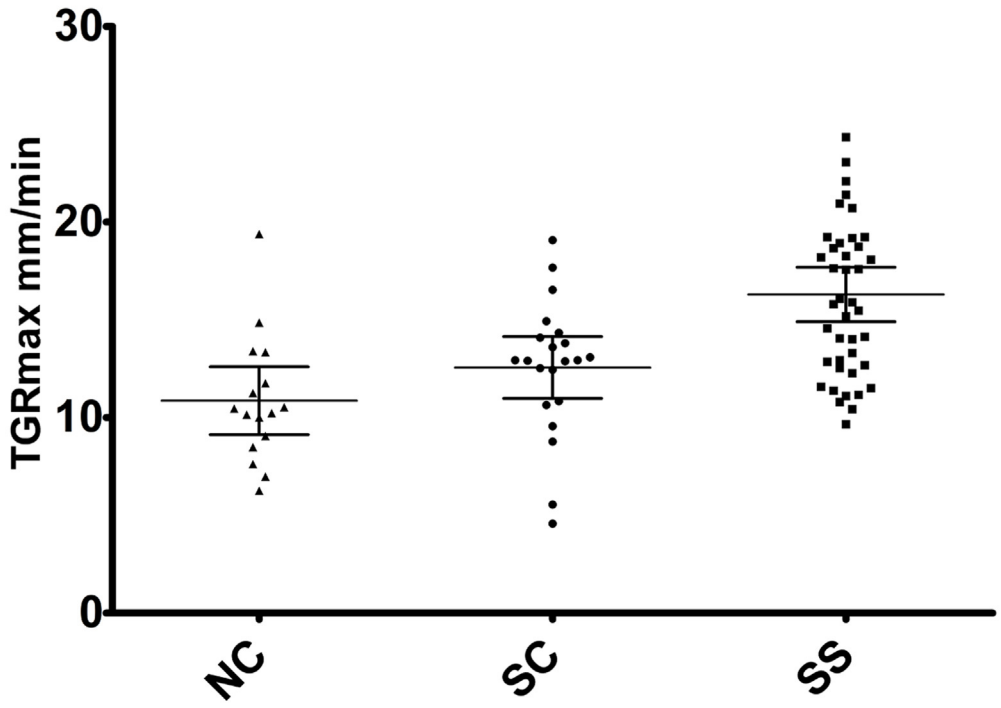


FIGURE 2 Thromboelastography (TEG) analysis. (A) Representative TEG tracings for sickle cell disease (SCD) patients and a normal healthy child (NC) show how response time (R), maximum amplitude (MA), and maximum rate of thrombus generation (TGRmax) parameter values indicate hypercoagulability. (B) Summary of results of TEG analysis: mean values with SD shown in brackets; pooled HbSS and HbSC data shown at bottom. No significant differences were noted between clinic, crisis, or follow-up subgroups of SCD patients or between pooled HbSC and healthy children. Asterisks indicate results of comparisons of pooled HbSS patient data with that for either pooled HbSC patients or healthy children, which were significant for all parameters. *$P < .05$; **$P < .01$; ***$P < .001$; ****$P < .0001$ for 1-way ANOVA with Bonferroni's multiple comparison test. (C) Graph of complete TGRmax datasets for NC and both patient groups; lines show means and 95% CIs. Hb, hemoglobin; SC, HbSC; SS, HbSS; NC, normal healthy controls.

A

Group (n)	Annexin A5	PAC-1
	% RBC	% platelets
SS Clinic (26)	1.8 (1.6)	1.7 (3.9)
SS Crisis (13)	1.4 (0.9)	1.6 (3.0)
SS Follow-up (8)	1.3 (0.8)	2.4 (3.8)
SC Clinic (10)	1.3 (1.0)	1.0 (1.5)
SC Crisis (5)	1.8 (1.1)	1.0 (0.7)
SC Follow-up (4)	2.4 (1.9)	1.6 (2.3)
Pooled SS (47)	1.6 (1.3)	1.8 (3.6)
Pooled SC (19)	1.6 (1.3)	1.1 (1.5)
Normal Healthy Controls (8)	0.8 (0.4)	0.4 (0.5)

