



# CD34+ Hematopoietic Stem Cell Count Is Predictive of Vascular Event Occurrence in Children with Sickle Cell Disease

Manoelle Kossorotoff<sup>1,2</sup>  · Mariane De Montalembert<sup>3,4</sup> · Valentine Brousse<sup>3</sup> · Dominique Lasne<sup>1,5</sup> · Emmanuel Curis<sup>4,6</sup> · David M. Smadja<sup>1,4,7</sup> · Romaric Lacroix<sup>8</sup> · Sebastien Bertil<sup>7</sup> · Elodie Masson<sup>8</sup> · Isabelle Desguerre<sup>2,4</sup> · Damien Bonnet<sup>4,9</sup> · Pascale Gaussem<sup>1,4,7</sup>

© Springer Science+Business Media, LLC, part of Springer Nature 2018

## Abstract

**Background/Objectives** Sickle cell disease (SCD) complications mostly result from vascular dysfunction, concerning systemic microvasculature and cerebral large vessels. The aim of this cohort study was to identify potential circulating biomarkers predictive for further vascular event occurrence in pediatric SCD.

**Methods** We consecutively enrolled 108 children with SCD at steady state, aged 3–18 years old (median 9.8 years). Hematology, coagulation, hemolysis, endothelial, platelet and vascular activation parameters were recorded at inclusion. Neurovascular and systemic vascular events were prospectively recorded during a mean follow-up period of 27 months.

**Results** Patients at steady state displayed significantly higher hemolysis and platelet activation markers, higher leukocyte, CD34+ hematopoietic stem cell and microvesicle counts, and a pro-coagulant profile compared to controls matched for age and ethnicity. Circulating endothelial cell or nucleosome level did not differ. During the follow-up period, 36 patients had at least one neurovascular ( $n = 12$ ) or systemic vascular event ( $n = 25$ ). In a multivariate model, high CD34+ cell count was the best predictor for the occurrence of a vascular event (OR 1.2 for 1000 cell/mL increase, 95% CI [1.049–1.4],  $p = 0.013$ , sensitivity 53%, specificity 84% for a threshold of 8675 cells/mL).

**Conclusion** CD34+ cell count at steady state is a promising biomarker of further vascular event in children with SCD.

**Keywords** Sickle-cell disease · Children · Vascular disease · Prognostic factors · CD34+ hematopoietic stem cell · Stroke

## Introduction

Sickle cell disease (SCD) complications usually result from induced vascular dysfunction. Among them, severe

complications such as acute chest syndrome and stroke represent important causes of mortality, further handicap, or altered quality of life in children with the most severe SCD genotypes (i.e. hemoglobin SS or S $\beta^0$ ). Therefore, identifying relevant

**Electronic supplementary material** The online version of this article (<https://doi.org/10.1007/s12015-018-9835-8>) contains supplementary material, which is available to authorized users.

✉ Manoelle Kossorotoff  
manoelle.kossorotoff@aphp.fr

<sup>1</sup> Inserm UMR-S1140, Faculté de Pharmacie, Paris, France

<sup>2</sup> AP-HP, Child Neurology, French center for pediatric stroke, Hôpital Universitaire Necker-Enfants malades, 149 rue de Sévres, 75015 Paris, France

<sup>3</sup> AP-HP, Pediatric Sickle Cell Clinic, Hôpital Universitaire Necker-Enfants malades and Laboratory of Excellence, GR-Ex, Paris, France

<sup>4</sup> Sorbonne Paris Cité, Université Paris Descartes, Paris, France

<sup>5</sup> AP-HP, Laboratoire d'hématologie, Hôpital Universitaire Necker-Enfants malades, Paris, France

<sup>6</sup> Laboratoire de biomathématiques, plateau iB2, Faculté de Pharmacie, Paris, France

<sup>7</sup> AP-HP, Service d'hématologie biologique, Hôpital Européen Georges Pompidou, Paris, France

<sup>8</sup> Inserm UMR-S1076, UFR de Pharmacie, Aix Marseille Université, Marseille, France

<sup>9</sup> AP-HP, M3C-Necker, Cardiologie Congénitale et Pédiatrique, Hôpital Universitaire Necker-Enfants malades, Paris, France

predictive markers for clinical vascular events is of utmost importance.

Systemic complications, i.e. bone/organ vaso-occlusive event (VOE), acute chest syndrome (ACS), pulmonary hypertension, acute splenic sequestration (ASS) or glomerular nephropathy, are attributed to microvasculature injury, while cerebral complications are related to both microvasculature and large vessel involvement. A combination of hypercoagulability, hemolysis, chronic arterial wall injury with altered regeneration pathways, and activation of circulating cells are hypothesized. To date, most studies focused on predictors of neurovascular events. Transcranial Doppler (TCD) velocities have been proven to be a very efficient predictor of overt stroke [1], but not of silent cerebral infarct or systemic vascular event. [2] On the contrary, no general marker has been proven predictive for systemic vascular events, although higher leukocyte count and markers of hemolysis suggested higher disease severity [3], while fetal hemoglobin (HbF) was suggested as a protective factor. [4, 5] Interestingly, reticulocyte count is known to be associated with ACS, with higher TCD velocities, and was proven to be an independent predictive risk factor for stroke, suggesting that a common marker may account for both systemic and neurovascular risks. [6–8] Actually, it may be hypothesized that injury mechanisms and excessive regenerative and scarring pathways could be similar in both small and large vessels in SCD patients, both in brain or in systemic organs (including bone). [9–12]

The aim of this study was to identify a predictive marker for the occurrence of vascular events in children with SCD included at steady state, using an original approach in considering together systemic and neurological vascular events, as they may have common pathophysiological determinants and risk factors.

