BRIEF REPORT

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Neutrophil extracellular trap regulators in sickle cell disease: Modulation of gene expression of PADI4, neutrophil elastase, and myeloperoxidase during vaso-occlusive crisis

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Abstract

Background: Recent evidence suggests that generation of neutrophil extracellular traps (NETosis), one of the components of immunothrombosis, is associated with the pathogenesis of both venous thromboembolism and sickle cell disease (SCD). NETosis is a complex process regulated by several proteins such as peptidyl arginine deaminase 4 (PADI4), neutrophil elastase (ELANE), and myeloperoxidase (MPO). Among these regulators, PADI4 is responsible of histone citrullination, an essential step for NETosis. Accordingly, its inhibition has been recently cited as a promising therapeutic strategy for diseases such as SCD. Although attractive, this strategy requires supportive evidence of its role in the pathogenesis of SCD.

Patients and Methods: Patients from two independent cohorts were enrolled in this study. Samples were obtained at steady state (53 patients) or during acute episodes of vaso-occlusive crisis (VOC; 28 patients) in patients from cohort 1. mRNA was extracted from granulocytes to analyze *PADI4*, *ELANE*, and *MPO* expression by qPCR. Furthermore, plasma activity of PADI4 was assessed from an independent cohort in 15 patients, within 24 hours from admission for VOC. Race-matched healthy individuals from the same geographic regions were used as controls for each cohort.

Results and Conclusions: Higher levels of gene expression of *PADI4* and *ELANE* were observed during VOC. Furthermore, plasma activity of PADI4 was higher in acute VOC when compared to healthy individuals. These results demonstrate that NETosis regulators are modulated during acute VOC, and pave the way for studies of *PADI4* inhibition as a therapeutic strategy for acute VOC in SCD.

Essentials

- Generation of neutrophil extracellular traps (NETosis) is a relevant trigger of the pathogenesis in sickle cell disease (SCD).
- Expression and activity of NETosis regulators were evaluated in two cohorts of SCD.
- Gene expression of *PADI4*, *ELANE*, and *MPO* was increased during SCD vaso-occlusive crisis.
- PADI4 enzymatic activity was also increased during SCD vaso-occlusive crisis.

1 | INTRODUCTION

Sickle cell disease (SCD) is a chronic hemolytic and multisystemic disorder characterized by chronic inflammation and a natural propensity to thrombotic events.[1-4] Recent findings suggest that hemostatic activation is a relevant mechanism in the pathogenesis of this disease.[5-8] This activation is part of a broader response to tissue injury involving innate immunity, hemostasis, and several proinflammatory processes that have been recently grouped under the umbrella term *immunothrombosis*.[9] This complex response, triggered by both pathogen-associated molecular patterns and danger-associated molecular patterns, is believed to contribute to an effective immune response to pathogens or tissue damage, by providing transient molecular cues for the recruitment of phagocytes into injured tissues.[7,10] However, if activated in a deregulated fashion, these same cellular programs are believed to support secondary tissue damage involving micro- and macrovascular thrombotic manifestations in inflammatory diseases.[9,11,12] Interestingly, neutrophils, monocytes, and platelets, which are critical in the pathogenesis of vaso-occlusive crisis (VOC) in SCD, are also key elements in immunothrombosis.[13,14]

One of the main elements of immunothrombosis is the release of neutrophil extracellular traps (NETs) by activated neutrophils, a process that has been associated with the pathogenesis of SCD in both animal models[15] and in humans,[16] with SCD patients presenting higher levels of markers of NET generation (NETosis) during acute VOC. More recently, it has also been demonstrated that proinflammatory cytokines are associated with NETosis during VOC crisis but not in steady state.[13] NETs are composed of DNA, histones, and antimicrobial components such as neutrophil elastase (ELANE) and contribute to pathogen clearance from the host. However, these components can also trigger thrombosis and inflammation in poorly regulated responses.[17] Accordingly, NETosis is a finely regulated process that involves the participation of several proteins such as peptidyl arginine deaminase 4 (PADI4), ELANE, and myeloperoxidase (MPO).

