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Association of Circulating Procoagulant Microvesicles with Painful Vaso-Occlusive Crisis in Sickle Cell Disease

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Keywords
Coagulation · Crisis · Microvesicles · Sickle cell disease

Abstract

Introduction: Thrombotic complication is one of the features of sickle cell disease (SCD), characterized by appearance of phosphatidylserine on the outer membrane of sickle-shaped red blood cells and most abundantly on membrane protrusions called microvesicles (MVs). However, the exact mechanism by which MVs may enhance coagulant activity in SCD patients has not been fully addressed. The aim of this study was to further investigate the procoagulant activity of circulating MVs in sickle cell crises. ***Materials and Methods:*** Subjects included in this cross-sectional study were 47 patients with SCD and 25 normal subjects with written informed consent obtained from all the participants. MV analysis was conducted by using CD61, CD235α, and Annexin-V monoclonal antibodies. The coagulant activity of MVs was determined by an ELISA-based procoagulant activity assay. ***Results:*** The majority of MVs were originated from platelets (CD61+) and erythrocytes (CD235+). These MVs demonstrated significantly enhanced levels during the painful crisis when compared with the steady-state period ($p < 0.001$) and controls ($p < 0.001$). Also, the procoagulant activity of MVs was significantly higher in crisis compared to those of steady state ($p < 0.001$) and positively correlated with the number of Annexin-V+ MVs ($p < 0.001$). Significant correlations were found between erythrocyte-derived MVs with hemolysis marker ($r = 0.51$, $p < 0.001$) and the hemoglobin level ($r = -0.63$, $p < 0.001$). ***Conclusion:*** The numbers of platelet- and erythrocyte-derived MVs are related to painful crisis, and their quantification in SCD may be helpful for identifying cases at increased risk of thrombotic complications.

Introduction

Sickle cell disease (SCD) is a type of hemoglobinopathy which is caused by a single nucleotide mutation in exon I of the β-globin gene and results in replacement of glutamic acid at amino acid position 6 by Valin (β6Glu→Val) [1]. The most severe form of SCD is homozygosity for the β^S allele. Under deoxygenated conditions, HbS molecules polymerize in erythrocytes and the affected cells are shaped like a sickle or a crescent moon. These fragile and rigid cells can get stuck in small blood vessels, resulting in either reducing or blocking the blood flow and the delivery of oxygen to the respective parts of the body [2, 3]. Recurrent HbS polymerization leads to chronic hemolysis and persistent vaso-occlusive episodes as the signature hallmark of SCD [3]. Additionally, SCD might get more complicated through chronic endothelial and coagulation activation, generating a hypercoagulable state [4]. It has become increasingly clear that chronic hemolysis and phosphatidylserine (PS) exposure on erythrocytes play a key role in triggering coagulation and oth-

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er sickle cell-related complications [5]. The vaso-occlusive episodes in SCD are unpredictable, and currently, there is no laboratory biomarker to precisely correlate the clinical symptom with the disease status. Some studies have been suggested a possible contribution between circulating microvesicles (MVs) and the hypercoagulable state in SCD [6–8].

MVs are submicron membrane-derived fragments (0.1–2.0 μm) that are shed from the surface of various cells including erythrocytes, platelets, leukocytes, and endothelial cells through budding upon activation or during apoptosis [9]. Phospholipids are the main elements of the cell and MVs' membranes. Upon cellular activation, cytosolic calcium increases and the leaflet membrane loses its distribution, leading to the exposure of PS on the outer surface and MVs formation [10]. The expression of these molecules plays a pivotal role in the activation of tenase and prothrombinase coagulation complexes and the subsequent elevation in thrombin generation [11]. In addition, MVs are considered to have other biological functions such as involvement in inflammation, vascular function, and angiogenesis [12]. Elevated numbers of circulating MVs have been reported in a broad spectrum of pathological conditions with vascular involvement and hypercoagulability such as SCD, suggesting that these MVs might be responsible for the adverse thrombotic events [13]. There is evidence indicating that MVs may be a useful prognostic and diagnostic biomarker in patients with SCD [14]. Despite recent improvements, there is still a lack of consensus among relevant studies to determine the exact mechanism by which circulating MVs trigger coagulation in SCD [15]. Therefore, in order to better characterization of various MVs populations and their clinical significance, more research is needed on circulating cell-derived MVs in different diseases.

In the present study, we established the cellular origin of circulating MVs and their procoagulant activity in patients with SCD during crises and steady-state periods. We also explored the relation of these MVs with blood cell count, hemolysis marker, and coagulation processes.

Materials and Methods

Subjects

In this cross-sectional study, we recruited 47 patients with SCD including 29 males and 18 females with a male-to-female ratio of 1.6:1. Patients were referred from the *Jahrom University of Medical Sciences* from April 2019 to March 2021. Forty-one patients were from both the individuals with pain crisis and the ones having stable conditions (baseline). Baseline was defined as a period without pain or any acute events for at least 4 weeks prior to blood sampling as well as being blood transfusion-free for at least 3 months before sampling. Six patients did not experience any painful crisis during the study period. Two blood samples were collected from patients, the first one during a painful crisis and the second during the steady state. Twenty-five sex- and age-matched healthy volunteers were enrolled as a control group, 15 males and 10 females (male-to-female ratio, 1.5:1). Written informed consent was obtained from all the patients and healthy controls prior to study inclusion. This study was approved by the Ethics Committee for the *Jahrom University of Medical Sciences* (IR.JUMS.REC.1398.081) and performed in accordance with the Declaration of Helsinki.