## Materials and Methods

### Study Population

Children with major SCD syndromes followed in the sickle-cell clinic of the University Hospital Necker-Enfants malades, Paris, France, and aged between 3 and 18 years old were consecutively included at steady state. Steady state was defined as the absence of a history of SCD complication leading to hospitalization or blood transfusion during the past two months. Patients were included during an 18 months period and were followed for the present study 18 to 36 months. During this period, all participants received standard age-appropriate care for SCD, including annual TCD, and screening for proteinuria and retinopathy. [13, 14] (Fig. 1).

The local institutional ethical review committee approved the study protocol (registered protocol 2011–01-04, Comité de Protection des Personnes Ile-de-France 2). Parents and children aged above 12 years old gave written informed consent.

### Characteristics at Steady State

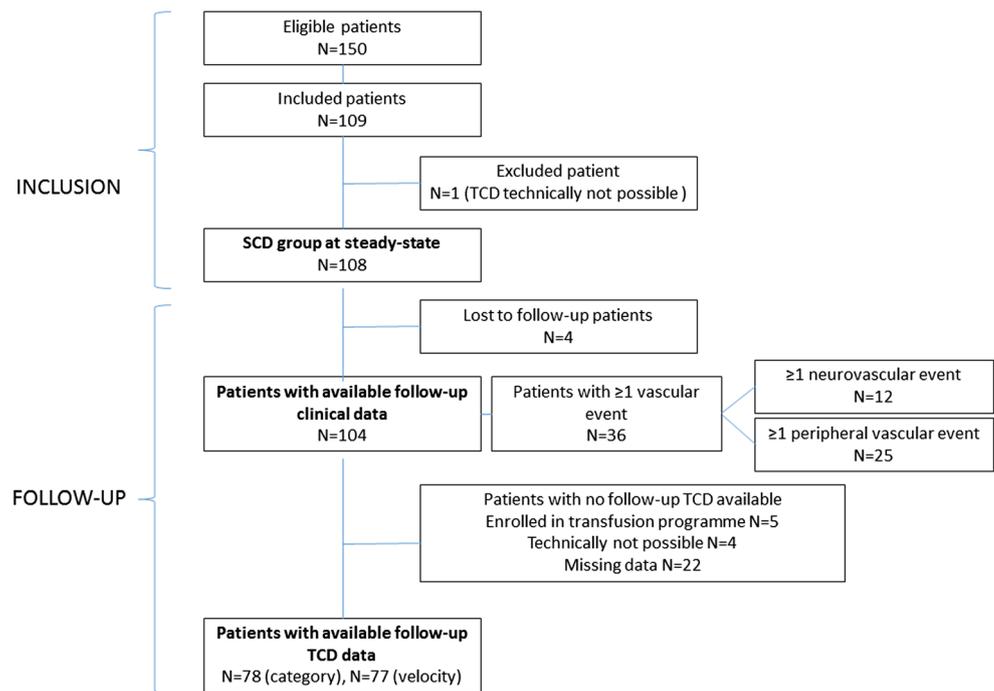
Clinical, vascular, and biological data were collected at inclusion.

Patients' personal and familial history of vascular condition, clinical parameters, and ongoing treatments were recorded.

Vascular data included TCD data according to the STOP study / NIH criteria [13, 14], issued from the patients' most recent routine TCD procedure, and systemic vascular parameters, such as blood pressure, markers of pulmonary hypertension (tricuspid regurgitation velocity) and mechanical functions (distensibility, elasticity). [11, 15] *Transcranial Doppler*: TCD procedure was performed annually with a Toshiba® Aplio 500 Ultrasound system with a probe sector transducer PST-25BT (3 MHz frequency), as recommended [13, 14]. Patients were classified in two categories at inclusion, i.e. normal or abnormal TCD, according to the results of their most recent routine TCD procedure. Normal TCD was defined according to the STOP study / NIH criteria (Time Averaged Mean Maximum Velocity TAMMV in the middle cerebral artery, anterior cerebral artery and internal carotid artery <170 cm/s). Patients with incomplete TCD procedure but with available velocities within normal values were also included in the normal TCD group. [13, 14] Abnormal TCD group comprised patients with STOP / NIH conditional (TAMMV  $\geq$ 170 cm/s) or abnormal (TAMMV  $\geq$ 200 cm/s) TCD. *Systemic vascular parameters*: Maximal tricuspid regurgitation velocity (TRV) at echocardiography, an indirect marker of pulmonary hypertension when TRV >2.8 m/s, was reported. Blood pressure and vascular function parameters, including intima-media thickness, mechanical properties of the common carotid artery (distensibility, elastic incremental modulus), endothelium-dependent vasodilation, and endothelium-independent vasodilation, were measured using previously described methods. [11, 15]