Among these regulators, PADI4 is responsible for histone citrullination, an essential step and initiator of NETosis. PADI4 knockout model animals have been shown to be protected from NETosis and from some thrombotic complications.[18,19] However, little is known about the role of PADI4 deregulation or its genetic modifications in SCD.[20] Upon activation, neutrophils undergo dynamic alteration of their cellular compartments and release reactive oxygen species (ROS) that promote membrane degradation and liberation of granule components such as ELANE and MPO. Before NET formation, ELANE released from azurophilic granules degrades F-actin to arrest actin dynamics[21] and is subsequently translocated to the nucleus,[22] where it degrades histone H1 linkers. By targeting histone H1, ELANE promotes chromatin decondensation,[22] facilitating PADI4 enzymatic action and nuclear component release. Evidence suggests that MPO is required for ELANE translocation to the nucleus,[21] contributing to chromatin partial degradation.[23]

Based on its importance in NETosis regulation, PADI4 inhibition has been recently cited as a potential therapeutic strategy for diseases in which NETosis could play a relevant pathogenic role.[19,24] Although attractive, the use of PADI4 inhibitors in SCD requires direct evidence of its role in the pathogenesis of this disease. Herein, we used two independent cohorts of patients to investigate the expression of NETosis regulators (*PADI4*, *ELANE*, and *MPO)* in SCD at both steady state and during acute VOC, and to assess whether PADI4 activity is increased during acute VOC.

2 | MATERIALS AND METHODS

2.1 | Patients and healthy volunteers

This was a cross-sectional study that included patients with SCD recruited in two independent centers in Brazil. Patients from cohort 1 were recruited at the Hematology and Hemotherapy Foundation of Pernambuco (HEMOPE) in either steady state (ie, at least 3 months from the last acute VOC), or within 24 hours from admission for an acute VOC. These patients provided samples for gene expression studies. Patients from cohort 2 were recruited at Hematology and Hemotherapy Foundation of Amazonas State (HEMOAM) within 24 hours from admission for acute VOC, as part of a previous study that obtained plasma samples to measure total heme levels during acute VOC. Accordingly, only plasma samples were available from patients from cohort 2. Race-matched ethnic healthy individuals were recruited independently for each study, from the same geographic region of each cohort. All of the healthy donors were homozygous for hemoglobin genotype and did not present any previous history of arterial or venous

thrombotic diseases. Furthermore, they were free of inflammatory or infectious diseases before sampling. The study was performed in accordance with the Declaration of Helsinki and approved by the Ethics Committees of State University of Campinas (CAAE: 46 853 115.4.0000.5404), HEMOPE (CEP: 13 175-667) and HEMOAM (CAAE: 71 147 817.3.0000.0009). Each participant provided written informed consent form before sample collection. Samples were immediately preprocessed at each site of sample collection, and only retro-transcribed cDNA (cohort 1) or plasma (cohorts 1 and 2) were transported to University of Campinas in dry ice.

2.2 | Quantitative PCR of NETosis regulators

Whole blood samples were obtained from healthy volunteers and patients from cohort 1 in tubes containing EDTA anticoagulant. Granulocytes were then isolated by two Ficoll gradients (Histopaque®-1.119, Histopaque®-1.077; Sigma-Aldrich, St. Louis, MO, USA). Briefly, Histopaque gradient was obtained in a centrifuge tube containing a layer of 3 mL of each Histopaque media. Then, 6 mL of diluted blood samples (dilution v/v with phosphate buffered saline 1X) were carefully layered onto the Histopaque and were centrifuged at 700 g for 30 minutes at 20°C with the brake turned off. After separation, granulocytes were collected, washed, and RNA extraction was then performed using the Trizol method; 1 μg of total RNA was used to perform cDNA transcription. Gene expression of *PADI4*, *ELANE*, and *MPO* were measured by quantitative PCR (qPCR). Cq values were obtained and normalized using the geometric mean of *ACTB* and *GAPDH* as reference genes.[25]

2.3 | PADI4 activity

Whole blood samples were collected in sodium citrate anticoagulant containing tubes from patients and healthy volunteers of cohort 2. Platelet-poor plasma was obtained after centrifugation (2000 g for 20 minutes) and used to measure PADI4 activity using a commercial kit (PADI4, PAD4 Inhibitor Screening Assay Kit; Cayman Chemical Co., Ann Arbor, MI, USA).