Blood Sample Collection

Peripheral blood samples were collected from the antecubital vein through a 21-gauge needle (BD Vacutainer needles). Blood samples were collected in ethylene diamine tetra-acetic acid (1.5 mg/mL) anticoagulant for complete blood count analysis. For evaluation of MVs by flow cytometry, blood samples were collected in sodium citrate (0.2 mL 3.8%) vials in a ratio of nine volumes of blood to one volume of anticoagulant. Serum samples for biochemical analyses were separated by centrifugation of clotted samples for 10 min at 1,200 *g*. To prevent in vitro formation of cell-derived MVs, cells were removed by centrifugation (10 min at 1,550 *g*) within 30 min after blood collection. The platelet-poor plasma prepared in this way is practically free of leukocytes and erythrocytes.

Reagents and Assays

Fluorescein isothiocyanate (FITC)-labeled anti-human CD235a, phycoerythrin (PE)-labeled anti-human CD61, FITC-conjugated Annexin-V, and appropriate isotype controls were obtained from Becton Dickinson (BD, San Jose, CA, USA). Complete blood counts were determined using an automated hematology analyzer (Sysmex XT 2000i; Diamond Diagnostics, USA). The procoagulant activity of MVs was measured by enzyme-linked immunosorbent assay (ELISA) kit (Zymuphen MP-activity; Hyphen BioMed, Neuville-sur-Oise, France). Assays were performed as described by the manufacturer.

MV Isolation

Patients and control samples were evaluated for the presence of circulating cell-derived MVs (platelet MVs, erythrocyte MVs, Procoagulant MVs). Initially, MVs were isolated from the samples using centrifugation. For this purpose, plasma was mixed with PBS, pH 7.4 (ratio, 5:1), and centrifuged at 2,000 *g* for 10 min at 10°C. The supernatant was centrifuged again with the same protocol to exclude residual cells and debris. The centrifuge brake was set to "off" to prevent mixing cell fragments with the supernatant. The top two-thirds of the double centrifuged plasma was then removed and stored at −70°C until later use.

MV Enumeration by Flow Cytometry

Flow cytometric analysis was performed with CyFlow Space flow cytometer (Partec PAS, Germany) using Flomax software. In this study, flow cytometric analysis was performed with a precise number of standard beads to determine MVs count, and specific conjugated antibodies were used to determine the origin cell of the MVs. Event size was identified using Fluoresbrite® Carboxylate Microspheres 1.0 μm (Polysciences, Warrington, Philadelphia).

FITC-labeled anti-human CD-235a (glycophorin A) (BD Pharmingen, San Diego, CA, USA) and PE-labeled anti-human CD61 (BD) were used to tag erythrocyte- and platelet-derived MVs, respectively. In addition, FITC-labeled Annexin-V (BD) was used to label PS on procoagulant MVs. As a negative control, mouse IgG2b k isotype controls (BD) were also used to differentiate the background noise of the cytometric analysis.

Table 1. Patients and healthy control characteristic

	Painful crisis	Steady state	Control	*p* value
n	41	47	25	
Sex ratio (M/F)	25/16	29/18	15/10	
Age, year	27.1 (14–39)	27.5 (14–40)	28 (17–38)	0.75
Specified genotype, %				
Hb SS	73.17	74.46	0	
S/$\beta^{0/+}$ thalassemia	26.83	25.54	0	
Blood parameters				
Hemoglobin, gr/dL	9.6 (7.6–10.9)	9.8 (7.9–10.9)	14.3 (11.0–17.1)	0.001
Hematocrit, %	29.9 (24.1–34.3)	31.1 (25.1–34.8)	42.1 (36.1–48.4)	0.001
Retic count, %	4.6 (3.9–6.6)	6.8 (4.2–10.6)	0.95 (0.91–1.4)	0.001
Leukocyte, $\times 10^3$/μL	9.9 (8.1–14.3)	8.0 (6.1–13.2)	6.8 (4.7–9.3)	0.01
Platelet, $\times 10^3$/μL	299 (105–410)	251 (143–363)	259 (179–418)	0.01
LDH, IU/L	422 (268–512)	297 (240–430)	208 (138–274)	0.001

The cellular origin and concentration of MVs were determined as follows; supernatants containing MVs (50 μL) were labeled with anti-CD235α (5 μL), anti-CD61 (5 μL), and PE-IgG2b isotype control (5 μL) for 30 min at 4°C in the dark. In addition, 50 μL of samples were added to test tubes containing 300 μL of binding buffer (0.1 M HEPES/pH 7.4, 25 mM $CaCl_2$, and 1.4 M NaCl), and subsequently FITC-conjugated Annexin-V (5 μL) was added. After 30 min of incubation (22°C in the dark), samples were analyzed with a flow cytometer. Before analysis, 5 μL of well-mixed 1.0 μm beads that were diluted in double-distilled water (1:500) was added to each sample [9].