Comprehensive biological testing was performed, including biochemical, hematology, and coagulation explorations. The following specific parameters were quantified: circulating endothelial cells, circulating CD34+ hematopoietic stem cells, leukocyte-platelet complexes, nucleosome level, and microvesicles derived from endothelial and blood cells (including subpopulations derived from platelets, erythrocytes, and leukocytes). Peripheral blood samples were collected into EDTA, citrate and lithium heparinate pediatric tubes. Biochemical, hematology, hemostasis explorations, and nitric oxide bioavailability estimation were performed. Basic hematology and hemolysis parameters were recorded. Coagulation tests were performed on ACLTop (Instrumentation Laboratory, Bedford, MA, USA) using reagents from Siemens® for Prothrombin time (PT), Factor VIII, and Factor IX, from Diagnostica Stago® (Asnières, France) for activated-partial prothrombin time (aPTT, CKPrest), von

Fig. 1 Study flowchart



Willebrand factor antigen (VWF), antithrombin, protein C, and protein S activities, and from Hyphen Biomed® (Neuville-sur-Oise, France) for Factor VII + X coagulant activities. D-dimer count and free Protein S antigen quantification used ELISA kit (Asserachrom Stago®). The ABO blood group, which is known to influence VWF level, was also determined. Thromboelastogram® (TEG Haemonetics, Brain tree, MA, USA) was performed using kaolin-activated citrated blood and provided the following data: R time (min) representing the enzymatic portion of coagulation, K time (min) representing clot kinetics, and maximum amplitude MA (mm) depending on platelet function/aggregation and fibrinogen level. A set of parameters generated from the mathematical first derivative of the standard TEG® tracing related to thrombin generation were obtained: thrombin generation (TEG G), time to maximum rate of thrombus generation (TMRTGG), maximum rate of thrombus generation (MRTGG), total thrombus generated (TGG). *Circulating Endothelial Cells*: CECs were counted after immunomagnetic separation using a monoclonal antibody raised against the endothelial antigen CD146 as recommended and described elsewhere. [16, 17] *Circulating Hematopoietic CD34+ stem Cells (CPCs)* were quantified by flow cytometry as recommended [18], and expressed as cells/mL, as detailed elsewhere. [19] *Leukocyte-platelet complexes (LPC)* were counted by flow cytometry (FACScalibur, Becton Dickinson) with CD45-CD41 labelling (Beckman Coulter and Caltag antibodies) and were expressed as a percentage of total leukocytes, as described elsewhere. [20] *Microparticles (MP)* derived from blood cells and endothelial cells were quantified by flow

cytometry using Annexin V labelling and cell-specific labelling. Platelet-derived MPs, erythrocyte-derived MPs, leukocyte-derived MPs and endothelial cell-derived MPs were quantified (mp/ $\mu$ L) using high-sensitivity flow cytometry, as previously described. [21] *Nucleosome* quantification, reflecting partly NETosis, was performed by ELISA (Cell death detection kit; Roche) as described by Fuchs et al. [22] One unit of nucleosomes referred to the average amount of nucleosomes quantified in plasma of the controls. Vascular markers were quantified using ELISA kits from R&D Systems (Minneapolis, MN, USA) for soluble E Selectin, and soluble P Selectin.

As pediatric laboratory reference values for most of these parameters were missing, especially for children with African or Caribbean ethnicity, a group of controls matched for age and ethnicity was concomitantly recruited.

## Follow-Up

The following vascular events occurring during the follow-up period were recorded: i) significant neurovascular events including stroke, transient ischemic event, intracranial hemorrhage, cerebral venous thrombosis, TCD switching from normal to abnormal categories, and intracranial aneurysm diagnosis, ii) significant systemic vascular events including ACS, ASS, priapism, symptomatic femoral osteonecrosis, proteinuria or retinopathy occurrence, and deep vein thrombosis, iii) significant treatment modification (i.e. initiating a transfusion program or hydroxycarbamide treatment) during the follow-up period because of neurologic clinical symptoms or TCD

abnormality was also considered as a neurovascular event, while significant treatment modification because of repeated VOE/ACS/ASS was considered as a systemic event. Bone and abdominal VOEs not leading to treatment modification were not considered as significant events, as they are common complications in patients with SCD.

## Statistical Analysis

All analyses were made using R and additional package coin [23] for exact inference with ties when using non-parametric tests. Confidence intervals are given for a 95% confidence level; tests were made with a maximal type I error set at 5% ( $p < 0.05$ ).

**Characteristics of the Study Populations** Differences between steady-state patients and controls were assessed using Mann-Whitney test. Association of parameters was evaluated using Spearman's rank correlation coefficient for quantitative predictors and Kruskal-Wallis test for qualitative predictors.

**Predictivity** A univariate analysis evaluated predictivity of parameters, corrected  $p$ -values for multiplicity were calculated according to Bonferroni's method. Then, a multivariate model, including selected parameters collected at inclusion was designed. Parameters selection relied on both previously published data and results found in the univariate analysis. Significance of these parameters was tested using likelihood ratio tests according to the nested models theory. The ability of the final model to predict the outcome was checked using the area under the ROC curve, and the model sensitivity and specificity.

## Results

The study enrolled 108 patients with SCD at steady state, 41 males and 67 females (sex ratio 0.61), aged 3.27–17.75 years old (median 9.76) (Fig. 1).