2.4 | D-dimer and von Willebrand factor plasma levels

D-dimer was measured in platelet-poor plasma obtained as describe above, from blood samples collected in 0.109M sodium citrate–containing tubes. The assay was performed using VIDAS D-dimer Exclusion kit (bioMérieux, Marcy l'Etoile, France) which is based on an automated two-step fluorescence test following the fabricant recommendations. Von Willebrand factor (VWF) antigen levels were determined in plasma by an in-house ELISA, as previously described.[26]

2.5 | Statistical Analysis

Results are presented as the median of each variable. Statistical analysis was performed using Prism 6 (GraphPad Software Inc., San Diego, California, USA). Mann-Whitney U tests were used to compare patients during VOC with healthy controls. Kruskal-Wallis tests were performed with Dunn postcomparison correction to compare gene expression among three groups. We also carried out a multivariable linear regression analysis of NETosis modulator expression and PADI4 plasma activity adjusting for white blood cell (WBC) count and absolute neutrophil count (ANC), respectively, using R, version 3.6.3 (R Foundation for Statistical Computing, Vienna, Austria). A P value < .05 was considered statistically significant.

3 | RESULTS AND DISCUSSION

In total, 53 patients in steady-state (51 with genotype SS and 2 SC), 28 in acute crisis (22 with genotype SS and 6 SC), and 40 healthy volunteers were recruited from cohort 1. In addition, 15 patients with genotype SS and 20 healthy volunteers were recruited from cohort 2. Clinical and laboratory characteristics of these patients are described in Table 1. Blood samples were obtained at admission before any treatment or blood transfusion.

qPCR analysis showed that the patients expressed higher levels of *PADI4* during VOC when compared to both healthy volunteers and patients in steady state (Figure 1A). No significant difference was observed between healthy volunteers and patients in steady-state (Figure 1A). To our knowledge, this is the first report addressing *PADI4* expression in a cohort of SCD. Our results suggest that *PADI4* could be involved in the pathogenesis of SCD during acute VOC and are in agreement with a recent study showing that NET production is higher when healthy neutrophils are incubated with plasma from SCD patients during VOC when compared to plasma obtained at steady state or from healthy volunteers.[13] Our group recently demonstrated, in a different cohort of patients with SCD, an association between single-nucleotide polymorphisms in the *PADI4* gene, which modify the expression of this gene, with the risk of acute chest syndrome.[20] Accordingly, even though our present results do not represent a direct demonstration of the contribution of PADI4 to NET release in SCD, we believe that they provide additional support to the concept that the upregulation of *PADI4* gene expression could be involved in the pathogenesis of SCD.

We also analyzed the expression of other genes involved in NETosis, namely, *MPO* and *ELANE*. As shown in Figure 1B and C, both were increased during acute VOC when compared to steady-state and healthy individuals (Figure 1 B and C). Evidence of increased neutrophil activation during acute VOC has been previously demonstrated, as patients in this condition present increased plasma level of elastase-α1-antitrypsin complexes. Interestingly, levels of these complexes were positively correlated with nucleosome levels.[16] In