The concentration of MVs was calculated by comparison to the bead concentration, and its number was measured concerning ten thousand bead events. To differentiate true events from the background noise, MVs were defined by size (less than 1.0 μm), cell origin (CD61, CD235α), and PS exposure (Annexin-V positive) as previously described [16, 17]. The absolute count of MVs per microliter was calculated as follows:

$$\text{MVs per } \mu\text{L} = \frac{\text{No. of events in gating containing MVs} \times \text{Absolute count of bead per tube}}{\text{No. of events in bead region} \times \text{test volume}}$$

MV Procoagulant Activity

The procoagulant activity of MVs was detected using a functional ELISA method (Zymuphen MP-activity; Hyphen BioMed) which was intended to measure PS expression. A micro-ELISA plate was pre-coated with Annexin-V-streptavidin to capture the cell-derived MVs. The calibrator (100 μL) and 1:20 diluted samples (100 μL) containing calcium, Factor Xa, and thrombin inhibitors were added to the plate wells and then incubated for 1 h at 37°C. Following washing steps, unbound materials were washed away.

Then, a mixture of clotting factors including FXa, FVa, calcium (100 μL), and prothrombin (50 μL) were added to the plate wells. Normally, procoagulant MVs in the sample bind to Annexin-V through PS and allow FXa-FVa to convert prothrombin to active thrombin. Therefore, following to incubation period of 10 min at 37°C, and addition of chromogenic substrate (50 μL) as well as acid citric as stopping reagent (2%, 50 μL), the amount of generated thrombin or FIIa that correlated with the number of procoagulant MVs was measured and the color was read at λ= 405 nm. Results were calculated according to the calibration curve and expressed as nano-molar PS equivalents.

Statistical Analysis

Data are presented as mean and standard deviation (SD). Also, categorical data are presented as either percentages or numbers. For data analysis, paired *t* test, Pearson correlation, and repeated measure ANOVA were used. For all statistical analyses, Stata software, version 16 (Stata Corp, College Station, TX, USA), was applied. Graphs were depicted by Excel and Stata software. The statistically significant difference was considered as p value <0.05.

Results

Patients

The patients and healthy control characteristics are presented in Table 1. The mean age of SCD patients in painful crisis and steady state were 27.1 ± 10.6 (range: 14–39 years) and 27.5 ± 11.1 years (range: 14–40 years), respectively, while this value was 28 ± 9.4 years (range: 17–38 years) for controls. Thirty-five patients had homozygote HbS disease, and 12 patients had sickle β-thalassemia (genotype S/$\beta^{+/0}$). In this study, none of the patients were treated with chronic transfusion therapy, nor did they develop complications such as acute chest syndrome, renal failure, or sepsis.

Blood Parameters

Patients with SCD were anemic and exhibited lower hemoglobin and hematocrit levels in relation to healthy controls ($p < 0.001$). Also, when compared to healthy controls, a number of blood parameters such as reticulocyte, leukocyte, and platelet counts, as well as lactate dehydrogenase (LDH) as a reliable hemolysis index, were significantly higher in SCD patients ($p < 0.001$). Additionally, the LDH levels were higher in painful crisis conditions (422 ± 68 IU/L) compared to steady-state (297 ± 41 IU/L) and control (208 ± 32 IU/L) groups ($p < 0.001$) (Table 1).