### Characteristics at Steady State (Supplemental Table 1)

As previously reported, compared with controls, patients with SCD displayed significantly higher levels of hemolysis and platelet activation markers, and higher leukocyte, CD34+ hematopoietic stem cell, and total microvesicle counts. Children with SCD had a pro-coagulant profile with an increased whole blood coagulant activity according to thromboelastography parameters, higher D-dimer, factor VIII, and von Willebrand Factor (VWF) levels. They also had lower levels of coagulation inhibitors.

Interestingly, patients with SCD did not differ from controls in terms of circulating endothelial cells ( $p = 0.88$ ),

although an endothelial activation was suggested by the increase in soluble (s) E-selectin level ( $p = 0.0032$ ) associated with a VWF increase. Cell nucleosomes, known as a leukocyte activation marker and a reflection of NETosis, did not differ between the two groups.

Patients with SCD had altered endothelial-dependent vasodilation (data obtained in 43 patients). No patient had pulmonary hypertension. Most patients ( $n = 102$ , 94.4%) had a normal TCD, while only six patients (5.6%) had an abnormal TCD (conditional  $n = 3$ , abnormal  $n = 3$ ).

## Follow-Up

Mean follow-up was 27 months (range 18–34.6 months), with data available for 104 patients, as four patients were lost to follow-up. During this period, 36 patients (35%) had at least one vascular event: 25 patients (24%) had one systemic vascular event or more, 12 patients (11.5%) had one neurovascular event or more, and one patient had both a neurovascular and a systemic event (Fig. 1, Table 1). Median delay for vascular event occurrence was 277 days from inclusion. Neurovascular events occurred at a younger age than systemic vascular events (median age 6.45 [4.65–10.2] vs 10.96 [8.61–14.18] years old,  $p = 0.0071$ ). Interestingly, patients treated with hydroxycarbamide at inclusion had significantly more systemic vascular events during the follow-up period ( $p = 0.021$ ).

## Predictive Factors for Vascular Events

The multivariate model was designed, including the following parameters selected both from the preliminary univariate analysis results and previously published data: age, reticulocyte count, treatment with hydroxycarbamide at inclusion, history of abnormal TCD, soluble E-selectin count, and CD34+ cell count at inclusion. In this model, higher total CD34+ cell count was the best predictor for the occurrence of a vascular complication (OR 1.2 for 1000/mL increase, 95%CI [1.049–1.4],  $p = 0.013$ , sensitivity 52.9%, specificity 84.6% for a threshold of 8675/mL) (Fig. 2, and Supplemental Fig. 1).

Potential association of this marker with abovementioned previously published risk factors, including markers of hemolysis and TCD values, was verified. As expected, an association with other markers of hemolysis was found, with hemoglobin level (negative association) and LDH (positive association). An association between reticulocyte and CD34+ cell counts was found but did not remain linear, i.e. CD34+ continued to increase when reticulocyte count had reached a steady state. (Supplemental Fig. 2). A non-significant association between higher TCD velocity and elevated CD34+ cell count was observed ( $p = 0.072$ ).

See Table 2.

**Table 1** First vascular complication recorded during the follow-up period

Type of first complication	Number of patients
<b>NEUROVASCULAR EVENTS</b>	
	N = 12, median age of occurrence 6.45 yrs. old (25–75 perc 4.65–10.2)
Ischemic stroke or transient ischemic event	N = 4
Intracranial hemorrhage and/or Intracranial aneurysm	N = 2
Cerebral venous thrombosis	N = 1
Transfusion program or HU started because of TCD abnormality	N = 5
<b>PERIPHERAL VASCULAR EVENTS</b>	
	N = 25, median age of occurrence 10.96 yrs. old (25–75 perc: 8.61–14.18)
Acute chest syndrome (ACS)	N = 13 (1 with concurrent deep vein thrombosis)
Priapism	N = 2
Acute splenic sequestration (ASS)	N = 1
Femoral head osteonecrosis	N = 1
Proteinuria occurrence	N = 5
Retinopathy	N = 0
HU started because of frequent VOE or repeated ACS/ASS	N = 3

Clinical data were available for 104/108 patients

HU Hydroxycarbamide

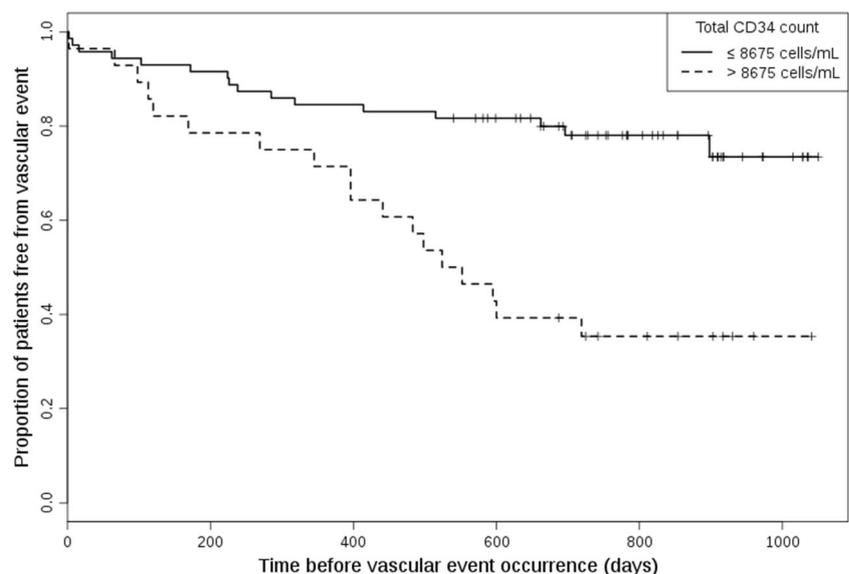
## Discussion

The main goal of this study was to identify a predictive marker for the occurrence of vascular events in children with SCD, using an original approach considering the occurrence of both neurovascular events and systemic events. We observed that a CD34+ hematopoietic stem cell count higher than 8675 cell/mL was a predictive factor of vascular complications.