TABLE 1 Clinical and laboratory characteristics of the study population

	Cohort 1				
	Healthy volunteers (n = 40)	Steady state (n = 53)	P^b	Acute VOC (n = 28)	P^c
Age, y	25 (23-30)	31 (25-38)	.004	28 (24-37)	.1
Sex, male/female[a]	19/21	29/24	.77	14	.83
Hemoglobin, g/dL	13.9 (12.7-15.6)	7.6 (6.9-8.8)	< .0001	8.1 (7.1-9.3)	< .0001
HbF %	-	6.5 (4.0-11.9)	-	6.5 (5.2-11.5)	.7[d]
Platelets, ×10⁹/L	252 (210-274)	338 (274-436)	< .0001	398 (311-508)	< .0001
ANC, ×10⁹/L	3.3 (2.11-4.65)	N/D	-	6.4 (5.0-9.6)	< .0001
WBC, ×10⁹/L	6 (4.7-8.0)	11.1 (9.4-14.5)	< .0001	13.9 (11.1-18.7)	< .0001
Cohort 2					
	Healthy volunteers (n = 20)	VOC admission (n = 15)	P		
Age, y	29.5 (25.25-38.75)	27.0 (15-34)	.17		
Sex, male/female [a]	7/13	7/8	.48		
Hemoglobin, g/dL	13.5 (13.03-15.43)	7.6 (6.5-8.5)	< .0001		
Platelet count, ×10⁹/L	277.5 (218.3-314.8)	411.0 (377-567)	< .0001		
ANC, ×10⁹/L	4 (3.32-4.7)	7.4 (5.46-9.6)	.0002		
WBC, ×10⁹/L	6.5 (5.77-7.39)	12.3 (9.15-16.1)	< .0001		

Note: Variables are presented as median (IQR).

Abbreviations: ANC, absolute neutrophil count; HbF, fetal hemoglobin; IQR, interquartile range; N/D, not available; VOC, vaso-occlusive crisis; WBC, white blood cell.

[a]Chi-square test.

[b]Comparison healthy volunteers versus steady state.

[c]Comparison healthy volunteers versus acute VOC.

[d]Comparison of HbF steady state versus acute VOC.

addition, both the expression[27,28] and the activity[29] of MPO, whose upregulation is crucial to the oxidative burst of neutrophils during inflammation, have been described in SCD. Accordingly, our results provide additional evidence of neutrophil activation in acute VOC in SCD. To account for the potential influence of differences in WBC counts between healthy individuals and patients with SCD (Table 1) in our gene expression assays, we carried out a multivariate analysis to adjust for this parameter. Of note, *PADI4* expression remained significantly elevated during VOC when compared to both steady-state and healthy individuals, supporting the relevance of this finding. Upregulation of *ELANE* was also confirmed during VOC when compared to healthy individuals, but not when compared to steady state. In contrast, no significant difference was observed in *MPO* expression between patients and healthy individuals after adjustment for WBC count.

We also analyzed the correlation between mRNA expressions of these three NETosis regulators with biomarkers of hemostatic activation in steady-state patients, since NET release promotes coagulation and endothelial activation as part of the immunothrombotic process in several inflammatory conditions, including SCD.[9,30] Of note, no association could be observed between PADI4 expression and these biomarkers, while only weak/moderate correlations were observed for *ELANE* and *MPO*, which were correlated with D-dimer

(R = 0.4, P = .004; and R = 0.32, P = .004, respectively), and with VWF levels (R = 0.27, P = .04; and R = 0.30, P = .04, respectively). These results support a modest relationship between *ELANE*, *MPO*, and immunothrombosis during steady state, and are in accordance with our observation that *PADI4* mRNA expression is increased only during VOC, and with other studies demonstrating higher levels of NET biomarkers only during VOC in SCD.[16] Additional studies are warranted to explore the association between PADI4 expression and coagulation and endothelial activation during acute VOC in SCD.

We then investigated whether PADI4 activity was also increased during acute VOC, in plasma samples from a different cohort. As shown in Figure 2, PADI4 activity increased during acute VOC when compared to healthy individuals (Figure 2). These results were also adjusted for ANC, and there was no association between ANC and PADI4 activity (ß estimate = 0.001; P = .1).