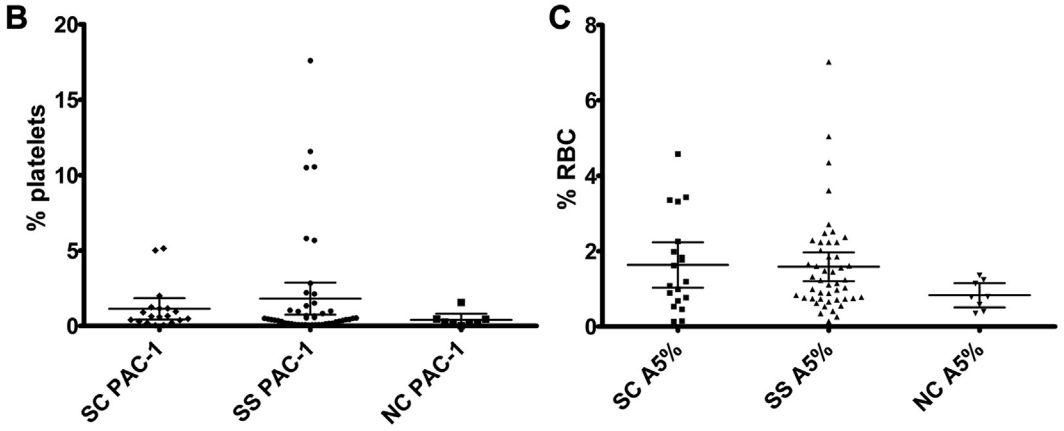


FIGURE 3 Flow cytometry analysis of procoagulant markers on platelets and red blood cells (RBCs). (A) Summary of data with mean values and SD (brackets), with pooled totals for HbSS (SS), HbSC (SC), and normal healthy controls (NC) at the bottom. No significant differences were noted among SCD patient subgroups or pooled data (Kruskal–Wallis test with Dunn's multiple comparison test). (B) Graph of total data for platelet PAC-1 binding indicative of fibrinogen receptor (integrin $\alpha_{IIb}\beta_3$) activation. (C) Graph of total data for annexin-A5 (A5) binding to phosphatidyl serine exposed RBCs. The data indicate a high variability of SCD patient results compared with normal controls; lines indicate mean values and 95% CIs. Hb, hemoglobin.

3) the maximum rate of thrombus generation (TGRmax, in mm/min), the peak value of the first derivative of the MA (ie, clotting velocity) curve, an indicator of clotting rate.

2.4 | Flow cytometry

Although it was not possible for all patients, blood from a subset of patients with HbSS, HbSC and NC, was also analyzed by flow cytometry. Buffered sodium citrate anticoagulated blood samples were incubated with combinations of fluorescence-conjugated antibodies or reagents reactive to platelet and RBC cell surface markers, and then analyzed on a BD FACSCalibur flow cytometer (BD Biosciences). Platelets and RBCs were identified by their characteristic light scatter and CD61 (clone Y2/51, Dako) or CD42a (clone SZ1, Beckman Coulter) and CD235a (clone 11E4B-7-6 [KC16], Beckman Coulter) expression, respectively. Platelet activation was assessed using PAC-1 (clone PAC-1, BD Biosciences) antibody to detect conformationally active surface integrin $\alpha_{IIb}\beta_3$ (glycoprotein IIb/IIIa) [12]. Annexin A5 (annexin V, Invitrogen) binding of RBCs was used as an indicator of cell surface PS.

2.5 | Data analysis

Data were collated and analyzed using Microsoft Excel and GraphPad Prism (GraphPad Software). Statistical testing is described in the text and figure legends.