The increase in CD34+ cells in SCD is usually thought to result from bone marrow stimulation due to chronic hemolysis and anemia. But in our patients with SCD, the association between reticulocyte and CD34+ cell counts did not remain

linear, and CD34+ continued to increase when reticulocyte count had reached a steady state. This pattern suggests that other mechanisms than hemolysis are involved in chronic CD34+ progenitor mobilization, such as hypoxia and regenerative pathways. While hematopoietic CD34+ cell mobilization through hypoxia-inducible angiogenic growth factor SDF-1 was found beneficial in acute hypoxic conditions (for example in the acute phase injury after stroke) [24] [25], chronic CD34+ cell mobilization may conversely have a detrimental effect in chronic vascular conditions. Circulating CD34+ cell population contains a scarce subpopulation of so-called endothelial progenitor cells, known to participate

**Fig. 2** CD34+ cell count predicts vascular event occurrence. Event-free survival is significantly different for patients with CD34+ cell count >8675 cells/mL,  $p \sim 0.0001$  (log-rank test)



**Table 2** Clinical and biological features associated with further clinical event occurrence, multivariate model

Parameters included in the multivariate model	Prediction of global vascular events (neurovascular + peripheral)	Prediction of neurovascular events
Age	ns	OR = 0.61 [0.38–0.86] for 1 year older, $p = 0.018^*$
Reticulocyte count	ns	OR = 2.03 [1.31–4.09] for $0.5 \cdot 10^9/L$ increase, $p = 0.034^*$
E-selectin dosage	<b>OR = 1.03 [1.01–1.05] for 1 ng/mL decrease, <math>p = 0.0044</math></b>	OR = 1.04 [1.01–1.09] for 1 ng/mL decrease, $p = 0.027$
Total CD34+ cell count	<b>OR = 1.2 [1.049–1.4], for 1000/mL increase <math>p = 0.013</math></b>	ns
Ongoing hydroxycarbamide treatment at inclusion	ns	ns
History of abnormal TCD at inclusion	ns	OR = 1.905 [1.46–1.99] for no history of abnormal TCD, $p = 0.018^*$

Bold font: significant result, ns: non significant, \*: previously published significant association/predictivity

in endothelium regeneration and stabilization. Those CD34+ cells in high number or with blocked maturation (as is the case in chronic hypoxic conditions), may display abnormal reparative functions and promote neointimal hyperplasia and arterial stenosis. [24–26] They are associated with poorer prognostic in heart failure adult patients. [27] Among these circulating CD34+ cell population, there is a subpopulation of very small embryonic-like stem cells (VSELs). [28, 29] VSELs are pluripotent stem cells with the phenotype of Lin-, CD34 ±, CD133 +, CD45-, CXCR4 +, SSEA-4 +, AP +, c-Met+, LIF-R +, HLA-DR-, MHC I -, CD90-, CD29-, CD105-, they can be isolated from the bone marrow, umbilical cord blood and peripheral blood. These small cells are deposited during embryogenesis in bone marrow and other organs as a backup population for adult tissue-committed stem cells and are involved in several processes related to tissue or organ regeneration. We and others have previously described that VSELs are able to give rise to endothelial cells and also perivascular cells, harboring the CD34 surface marker [30–32]. Thus, in patients with SCD, VSELs subset in total CD34+ cells may be associated to vascular events as a consequence of a multilineage differentiation of potential cells that would modify vascular structure and/or remodeling process leading to vascular adverse events. In line with this, Croizat et al. showed that patients with SCD (SS hemoglobin) had a chronically mobilized hematopoietic progenitor phenotype, probably related to exposure to elevated levels of IL-3, GM-CSF, etc. [33] These chronically detrimental effects might be a potential explanation of the predictive role of CD34+ cell count for clinical vascular events in our study population. Interestingly, it was recently shown that, in patients with SCD with vascular damage, the high count of hematopoietic and progenitor cells may be reduced by angiotensin II inhibitors, suggesting a potential therapeutic strategy. [34]