While limited by a relatively small sample size, our results represent, to our knowledge, the first direct evidence that PADI4 levels are modulated during acute VOC in SCD. Modulation of PADI4 expression and/or activity might contribute to immunothrombosis in SCD by promoting NET release.[31] Moreover, if NET release in SCD is confirmed to be PADI4 dependent, PADI4 inhibition can be explored as a therapeutic target for this condition.[24] However, it should be noted that NETosis regulation also involves PADI4-independent

(A) P = 0.0002, P < 0.0001

(B) P < 0.0001, P = 0.04, P < 0.0001

(C) P = 0.002, P = 0.009

(D)

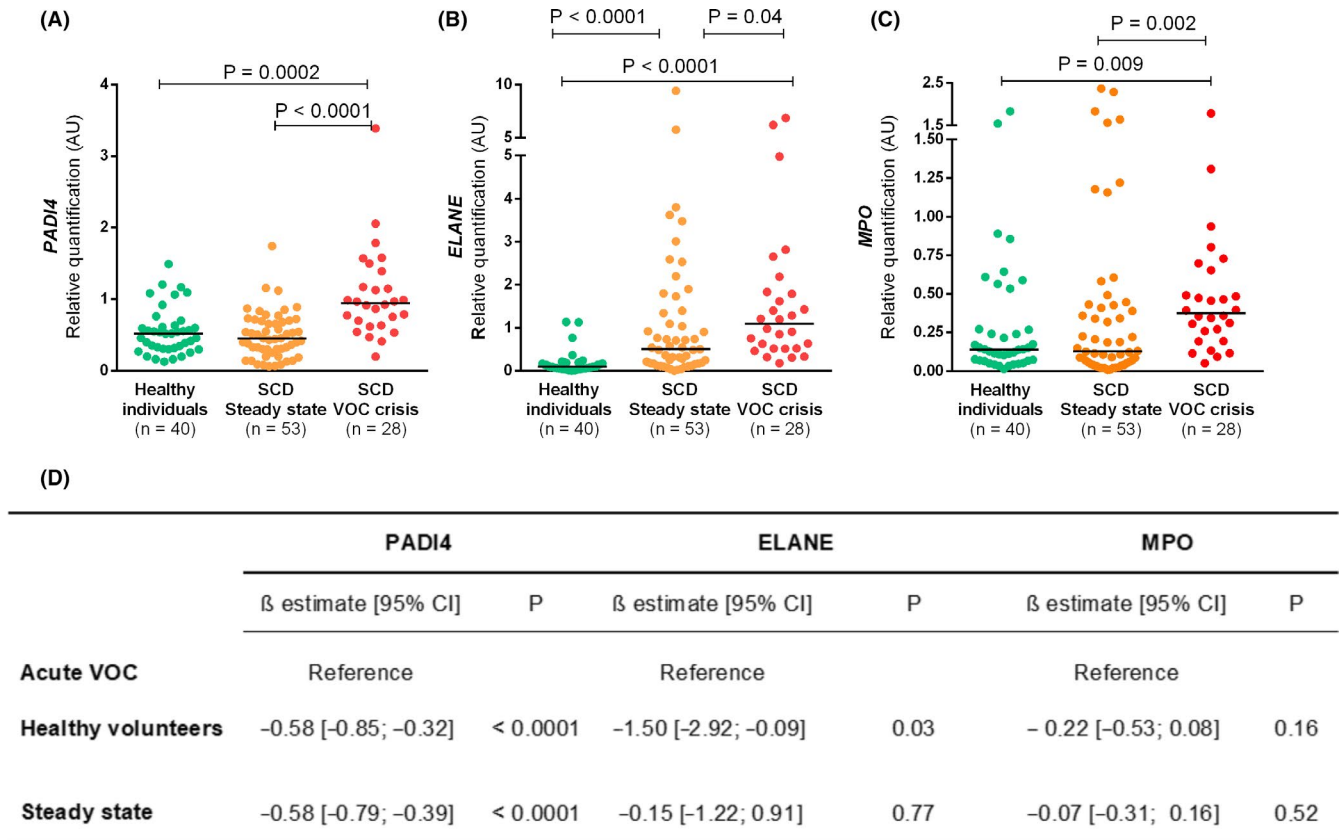|	PADI4		ELANE		MPO	
	ß estimate [95% CI]	P	ß estimate [95% CI]	P	ß estimate [95% CI]	P
Acute VOC	Reference		Reference		Reference	
Healthy volunteers	−0.58 [−0.85; −0.32]	< 0.0001	−1.50 [-2.92; −0.09]	0.03	− 0.22 [−0.53; 0.08]	0.16
Steady state	−0.58 [−0.79; −0.39]	< 0.0001	−0.15 [-1.22; 0.91]	0.77	−0.07 [−0.31; 0.16]	0.52

