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ORIGINAL ARTICLE

Red blood cells microparticles are associated with hemolysis markers and may contribute to clinical events among sickle cell disease patients

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Abstract

Microparticles are sub-micron vesicles possessing protein and other materials derived from the plasma membrane of their parent cells, and literature suggests that they may have a role in the pathophysiology and downstream manifestations of sickle cell disease (SCD). The contributions of red blood cells microparticles (RMP) to the pathogenic mechanisms and clinical phenotypes of SCD are largely unknown. There is a controversy as to whether the proportions of intravascular hemolysis (approximately ≤ 30% of total hemolysis) would be enough to explain some complications seen in patients with SCD. We investigated RMP among 138 SCD patients and 39 HbAA individuals. Plasma RMPs were quantified by flow cytometry, plasma hemoglobin and heme by colorimetric assays, and haptoglobin and hemopexin by ELISA. The patients had higher RMP, plasma hemoglobin, and heme compared to the controls. On the contrary, haptoglobin and hemopexin were depleted in the patients. The RMP correlated positively with heme, lactate dehydrogenase, plasma hemoglobin, serum bilirubin, reticulocyte counts, and tricuspid regurgitant jet velocity of the patients. Contrarily, it correlated negatively with HbF, hemopexin, red blood cells counts, hemoglobin concentration, and haptoglobin. Although patients treated with hydroxyurea had lower RMP, this did not attain statistical significance. Patients with sickle leg ulcer and elevated tricuspid regurgitant jet velocity had higher levels of RMP. In conclusion, these data suggest that RMPs are associated with hemolysis and may have important roles in the pathophysiology and downstream complications of SCD.

Keywords Sickle cell disease · Red blood cells microparticles · Hemolysis · Clinical manifestations

Introduction

Sickle cell disease (SCD) is a common genetic disorder of man that is caused by a mutation in the β-globin gene [1–3]. As a result of this mutation, there is formation of sickle hemoglobin (HbS) which polymerizes at low oxygen tension and this is the primary step in the pathophysiology of sickle cell disease [1–3]. The polymerization of deoxy-HbS inside red blood cell leads to some complex interactions with the cell membrane and other molecules [1–3]. These interactions eventually cause hemolysis, vaso-occlusion, and tissue lesions. Despite the seemingly common genetic basis of SCD, the clinical manifestations of the disease are not uniform [1–3]. Studies have shown that SCD is clinically pleiotropic both within an individual and among groups of patients [1–3]. Several hypotheses have been proposed for the phenotypic diversity in SCD. These include the influences of genetic modifiers [4] and hemolysis intensity [5, 6].

Chronic hemolysis occurs both in the extravascular and intravascular compartments in patients with SCD. The exact proportion of intravascular hemolysis is not known, but studies suggest that this is probably less than 30% of the entire

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hemolysis component [6, 7]. Also, there is a controversy as to whether this minor contribution would be enough to explain some predicted complications in SCD patients such as pulmonary hypertension, leg ulcer, stroke, and priapism ascribable to intravascular hemolysis [6, 7]. Nevertheless, there is increasing evidence of endothelial lesions from free hemoglobin and heme in patients with SCD, which are capable of driving sterile inflammation, oxidative stress, and vasculopathy [5, 6, 8]. In a bid to further buttress the contributions of hemolysis to the clinical manifestations seen in SCD patients, some subphenotypes of SCD manifestations, e.g., stroke, sickle leg ulcers (SLU), pulmonary hypertension, and priapism, have been linked to higher hemolysis rate [5, 6, 9]. In further pursuance of this view, several biologic markers such as reticulocyte count, lactate dehydrogenase (LDH), aspartate transaminase, serum bilirubin, heme, haptoglobin, hemopexin, and cell-free plasma hemoglobin have been used to predict intravacular hemolysis and, by extension, elucidate the complications seen in patients with SCD [5, 6, 9–12]. However, not all these parameters are completely consequences of intravascular hemolysis, as LDH elevation can also been linked to tissue destruction in SCD patients [6, 13]. In view of these observations, the quest to further understand and explain the clinical events in patients with SCD is on-going and has led to the search for more candidate markers of the disease.

Microparticles are sub-micron, unilamellar vesicles possessing protein and other materials derived from the plasma membrane of their parent cells [14, 15]. Literature data suggest that they may have a role in the pathophysiology of SCD [14, 15]. Red blood cell microparticles (RMP) are derived from the red blood cells (RBC) as a result of shedding of unwanted surface features during reticulocytes maturation, apoptosis, and activation by endogenous triggers [14–16]. In addition, they have been shown to carry trapped heme and hemoglobin upon their release [17]. However, the difficulties often encountered with the methods currently available to quantify RMP had hampered their been frequently used as markers in SCD [14–16]. Nevertheless, the introduction of modern flow cytometers now allows a more reliable procedure to measure RMP [14–16].