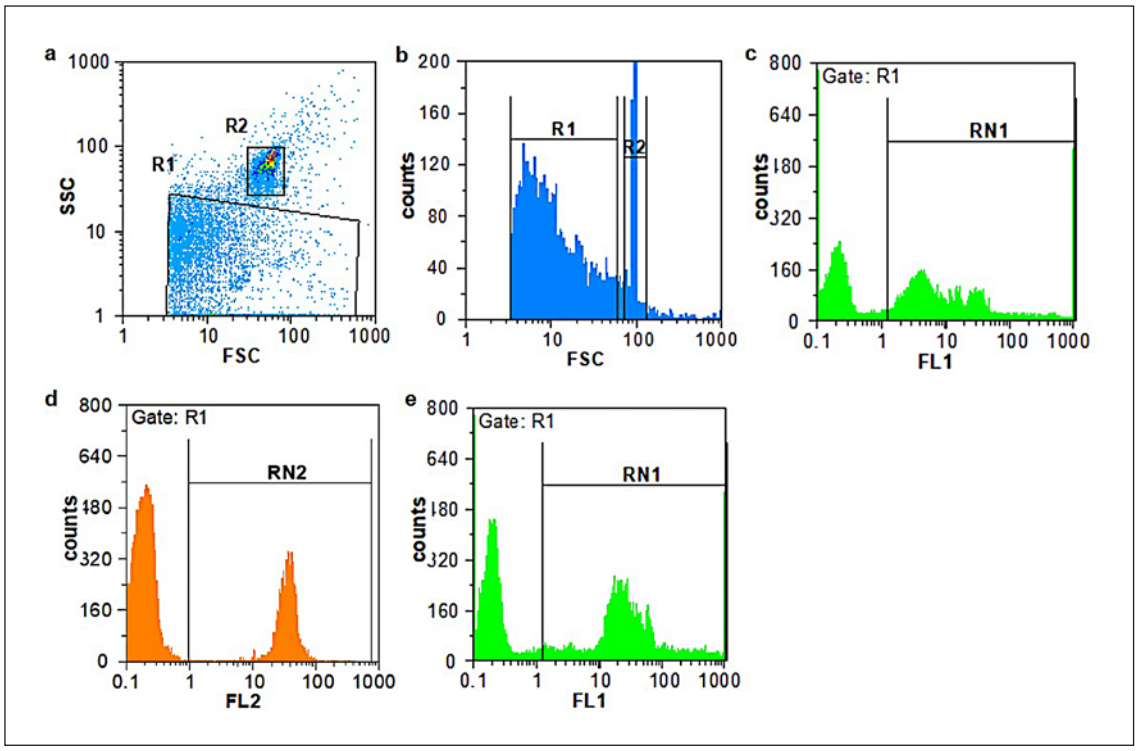

Fig. 1. Flow cytometry plots of MVs in SCD patients. FSC and SSC indicate, respectively, size and granularity. The MV size gate was set by 1.0 μm beads. **a** Two gating regions are region R1 and R2 that represents MVs and 1.0 μm beads, respectively. Based on the FSC and SSC, MVs are located lower than 1.0 μm beads. **b** A histogram of logarithmic FSC vs. count, showing the distribution of MVs in comparison to the beads. **c** Region RN1 represents region R1 events that were labeled with conjugated FITC anti-CD235a and indicates erythrocyte-derived MVs. **d** Region RN2 represents region R1 events that were labeled with conjugated PE anti-CD42b and indicates platelet-derived MVs. **e** Region RN1 represents region R1 events that were labeled with conjugated FITC Annexin-V. Nonstained events are demonstrated by the left peak in **c–e**.

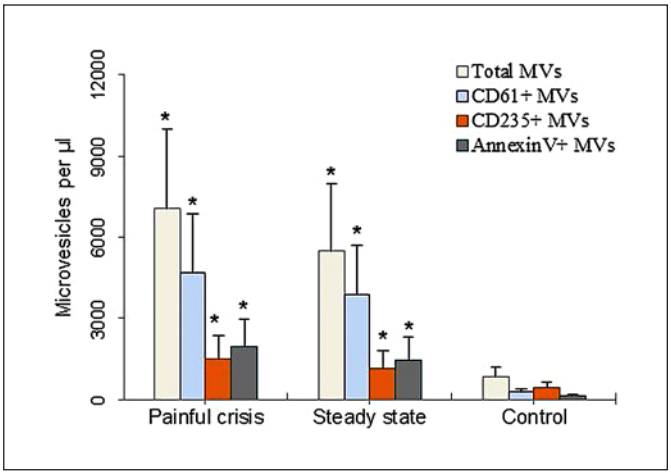

Fig. 2. Quantification of different MVs in SCD patients and controls. Total count of different MVs according to the expression of CD61, CD235a, and Annexin-V that represents platelet, erythrocyte, and procoagulant MVs, respectively. Plot revealed a statistically significant increase of MVs in painful crisis and steady state compared to controls (*$p < 0.001$ by ANOVA). Results are mean ± SD.

MV Enumeration Results

In this study, MVs were gated based on their specific forward scatter (FSC) and sideward scatter (SSC) pattern (Fig. 1a, b). As it is demonstrated in Figure 1, MVs were found to be the population with FSC and SSC levels of less than 1.0 μm beads. Afterward, MVs were further identified by staining with conjugated CD235α (Fig. 1c), CD61 (Fig. 1d), and Annexin-V (Fig. 1e) markers.

The number of total MVs that were counted based on the forward and side scatter, as well as three measured subpopulations, was significantly elevated in SCD patients compared to the controls. In SCD patients (steady state and painful crisis) and healthy controls, the majority of MVs originated from platelets (CD61+) and erythrocytes (CD235+). The number of CD61+ and CD235+ MVs was significantly different between patients and controls. This difference was mainly noticeable between controls and patients during a painful crisis ($p < 0.001$). The mean and SD of MVs counts during the painful crisis, steady state as well as healthy controls have been shown in Figure 2.