Most of our results confirmed previous reports, as TCD data distribution. [2, 35] Predictive factors or neurovascular events only replicated previously published results in children with SCD, i.e. abnormal TCD, younger age, increased hemolysis (especially elevated reticulocyte count), and history of

stroke or abnormal TCD. [1, 6, 7] Interestingly, some other factors that may be hypothesized or have been reported involved in vascular complications in children with SCD were not significant in this study. First, considering markers of endothelial function, lower soluble E-selectin was pointed by univariate analysis but was not retained in the multivariate analysis (Supplemental Table 1). No increase in Circulating Endothelial Cell (CEC) count was found in children with SCD, contrary to what Solovey et al. found in adults with SCD. [36] The technique we used for CEC count is the recommended method published by Woywodt et al. in 2006 [16], relying on CEC count after immunomagnetic separation using a monoclonal antibody recognizing a different epitope than the PIH12 mAb used by Solovey et al. We have also chosen to present results as median values rather than means, because the values were not normally distributed, which is, to our opinion, another cause of difference between our results and those reported by Solovey et al. Finally, they quantified cells in buffy coat and might have included progenitor endothelial cells as well as circulating endothelial cells in their count. [36] The absence of CEC count elevation in SCD patients highlights the potential of CEC count to differentiate endothelial lesion from endothelial regeneration. In previous studies, our team found that CEC count reflected more a strong remodeling process than endothelial lesion. In pulmonary artery hypertension (idiopathic or associated with congenital cardiopathy), CEC count elevation was related to intimal proliferation and subsequent PAH non-reversibility. [17, 37, 38] In oncology, CEC count is considered as a surrogate biomarker of angiogenic activity, especially in lung and colorectal cancers, and has potential therapeutic interest for the monitoring of anti-angiogenic treatments. [39–41] In the present study, normal CEC count observed either during vaso-occlusive event (data not shown) or not, suggests that SCD patients actually have endothelial activation, with increased soluble E-selectin and VWF levels, but no vascular remodeling or intima proliferation process. Second, no parameter of hemolysis reached significance in this study. The use of multiplicity corrections, although necessary when analyzing multiple parameters, may

have limited statistical power. Third, although hypercoagulability and thrombin generation activation were observed in more than 90% of children with SCD enrolled in the present study, they were not predictive of vascular event occurrence. Results published by Noubouossie et al. [42] were not replicated in our study, as increase in thrombin generation parameters was not associated with further neurovascular event occurrence, although patients' characteristics were comparable. Finally, in a subgroup of patients with available results ( $n = 43$ ), parameters reflecting a loss of arterial wall distensibility were found to be predictive of systemic vascular event occurrence. As they represent chronic vascular changes since childhood, which may lead to early atherosclerosis [43], further explorations may be encouraged.

## Conclusion

CD34+ cell count appears as a promising marker of global vascular event occurrence in children with SCD, including both systemic and neurovascular events. A large prospective study including CD34+ count in the biological follow-up of patients with SCD is now warranted to validate these findings. Risk stratification in these patients is challenging and could lead to modify treatments in order to prevent severe complications leading to pain, irreversible organ damage, or handicap.

**Acknowledgements** The authors would like to thank Florence Desvard, Yann Brunel, Laurent Garcia, Aurélie Dumont, and Peggy Tournoux for their excellent technical assistance. The French Institute for Medical Research INSERM funded Dr. M. Kossorotoff for this study.