FIGURE 1 Gene expression of NETosis regulators *PADI4*, *ELANE*, and *MPO* in granulocytes from patients with SCD and healthy individuals. Results are presented as median relative quantifications of gene expression normalized by *ACTB* and *GAPDH* as reference genes. Both *PADI4* (A) and *MPO* (C) mRNA expression increased only during acute VOC when compared with healthy volunteers. In contrast *ELANE* (B) mRNA expression increased in both VOC and steady state when compared with healthy volunteers. Kruskal-Wallis test was performed with Dunn postcomparison correction. The numbers of patients studied are indicated in each panel. (D) Regression coefficients from multivariable linear regression analysis of the association of patient group with gene expression of each NETosis regulator, adjusted for white blood cell count. CI, confidence interval; ELANE, neutrophil elastase; MPO, myeloperoxidase; PADI4, peptidyl arginine deaminase 4; SCD, sickle cell disease; VOC, vaso-occlusive crisis
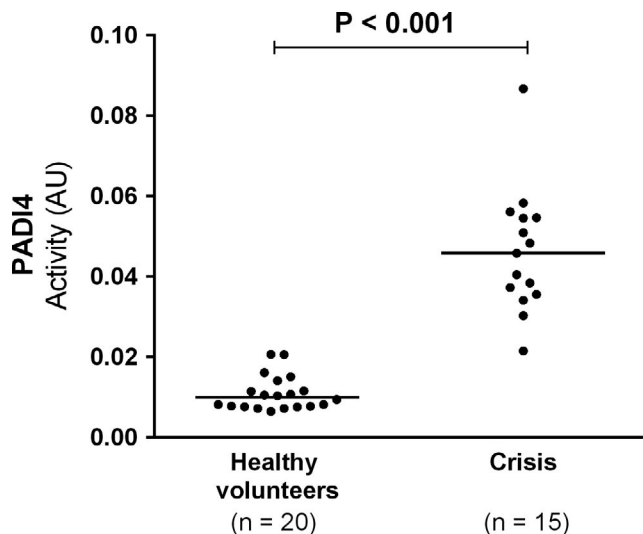

FIGURE 2 PADI4 plasma activity at admission for an acute vaso-occlusive crisis was compared with healthy volunteers. Horizontal bars indicate median PADI4 activity. Mann-Whitney test was performed. AU, arbitrary unit; PADI4, peptidyl arginine deaminase 4

pathways in some specific settings (eg, specific pathogens), which include the production of reactive oxygen species (ROS) by mitochondria or nicotinamide adenine dinucleotide phosphate (NADPH) oxidase.[32,33] While SCD environment is a potent trigger of NADPH oxidase–mediated ROS production, there is no evidence that SCD is a condition in which NET release is independent of PADI4 activation. Moreover, NET release has already been shown to be PADI4 dependent in several other inflammatory diseases such as diabetes[17] and can also involve both pathways.[34] Experiments using PADI4 knockout and SCD mice would be important to answer this question. Finally, since infections are very common in patients with SCD, and NET release is an important element of innate immunity,[35] studies involving PADI4 inhibition in the context of SCD should also investigate whether this strategy increases risk of infections. This concern was addressed in a recent study that reported that PADI4 deficiency did not increase host vulnerability to bacterial infections.[36]