As expected, the MVs were also positive for Annexin-V staining (27.7% Fig. 1c). Annexin-V is a useful

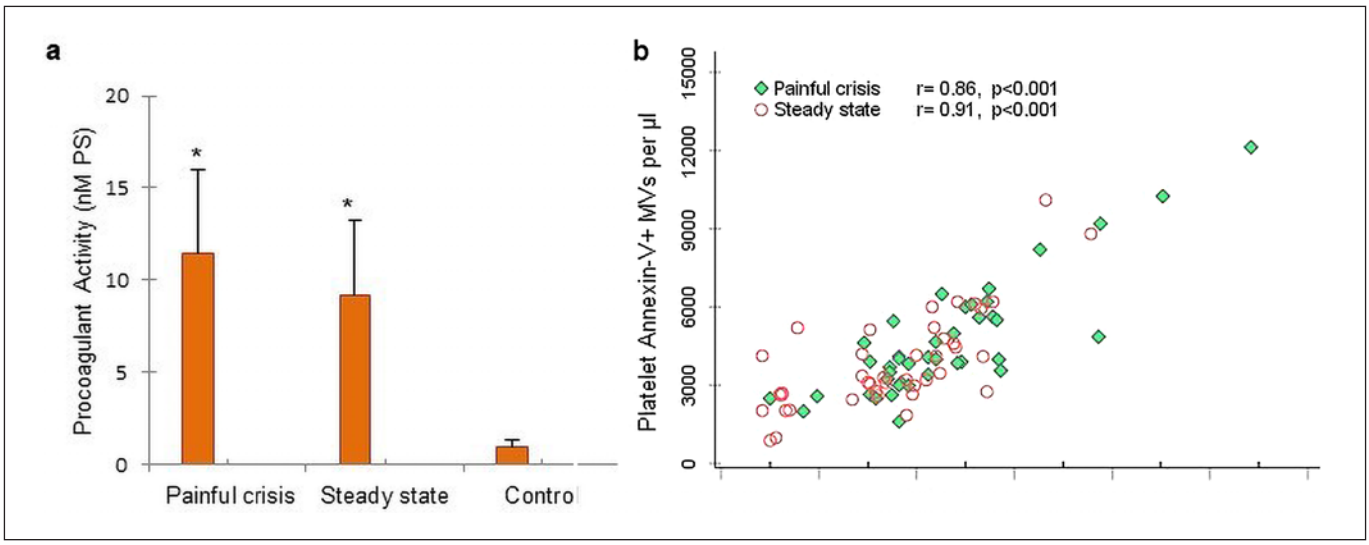

Fig. 3. Assessment of procoagulant activity due to PS-expressing MVs and its correlation with the number of Annexin-V+ MVs in SCD patients. **a** Procoagulant activity of collected samples in SCD patients compared to control. There was significant difference between procoagulant activity in painful crisis compared to steady state and control (*$p < 0.001$; by paired *t* test). **b** Correlation of Annexin-V+ MVs and procoagulant activity measurements. Change in the number of Annexin-V+ MVs correlated with the change in procoagulant activity in painful crisis ($r = 0.86$, $p < 0.001$ by Pearson's test) and steady state ($r = 0.91$, $p < 0.001$ by Pearson's test).

marker to determine the general levels of procoagulant MVs. During the study, patients in painful crisis demonstrated higher levels of procoagulant MVs compared to those of steady-state ($p < 0.001$) and control ($p < 0.001$) groups.

MV Procoagulant Activity

The results of the procoagulant activity assays are depicted in Figure 3. In this study, the procoagulant activity of MVs was significantly enhanced in painful crisis than those in steady-state ($p < 0.001$) and control ($p < 0.001$) groups (Fig. 3a). The procoagulant activity was correlated with the number of Annexin-V+ MVs in the crisis ($r = 0.86$, $p < 0.001$), and steady-state ($r = 0.91$, $p < 0.001$), patient groups (Fig. 3b).

Correlation Analysis

Pearson correlation analysis was carried out to determine the relationship between the concentrations of MVs with blood parameters in SCD patients (Table 2). Correlation studies revealed a significant positive correlation between CD61+ MVs and leukocyte count ($r = 0.43$, $p < 0.05$), platelet ($r = 0.74$, $p < 0.001$), and reticulocyte count ($r = 0.44$, $p < 0.001$). The CD235+ MVs were strongly correlated with levels of LDH ($r = 0.51$, $p < 0.001$) and platelet count ($r = 0.41$, $p < 0.05$). Moreover, significant negative correlation was observed between CD235+ MVs and hemoglobin level ($r = -0.63$, $p < 0.001$) (Table 2).

Table 2. Correlations between blood parameters and MVs count

	Total MVs	CD61+ MVs	CD235+ MVs
Hemoglobin	0.03	0.09	−0.63[b]
Reticulocyte	0.55[a]	0.44[b]	0.35
Leukocyte	0.66[b]	0.43[a]	0.38
Platelet	0.69[b]	0.74[b]	0.41[a]
LDH	−0.09	0.10	0.51[b]

[a] $p < 0.05$. [b] $p < 0.001$.

Discussion

The hallmark complication associated with SCD is the vaso-occlusive painful crisis, leading to chronic hemolytic anemia [18]. However, the pathophysiology of vaso-occlusive complications in SCD is only partially understood [19]. Several studies reported the activation of coagulation as a prominent feature of SCD [20] and proposed the involvement of circulating cell-derived MVs in pathophysiological processes of SCD including coagulation, inflammation, and abnormal cellular adherence [15]. In this study, we compared the pattern of circulating MVs between SCD patients during painful crises and steady-state and healthy subjects and studied the association of MVs with complete blood count indices, hemolysis marker, and procoagulant activity. We found that circulating levels of procoagulant MVs could predict the clinical consequences in patients with SCD.