3 | RESULTS AND DISCUSSION

Consistent with previous studies [25], white blood cell counts for the SCD patient cohort were elevated and hemoglobin levels were reduced relative to healthy children (Figure 1). There was no evident difference between HbSS and HbSC patients; platelet counts were within normal ranges for all groups. Representative tracings are shown in Figure 2A for TEG assays of blood from HbSS and HbSC patients and a normal child, showing the expected trend that samples from SCD patients often yielded clotting profiles with a shorter R (in minutes) and greater MA (in mm) and TGRmax (in mm/min) relative to samples from healthy children. TEG assay results are summarized in Figure 2B. Comparisons showed no significant differences in TEG data

for clinic, crisis, and follow-up subgroups for either patient genotype, while comparisons of pooled data indicated all TEG parameters (R, MA, and TGRmax) for HbSS patients differed significantly from those for HbSC patients and healthy children (the latter 2 groups did not differ significantly). Full TGRmax datasets are shown in Figure 2C. These results are indicative of elevated constitutive hemostatic activation in HbSS patients.

Flow cytometry was used to assess potential procoagulant aspects of platelets and RBCs in SCD patients; the results of this analysis are summarized in Figure 3A. PAC-1 binding indicates activation of platelet fibrinogen receptors (integrin $\alpha_{IIb}\beta_3$), for which we observed a nonsignificant trend toward elevation and high variability in both HbSS and HbSC patients relative to healthy children (Figure 3B). Annexin A5 binding of RBCs was used to detect exposure to procoagulant PS, which was also not significantly elevated and highly variable for SCD patients relative to healthy children (Figure 3C). Unlike TEG parameters, there were no evident differences observed among HbSS and HbSC patients and healthy children. As with the TEG data, there was no indication of consistent differences between patients in clinic, crisis, or follow-up.

Among previous TEG studies of pediatric SCD patients, the most relevant comparison is with Yee et al. [23], who reported TEG results indicative of hypercoagulability in steady-state HbSS and HbSC patients relative to normal children and elevated MA and CI values in HbSS crisis patients relative to steady-state. Our TEG results also indicated a hypercoagulable state in both groups of SCD patients, with HbSS patients showing significant differences from normal children (Figure 2B); we did not detect significant differences between clinic, crisis, and follow-up patient groups for HbSS or HbSC patients. While both studies are in overall agreement, there are differences in specific findings, which may be associated with variations between the patient cohorts examined in each study.

It is well established that SCD patients are at increased risk for thromboembolic events, and aspects of the hemostatic system that have been implicated include platelets, RBCs, and fibrinolysis [26,27]. PS on RBC membranes can contribute to thrombin generation [28] as can increased circulating tissue factor, which has been observed in SCD and other hypercoagulable disease states [29]. For example, a previous study [12] reported increased binding of annexin-A5 to PS on RBCs in adult SCD patients. However no correlation was found with thrombin–antithrombin complex levels in that study. In contrast, our flow cytometry results failed to reveal notable increases in binding of Annexin-A5 to RBCs in pediatric SCD patient samples nor did we observe increased PAC-1 binding to platelets. These results may indicate a relatively minor role for these cellular aspects of hemostasis in driving the constitutive hypercoagulable state in children with SCD.

In conclusion, our TEG results indicate that pediatric SCD patients of the HbSS genotype show a constitutive hypercoagulable state that persists in both steady state and vaso-occlusive crisis. This highlights a need for monitoring and treating these patients in a preventative context, where TEG could be useful for assessing and monitoring the effects of treatments, such as medications and blood transfusions. In the current era of increasing treatment options and curative approaches to SCD, including pharmacologic interventions, hematopoietic stem cell transplantation, gene editing [30], and other gene therapy modalities [31], TEG may also prove useful in assessing efficacy in alleviating constitutive hypercoagulability.

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AUTHOR CONTRIBUTIONS

W.K. conceived and designed the study. W.K. and F.P. acquired and analyzed data. R.S. and J.M. interpreted data and wrote the manuscript. M.S., J.F., R.G., M.K., and S.W. recruited patients for the study and edited the manuscript. A.B. did the flow cytometry. F.P. did the thromboelastography. M.R. and V.B. facilitated laboratory resources and edited the manuscript. R.S., F.P., and W.K. edited the final version of the manuscript.

RELATIONSHIP DISCLOSURE

The authors have no conflicts of interest to declare.

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