## Compliance with Ethical Standards

**Conflict of Interest Statement** The authors disclose any conflict of interest.

## References

- Adams, R., McKie, V., Nichols, F., Carl, E., Zhang, D. L., McKie, K., Figueroa, R., Litaker, M., Thompson, W., & Hess, D. (1992). The use of transcranial ultrasonography to predict stroke in sickle cell disease. *The New England Journal of Medicine*, *326*(9), 605–610.
- Wang, W. C., Gallagher, D. M., Pegelow, C. H., Wright, E. C., Vichinsky, E. P., Abboud, M. R., Moser, F. G., & Adams, R. J. (2000). Multicenter comparison of magnetic resonance imaging and transcranial Doppler ultrasonography in the evaluation of the central nervous system in children with sickle cell disease. *Journal of Pediatric Hematology/Oncology*, *22*(4), 335–339.
- Miller, S. T., Sleeper, L. A., Pegelow, C. H., Enos, L. E., Wang, W. C., Weiner, S. J., Wethers, D. L., Smith, J., & Kinney, T. R. (2000). Prediction of adverse outcomes in children with sickle cell disease. *The New England Journal of Medicine*, *342*(2), 83–89.
- Stevens, M. C., Hayes, R. J., Vaidya, S., & Serjeant, G. R. (1981). Fetal hemoglobin and clinical severity of homozygous sickle cell disease in early childhood. *The Journal of Pediatrics*, *98*(1), 37–41.
- Lebensburger, J., Johnson, S. M., Askenazi, D. J., Rozario, N. L., Howard, T. H., & Hilliard, L. M. (2011). Protective role of hemoglobin and fetal hemoglobin in early kidney disease for children with sickle cell anemia. *American Journal of Hematology*, *86*(5), 430–432.
- Silva, C. M., Giovani, P., & Viana, M. B. (2011). High reticulocyte count is an independent risk factor for cerebrovascular disease in children with sickle cell anemia. *Pediatric Blood & Cancer*, *56*(1), 116–121.
- Meier, E. R., Wright, E. C., & Miller, J. L. (2014). Reticulocytosis and anemia are associated with an increased risk of death and stroke in the newborn cohort of the cooperative study of sickle cell disease. *American Journal of Hematology*, *89*(9), 904–906.
- DeBaun, M. R., Sarnaik, S. A., Rodeghier, M. J., Minniti, C. P., Howard, T. H., Iyer, R. V., Inusa, B., Telfer, P. T., Kirby-Allen, M., Quinn, C. T., Bernaudin, F., Airewele, G., Woods, G. M., Panepinto, J. A., Fuh, B., Kwiatkowski, J. K., King, A. A., Rhodes, M. M., Thompson, A. A., Heiny, M. E., Redding-Lallinger, R. C., Kirkham, F. J., Sabio, H., Gonzalez, C. E., Saccente, S. L., Kalinyak, K. A., Strouse, J. J., Fixler, J. M., Gordon, M. O., Miller, J. P., Noetzel, M. J., Ichord, R. N., & Casella, J. F. (2012). Associated risk factors for silent cerebral infarcts in sickle cell anemia: Low baseline hemoglobin, sex, and relative high systolic blood pressure. *Blood*, *119*(16), 3684–3690.
- Switzer, J. A., Hess, D. C., Nichols, F. T., & Adams, R. J. (2006). Pathophysiology and treatment of stroke in sickle-cell disease: Present and future. *The Lancet. Neurology*, *5*(6), 501–512.
- Platt, O. S. (2005). Preventing stroke in sickle cell anemia. *The New England Journal of Medicine*, *353*(26), 2743–2745.
- de Montalembert, M., Aggoun, Y., Niakate, A., Szezepanski, I., & Bonnet, D. (2007). Endothelial-dependent vasodilation is impaired in children with sickle cell disease. *Haematologica*, *92*(12), 1709–1710.
- Ataga, K. I., Moore, C. G., Jones, S., Olajide, O., Strayhorn, D., Hinderliter, A., & Orringer, E. P. (2006). Pulmonary hypertension in patients with sickle cell disease: A longitudinal study. *British Journal of Haematology*, *134*(1), 109–115.
- National Institutes of Health. (2002). The Management of Sickle Cell Disease. [http://www.nhlbi.nih.gov/files/docs/guidelines/sc\\_mngt.pdf](http://www.nhlbi.nih.gov/files/docs/guidelines/sc_mngt.pdf)
- NHS. (2010). Sickle cell disease in childhood, standards and guidelines for clinical care. <http://sct.screening.nhs.uk/getdata.php?id=11164>
- Tounian, P., Aggoun, Y., Dubern, B., Varille, V., Guy-Grand, B., Sidi, D., Girardet, J. P., & Bonnet, D. (2001). Presence of increased stiffness of the common carotid artery and endothelial dysfunction in severely obese children: A prospective study. *Lancet*, *358*(9291), 1400–1404.
- Woywodt, A., Kirsch, T., & Haubitz, M. (2006). Immunomagnetic isolation and FACS-competing techniques for the enumeration of circulating endothelial cells. *Thrombosis and Haemostasis*, *96*(1), 1–1), 2.
- Smadja, D. M., Gaussem, P., Mauge, L., Israël-Biet, D., Dignat-George, F., Peyrard, S., et al. (2009). Circulating endothelial cells: A new candidate biomarker of irreversible pulmonary hypertension secondary to congenital heart disease. *Circulation*, *119*(3), 374–381.
- Sutherland, D. R., Anderson, L., Keeney, M., Nayar, R., & Chin-Yee, I. (1996). The ISHAGE guidelines for CD34+ cell determination by flow cytometry. International Society of Hematology and Graft Engineering. *Journal of Hemotherapy*, *5*(3), 213–226.
- Mauge, L., Sabatier, F., Boutouyrie, P., D'Audigier, C., Peyrard, S., Bozec, E., et al. (2014). Forearm ischemia decreases endothelial colony-forming cell angiogenic potential. *Cytotherapy*, *16*(2), 213–224.