Our finding illustrated considerable differences in the number of MVs in sickling painful crisis patients. Using flow cytometry, MVs were detected based on their size and density, while they were also verified using standard beads and characterized by monoclonal antibodies. It seems that MVs events are distributed near to the device noise and debris [21]. It should be noted that the small size of MVs is the main limitation that challenges the reproducibility of the flow cytometry technique. Accordingly, when using this assay, a limited number of MVs might be counted [22]. Almost all circulating MVs in SCD were found to be derived from erythrocytes and platelets in addition to the fact that the total count of MVs recognized to vary significantly between baseline conditions and during a painful crisis.

Although the numbers of all types of MVs were lower in healthy controls in relation to patients during crisis and baseline conditions, considerably higher erythrocyte-derived MVs and platelet-derived MVs levels were discovered in patients with painful crises compared to the baseline situations and control groups. These data are in line with previous observations that high numbers of both erythrocyte-derived and platelet-derived MVs present in patients with SCD [23, 24]. Our findings are in consistency with a recent study by Kasar et al. [18] and Tantawy et al. [24] who reported a higher number of MVs in crisis versus steady state and control. However, our results were incongruent with findings in two other studies [19, 25], with a relevant discrepancy on absolute MVs numbers in SCD crises and a significant increase in crises in our study. Also, in another study, in contrast to our finding, Nouboussie et al. [25] recently depicted higher number of MVs in steady state. MVs derived from erythrocytes were reported to be most commonly present in SCD [26]. Concerning erythrocyte-derived MVs, a number of studies reported significant increases in this MVs subpopulation in painful crisis [18, 27]. Level of MVs can reach up to 5- to 6-fold increase in SCD patients versus healthy controls [28], although it might even further increase during vaso-occlusive complications [18].

Furthermore, we found statistical associations between erythrocytic MVs levels and LDH as hemolysis marker as well as the hemoglobin concentration. These findings are in agreement with a study by Van Beers et al. [27], who reported the majority of MVs being originated from platelets and erythrocytes, even though the number of MVs did not vary significantly between crisis and steady state. The role of MVs in SCD has recently been reviewed in detail [29], which highlights their important role as biomarkers in coagulation activation. Several studies found positive and strong correlations between erythrocyte-derived MVs count and markers of hemolysis (e.g., LDH) in SCD [30]. It should be noted that chronic hemolysis in SCD patients can also trigger the production of erythrocyte-derived MVs [28].

Association of elevated level of circulating MVs with increased risk of thrombotic complications has been reported in several diseases [31]. One of the key processes contributing to the hypercoagulable state could occur due to the changes in the cell membrane and subsequent MVs formation [32]. Expression of PS on the outer leaflet can enhance the procoagulant activity and the adhesive properties of sickle cells [5, 33]. Increased expression of such negatively charged PS acts as a docking site for enzymatic complexes, such as the serine proteases involved in coagulation. SCD patients also have high levels of circulating PS-positive erythrocytes due to the impairment of spleen function [34]. Thus, PS externalization plays a key role in the occurrence of thrombotic events [4]. PS can be exposed on the surface of mature sickle cells [35] and the surface of sickle cell-derived MVs [8].

For measuring the level of procoagulant MVs, FITC-Annexin-V was used that binds to PS. In our assay, flow cytometry analysis showed that the sickling patients had higher procoagulant MVs levels compared to the healthy controls. The procoagulant MVs levels were significantly elevated in patients during the crisis when compared to baseline conditions and controls. Significantly higher levels of Annexin-V were observed on the surface of erythrocytes from SCD patients compared to erythrocytes from control subjects suggesting an augmented loss of phospholipid asymmetry in these patients [4]. Interestingly, a recent study presented evidence that the density of PS is higher for erythrocyte-derived MVs compared to platelet-derived MVs [36].

In the current study, we also investigated the impact of MVs, especially Annexin-V+ MVs on the procoagulant activity assay or thrombin generation. The procoagulant activity of MVs was significantly increased during the crisis. Our findings depicted a direct and significant association between procoagulant activity and the levels of Annexin-V+ MVs in sickling crisis and steady state. Our data however are in contrast to previous findings by Whelihan et al. [20] who found an inverse correlation between thrombin generation and erythrocyte PS exposure.

MVs exhibit procoagulant properties through different mechanisms depending on the presence of PS and tissue factor at their surface [37]. Both tissue factor and PS exposed on MVs increase the procoagulant activity in SCD [38]. A majority of circulating MVs in SCD provide docking sites for activated coagulation factors; indeed, an accelerating effect on thrombin generation was observed with erythrocyte-derived MVs [25]. In our study, MVs surfaces were enriched with PS, and based on this fact, MVs could support coagulant activities highlighting that a high number of MVs may induce the risk of thrombotic complications [39]. Accordingly, in another study, positive correlations were demonstrated between erythrocyte-derived MVs concentrations, the expression of coagulation markers as well as acceleration in the propagation phase of thrombin generation [25]. Several markers of coagulation

activation like D-dimer, and prothrombin fragment, were also found to positively correlate with MVs numbers, including erythrocyte-derived MVs and total MVs [27].