This study has limitations that need to be considered. First, there was significant heterogeneity between the two cohorts in regard to hydroxyurea use, which can have complex and heterogeneous effects in inflammation in SCD, including a decrease in ROS generation

TABLE 2 Subgroups of patients with acute VOC from cohort 1 stratified by hydroxyurea use

	No hydroxyurea (n = 15)	Hydroxyurea users (n = 13)	P
Hemoglobin, g/dL	8.2 (7.6-9.9)	8.1 (6.65-9.05)	.84
HbF %	6.7 (5.3-9.7)	6.3 (3.25-12.5)	.93
ANC, $\times10^9$/L	6.3 (4.99-9.95)	6.4 (4.33-9.46)	.77
WBC, $\times10^9$/L	14.4 (11.6-16.6)	12.1 (11.1-18.75)	.6
PADI4 mRNA, AU	0.97 (0.62-1.15)	0.91 (0.72-1.68)	.7
ELANE mRNA, AU	1.28 (0.63-2.65)	0.75 (0.38-1.50)	.1
MPO mRNA, AU	0.36 (0.27-0.65)	0.39 (0.16-0.61)	.8

Note: Variables are presented as median (IQR).

Abbreviations: ANC, absolute neutrophil count; AU, arbitrary unit; HbF, fetal hemoglobin; IQR, interquartile range; WBC, white blood cell.

and free heme levels,[37] and hence influence NETosis. While all patients from cohort 2 were under hydroxyurea treatment, only a subgroup of patients from cohort 1 were hydroxyurea users (13/28 patients). Accordingly, we performed a subgroup analysis of patients with acute VOC from cohort 1 and demonstrated that they were similar in regard to baseline factors (hemoglobin, ANC, WBC, and platelets), and that no significant difference could be observed in gene expression levels of *PADI4*, *ELANE* and *MPO* between these two subgroups (Table 2). However, this subgroup comparison should be interpreted with caution considering the small sample size, low power to demonstrate significant differences, and the fact that variations in dose and adherence to hydroxyurea therapy could also have influenced these results.

A second limitation is the fact that gene expression and functional studies were not measured in both cohorts. While the use of different methods strengthens our conclusions about PADI4 modulation during acute VOC, an independent validation of each of these observations is lacking. Of note, an analysis of a public database (GSE139912) of RNA sequencing data from patients with SCD during acute VOC also demonstrated an increase in *PADI4* expression (Figure S1), although these data were obtained from whole blood RNA, while in our study RNA was obtained from neutrophils. Third, our data do not exclude the possibility that upregulation of PADI4 activity is not specific to acute VOC and could be observed in other forms of acute illness. Studies exploring PADI4 modulation in other acute conditions are warranted to address this question. Finally, data on the association of NETosis regulators with a more extensive panel of markers of inflammatory and hemostatic activation, also including samples from acute VOC, could provide additional insights into the role of PADI4 in immunothrombosis in SCD.

In summary, we have demonstrated that the mRNA expression of NETosis regulators, including *PADI4* is increased in SCD during acute VOC but not in steady state. PADI4 protein activity was also increased in an independent cohort during acute VOC, supporting the concept that PADI4 inhibition could be explored as a therapeutic target for SCD during acute VOC. Additional studies are warranted to confirm these results in independent populations and further elucidate their clinical implications.

RELATIONSHIP DISCLOSURE

AUTHOR CONTRIBUTIONS

BWH and FC performed experiments; BWH analyzed and interpreted data and drafted the manuscript; IFD, MJVCS, ASA, ARLANAF, MCB, and MNNS recruited and preprocessed patients from the SCD cohort 1; ECC, PVSN, AM, and NAF recruited and preprocessed patients from the SCD cohort 2, FFC contributed with reagents and laboratory infrastructure; EV designed the study, reviewed and analyzed data, and drafted the manuscript. All authors revised and approved all submitted versions of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.