20. Le Manach, Y., Kahn, D., Bachelot-Loza, C., Le Sache, F., Smadja, D. M., Remones, V., et al. (2014). Impact of aspirin and clopidogrel interruption on platelet function in patients undergoing major vascular surgery. *PLoS One*, *9*(8), e104491.
21. Robert, S., Lacroix, R., Poncelet, P., Harhour, K., Bouriche, T., Judicone, C., Wischhusen, J., Arnaud, L., & Dignat-George, F. (2012). High-sensitivity flow cytometry provides access to standardized measurement of small-size microparticles—brief report. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *32*(4), 1054–1058.
22. Fuchs, T. A., Kremer Hovinga, J. A., Schatzberg, D., Wagner, D. D., & Lämmle, B. (2012). Circulating DNA and myeloperoxidase indicate disease activity in patients with thrombotic microangiopathies. *Blood*, *120*(6), 1157–1164.
23. Torsten Hothorn, Hornik, K., Van de Wiel, M. A., & Zeileis, A. (2008). Implementation of a class of permutation tests: the coin package. *Journal of Statistical Software*, *28*(8), 1–23.
24. Ofori-Acquah, S. F., Buchanan, I. D., Osunkwo, I., Manlove-Simmons, J., Lawal, F., Quarshie, A., et al. (2012). Elevated circulating angiogenic progenitors and white blood cells are associated with hypoxia-inducible angiogenic growth factors in children with sickle cell disease. *Anemia*, *2012*, 156598.
25. Patel, S. D., Humphries, J., Mattock, K., Wadoodi, A., Modarai, B., Ahmad, A., Burnand, K. G., Waltham, M., & Smith, A. (2012). Hematopoietic progenitor cells and restenosis after carotid endarterectomy. *Stroke; a Journal of Cerebral Circulation*, *43*(6), 1663–1665.
26. Wierenga, A. T. J., Vellenga, E., & Schuringa, J. J. (2014). Convergence of hypoxia and TGF $\beta$  pathways on cell cycle regulation in human hematopoietic stem/progenitor cells. *PLoS One*, *9*(3), e93494.
27. Lee, P. S. S., & Poh, K. K. (2014). Endothelial progenitor cells in cardiovascular diseases. *World Journal of Stem Cells*, *6*(3), 355–366.
28. Ratajczak, M. Z., Ratajczak, J., Suszynska, M., Miller, D. M., Kucia, M., & Shin, D.-M. (2017). A novel view of the adult stem cell compartment from the perspective of a quiescent population of very small embryonic-like stem cells. *Circulation Research*, *120*(1), 166–178.
29. Smadja, D. M. (2017). Bone marrow very small embryonic-like stem cells: New generation of autologous cell therapy soon ready for prime time? *Stem Cell Reviews*, *13*(2), 198–201.
30. Guerin, C. L., Loyer, X., Vilar, J., Cras, A., Mirault, T., Gaussem, P., Silvestre, J. S., & Smadja, D. M. (2015). Bone-marrow-derived very small embryonic-like stem cells in patients with critical leg ischaemia: Evidence of vasculogenic potential. *Thrombosis and Haemostasis*, *113*(5), 1084–1094.
31. Guerin, C. L., Rossi, E., Saubamea, B., Cras, A., Mignon, V., Silvestre, J.-S., & Smadja, D. M. (2017). Human very small embryonic-like cells support vascular maturation and therapeutic revascularization induced by endothelial progenitor cells. *Stem Cell Reviews*, *13*(4), 552–560.
32. Zhang, S., Zhao, L., Wang, J., Chen, N., Yan, J., & Pan, X. (2017). HIF-2 $\alpha$  and Oct4 have synergistic effects on survival and myocardial repair of very small embryonic-like mesenchymal stem cells in infarcted hearts. *Cell Death & Disease*, *8*(1), e2548.
33. Croizat, H., Ponchio, L., Nicolini, F. E., Nagel, R. L., & Eaves, C. J. (2000). Primitive haematopoietic progenitors in the blood of patients with sickle cell disease appear to be endogenously mobilized. *British Journal of Haematology*, *111*(2), 491–497.
34. Chang, K. H., Nayak, R. C., Roy, S., Perumbeti, A., Wellendorf, A. M., Bezold, K. Y., Pirman M., Hill S. E., Stames J., Loberg A., Zhou X., Inagami T., Zheng Y., Malik P., Cancelas J. A. (2015). Vasculopathy-associated hyperangiotensinemia mobilizes haematopoietic stem cells/progenitors through endothelial AT<sub>2</sub>R and cytoskeletal dysregulation. *Nature Communications*, *6*, 5914.
35. Adams, R. J., Brambilla, D. J., Granger, S., Gallagher, D., Vichinsky, E., Abboud, M. R., Pegelow C.H., Woods G., Rohde E.M., Nichols F.T., Jones A., Luden J.P., Bowman L., Hagner S., Morales K.H., Roach E.S., STOP Study. (2004). Stroke and conversion to high risk in children screened with transcranial Doppler ultrasound during the STOP study. *Blood*, *103*(10), 3689–3694.
36. Solovey, A., Lin, Y., Browne, P., Choong, S., Wayner, E., & Hebbel, R. P. (1997). Circulating activated endothelial cells in sickle cell anemia. *The New England Journal of Medicine*, *337*(22), 1584–1590.
37. Smadja, D. M., Mauge, L., Sanchez, O., Silvestre, J.-S., Guerin, C., Godier, A., Henno, P., Gaussem, P., & Israel-Biet, D. (2010). Distinct patterns of circulating endothelial cells in pulmonary hypertension. *The European Respiratory Journal*, *36*(6), 1284–1293.
38. Levy, M., Bonnet, D., Mauge, L., Celermajer, D. S., Gaussem, P., & Smadja, D. M. (2013). Circulating endothelial cells in refractory pulmonary hypertension in children: Markers of treatment efficacy and clinical worsening. *PLoS One*, *8*(6), e65114.
39. Manzoni, M., Comolli, G., Torchio, M., Mazzini, G., & Danova, M. (2015). Circulating endothelial cells and their subpopulations: Role as predictive biomarkers in antiangiogenic therapy for colorectal cancer. *Clinical Colorectal Cancer*, *14*(1), 11–17.
40. Liu, Y., Yuan, D., Ye, W., Lv, T., & Song, Y. (2015). Prognostic value of circulating endothelial cells in non-small cell lung cancer patients: A systematic review and meta-analysis. *Translational Lung Cancer Research*, *4*(5), 610–618.
41. Zhou, F., Zhou, Y., Dong, J., & Tan, W. (2017). Circulating endothelial cells and their subsets: Novel biomarkers for cancer. *Biomarkers in Medicine*.
42. Noubouossie, D. C. F., Lê, P. Q., Rozen, L., Ziereisen, F., Willems, D., Demulder, A., & Ferster, A. (2013). Thrombin generation in children with sickle cell disease: Relationship with age, hemolysis, transcranial Doppler velocity, and hydroxyurea treatment. *European Journal of Haematology*, *91*(1), 46–54.
43. Bartolucci, P., & Galactéros, F. (2012). Clinical management of adult sickle-cell disease. *Current Opinion in Hematology*, *19*(3), 149–155.