In conclusion, we suggest that cell-derived MVs especially platelet and erythrocyte-derived MVs may be involved in painful crisis, thereby providing further insights on the pathophysiology of SCD. Circulating MVs may be considered as a potential biomarker for disease severity in SCD patients. Their levels are closely related to the vaso-occlusive crisis, markers of hemolysis, and coagulation. Although the knowledge on cell-derived MVs in SCD is confined to few studies, quantification of MVs in SCD could be a potentially valuable tool to identify patients who are at increased risk of thrombotic events.

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Statement of Ethics

The study complied with the guidelines for human studies. The study was approved by the *Jahrom University of Medical Sciences* Ethics Committee (IR.JUMS.REC.1398.081). Written informed consent was obtained from subjects and healthy volunteers prior to participation in this study.

Conflict of Interest Statement

The authors declare no conflicts of interest.

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Author Contributions

Akbar Hashemi Tayer participated in the design of the study, carried out the experiments, analyzed and interpreted the results, and drafted the manuscript; Reza Ranjbaran and Mojdeh Abbasi revised the manuscript critically and contributed to the writing of the manuscript; Maryam Kamravan was responsible for informed consent and created the tables and figures; Reyhaneh Zareian conducted the experiments. All of the authors approved the final version of this paper for publication.

Data Availability Statement

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

References

1 Kato GJ, Piel FB, Reid CD, Gaston MH, Ohene-Frempong K, Krishnamurti L, et al. Sickle cell disease. Nat Rev Dis Primers. 2018;4(1):18010–22.
2 Ware RE, de Montalembert M, Tshilolo L, Abboud MR. Sickle cell disease. Lancet. 2017;390(10091):311–23.
3 Sundd P, Gladwin MT, Novelli EM. Pathophysiology of sickle cell disease. Annu Rev Pathol. 2019;14:263–92.
4 Faes C, Sparkenbaugh EM, Pawlinski R. Hypercoagulable state in sickle cell disease. Clin Hemorheol Microcirc. 2018;68(2–3):301–18.
5 Kennedy JR. Attenuating a sickle cell crisis with annexin V. Med Hypotheses. 2015;84(5):434–6.
6 Shet AS, Lizarralde-Iragorri MA, Naik RP. The molecular basis for the prothrombotic state in sickle cell disease. Haematologica. 2020;105(10):2368–79.
7 Ridger VC, Boulanger CM, Angelillo-Scherrer A, Badimon L, Blanc-Brude O, Bochaton-Piallat M-L, et al. Microvesicles in vascular homeostasis and diseases. Position paper of the European Society of Cardiology (ESC) Working Group on atherosclerosis and vascular biology. Thromb Haemost. 2017;117(7):1296–316.
8 Zahran AM, Elsayh KI, Saad K, Embaby MM, Youssef MA, Abdel-Raheem YF, et al. Circulating microparticles in children with sickle cell anemia in a tertiary center in upper Egypt. Clin Appl Thromb Hemost. 2019;25:1076029619828839.
9 Hashemi Tayer A, Amirizadeh N, Ahmadinejad M, Nikougoftar M, Deyhim MR, Zolfaghari S. Procoagulant activity of red blood cell-derived microvesicles during red cell storage. Transfus Med Hemother. 2019;46(4):224–30.
10 Tayer AH, Amirizadeh N, Mghsodlu M, Nikogoftar M, Deyhim M, Ahmadinejad M. Evaluation of blood storage lesions in leuko-depleted red blood cell units. Iran J Pediatr Hematol Oncol. 2017;7:171–9.
11 Westerman M, Porter JB. Red blood cell-derived microparticles: an overview. Blood Cells Mol Dis. 2016;59:134–9.
12 Cointe S, Lacroix R, Dignat-George F. Platelet-derived microparticles. Platelets in thrombotic and non-thrombotic disorders. Springer; 2017. p. 379–92.
13 Olatunya OS, Lanaro C, Longhini AL, Penteado CFF, Fertrin KY, Adekile A, et al. Red blood cells microparticles are associated with hemolysis markers and may contribute to clinical events among sickle cell disease patients. Ann Hematol. 2019;98(11):2507–21.
14 Kato GJ. Sickle particulars of microparticles. Blood. 2020;136(2):154–5.
15 Romana M, Connes P, Key NS. Microparticles in sickle cell disease. Clin Hemorheol Microcirc. 2018;68(2–3):319–29.
16 Ayers L, Kohler M, Harrison P, Sargent I, Dragovic R, Schaap M, et al. Measurement of circulating cell-derived microparticles by flow cytometry: sources of variability within the assay. Thromb Res. 2011;127(4):370–7.
17 Grisendi G, Finetti E, Manganaro D, Cordova N, Montagnani G, Spano C, et al. Detection of microparticles from human red blood cells by multiparametric flow cytometry. Blood Transfus. 2015;13(2):274–80.
18 Kasar M, Boğa C, Yeral M, Asma S, Kozanoglu I, Ozdogu H. Clinical significance of circulating blood and endothelial cell microparticles in sickle-cell disease. J Thromb Thrombolysis. 2014;38(2):167–75.
19 Piccin A, Murphy C, Eakins E, Kunde J, Corvetta D, Di Pierro A, et al. Circulating microparticles, protein C, free protein S and endothelial vascular markers in children with sickle cell anaemia. J Extracell Vesicles. 2015;4:28414.
20 Whelihan MF, Lim MY, Mooberry MJ, Piegore MG, Ilich A, Wogu A, et al. Thrombin generation and cell-dependent hypercoagulability in sickle cell disease. J Thromb Haemost. 2016;14(10):1941–52.

21 Macey MG, Enniks N, Bevan S. Flow cytometric analysis of microparticle phenotype and their role in thrombin generation. Cytometry B Clin Cytom. 2011;80(1):57–63.
22 Nielsen MH, Beck-Nielsen H, Andersen MN, Handberg A. A flow cytometric method for characterization of circulating cell-derived microparticles in plasma. J Extracell Vesicles. 2014;3.
23 Shet AS, Aras O, Gupta K, Hass MJ, Rausch DJ, Saba N, et al. Sickle blood contains tissue factor-positive microparticles derived from endothelial cells and monocytes. Blood. 2003;102(7):2678–83.
24 Tantawy AA, Adly AA, Ismail EA, Habeeb NM, Farouk A. Circulating platelet and erythrocyte microparticles in young children and adolescents with sickle cell disease: relation to cardiovascular complications. Platelets. 2013;24(8):605–14.
25 Noubouossie DC, Lê PQ, Rozen L, Debaugnies F, Ferster A, Demulder A. Evaluation of the procoagulant activity of endogenous phospholipids in the platelet-free plasma of children with sickle cell disease using functional assays. Thromb Res. 2012;130(2):259–64.
26 Hierso R, Lemonne N, Villaescusa R, Lalanne-Mistrih ML, Charlot K, Etienne-Julan M, et al. Exacerbation of oxidative stress during sickle vaso-occlusive crisis is associated with decreased anti-band 3 autoantibodies rate and increased red blood cell-derived microparticle level: a prospective study. Br J Haematol. 2017;176(5):805–13.
27 van Beers EJ, Schaap MC, Berckmans RJ, Nieuwland R, Sturk A, van Doormaal FF, et al. Circulating erythrocyte-derived microparticles are associated with coagulation activation in sickle cell disease. Haematologica. 2009;94(11):1513–9.
28 Camus SM, De Moraes JA, Bonnin P, Abbyad P, Le Jeune S, Lionnet F, et al. Circulating cell membrane microparticles transfer heme to endothelial cells and trigger vasoocclusions in sickle cell disease. Blood. 2015;125(24):3805–14.
29 Hebbel RP, Key NS. Microparticles in sickle cell anaemia: promise and pitfalls. Br J Haematol. 2016;174(1):16–29.
30 Nouraie M, Lee JS, Zhang Y, Kanias T, Zhao X, Xiong Z, et al. The relationship between the severity of hemolysis, clinical manifestations and risk of death in 415 patients with sickle cell anemia in the US and Europe. Haematologica. 2013;98(3):464–72.
31 Chew HK, Wun T, Harvey D, Zhou H, White RH. Incidence of venous thromboembolism and its effect on survival among patients with common cancers. Arch Intern Med. 2006;166(4):458–64.
32 Provost P. The clinical significance of platelet microparticle-associated microRNAs. Clin Chem Lab Med. 2017;55(5):657–66.
33 Semeraro F, Ammollo CT, Esmon NL, Esmon CT. Histones induce phosphatidylserine exposure and a procoagulant phenotype in human red blood cells. J Thromb Haemost. 2014;12(10):1697–702.
34 Whelihan MF, Zachary V, Orfeo T, Mann KG. Prothrombin activation in blood coagulation: the erythrocyte contribution to thrombin generation. Blood. 2012;120(18):3837–45.
35 Tait JF, Gibson D. Measurement of membrane phospholipid asymmetry in normal and sickle-cell erythrocytes by means of annexin V binding. J Lab Clin Med. 1994;123(5):741–8.
36 Garnier Y, Ferdinand S, Etienne-Julan M, Elana G, Petras M, Doumdo L, et al. Differences of microparticle patterns between sickle cell anemia and hemoglobin SC patients. PLoS One. 2017;12(5):e0177397.
37 Haghbin M, Hashemi Tayer A, Kamravan M, Sotoodeh Jahromi A. Platelet-derived procoagulant microparticles as blood-based biomarker of breast cancer. Asian Pac J Cancer Prev. 2021;22(5):1573–9.
38 Klaihmon P, Phongpao K, Kheansaard W, Noulsri E, Khuhapinant A, Fucharoen S, et al. Microparticles from splenectomized β-thalassemia/HbE patients play roles on procoagulant activities with thrombotic potential. Ann Hematol. 2017;96(2):189–98.
39 Van Der Meijden PE, Van Schilfgaarde M, Van Oerle R, Renné T, ten Cate H, Spronk HM. Platelet- and erythrocyte-derived microparticles trigger thrombin generation via factor XIIa. J Thromb Haemost. 2012;10(7):1355–62.