The contribution of RMP to endothelial cell lesions and clinical phenotypes of SCD is largely unknown. However, there are some data that suggest that, due to the repeated sickling-unsickling cycles and polymerization of HbS, patients with SCD are likely to generate more RMP in their blood than healthy subjects [14–17]. Furthermore, the generated RMP has been speculated to be able to contribute to the vascular, oxidative, and inflammatory lesions and other complications of SCD [14–18]. Hence, the aim of this study was to study RMP relationships with hemolysis markers in steady state and clinical events in a cohort of SCD patients.

Patients and methods

Study participants

This study involved 138 adults with SCD (78-SS, 12-SBeta0, 12-SBeta+ and 36-SC) on regular follow-up and 39 healthy AA-matched controls at the hematology and hemotherapy center (Hemocentro), University of Campinas, (UNICAMP), Brazil. The patients were of both sexes, aged 18–60 years, with diagnostic testing by high-pressure liquid chromatography (HPLC) (Bio-Rad, Hercules, CA, USA) and genetic studies to confirm the SCD. They were all in steady state defined as absence of any acute event such as painful crisis, and or infection, within a month and without blood transfusion for at least 3 months to recruitment period. Seventy-five patients made up of 71SS/SBeta0 and 4 S-Beta+ patients were on stable hydroxyurea treatment with an average dose of 25.3 ± 2.3 mg/kg/day, range (20–35 mg/kg/day) for more than 6 months prior recruitment. None of the SC patients was on hydroxyurea. The indications for hydroxyurea therapy were three or more severe episodes of bone pain crisis (VOC) per year and/or presence of any one or more of the following: stroke, leg ulcer, priapism, acute chest syndrome, or persistently elevated tricuspid regurgitant jet velocity (risk for pulmonary hypertension) or recurrent low hemoglobin (Hb < 6.5 g/100 mL). The control group consisted of healthy volunteers, and staff members at the Hemoglobin and Genome Laboratory of the Hematology and Hemotherapy Center of UNICAMP, aged 18–60 years. All the controls had HbAA genotype confirmed by high-performance liquid chromatography (HPLC) and genetic studies. All the participants gave written informed consents. Participants who did not give written informed consents as well as patients who had any crisis or pain crisis in the last 1 month before the study were excluded. Also, excluded were patients hospitalized for any medical condition in the last 3 months prior to the study or on chronic blood transfusion or anti-inflammatory/cancer drugs or those who have received blood transfusion in the last 3 months prior to recruitment. In addition, patients with pregnancy, acute or chronic inflammatory disease, diabetes mellitus, chronic kidney diseases, other chronic diseases, and malignancies were excluded.

Ethical approval

The study was approved by the institutional review board of UNICAMP no. CAAE 54031115.9.0000.5404.

Data collection

Laboratory parameters

Colorimetric assays (plasma hemoglobin and heme) Plasma samples obtained from each participant was used to quantify

their plasma hemoglobin and heme levels through colorimetric assays using the QuantiChrom™ Hemoglobin Assay Kit (DIHB-250) and the QuantiChrom™ Heme Assay Kit (DIHM-250), BioAssay Systems USA respectively as specified by the manufacturers.

ELISA studies (haptoglobin and hemopexin) The plasma haptoglobin and hemopexin levels were quantified through enzyme-linked immunosorbent assays (ELISA) using the Quantikine Human Haptoglobin (DHAPGO) ELISA kit, R & D Systems USA, and the Abcam Human Hemopexin (ab171576) USA ELISA kits respectively as specified by the manufacturers.

RMP measurement Identification and quantification of RMP were done by differential centrifugation followed by staining and fluorescence flow cytometry analysis as earlier described by Wisgrill et al. [19], and RMPs were measured by numbers per milliliter of blood.

Pre-staining preparation Briefly, 3.5 mL of citrated venous sample collected by 21 gauge syringe was drawn from each participants after taking the first 8 mL of blood into two EDTA sample tubes (4 mL each) for plasma separation and the other for hematological analysis with very light tourniquets applied. The citrate samples were not mixed or rocked and were transported securely in upright positions inside the sample carrier to the laboratory immediately. Thereafter, the plasma separation was done within 30 min of collection. To obtain the plasma, the topmost 1 mL of the citrated whole blood sample was pipetted out and discarded, and the remaining was processed through a two-stage centrifuging, the first at 2500*g* × 15 min at 22 °C thereafter; the topmost 1 mL of the plasma from this first centrifugation was pipetted out and the remaining discarded. A second centrifugation at 13,000*g* × 5 min at 22 °C was done for the topmost 1 mL plasma obtained from the first centrifugation. The topmost 700 μL of the product of the second centrifugation was pipetted out into a separate polypropylene tube, and the platelet count of the plasma was checked using Beckman Coulter hematologic counter (Model 8246-EN, SN-AN37824), USA, to ensure it was platelet depleted. Thereafter, the samples were aliquoted into polypropylene tubes and frozen at − 80 °C until analysis.

Staining and quantification Briefly, the staining reagents (a. Calcein violet AM (Molecular probes-Invitrogen: 3125 μg (5 μL), b. Bovine Lactadherin FITC (Haematologic Technologies, Inc. 0.83 μg (10 μL), c. Anti-CD235a R-PE (Life Technologies: 2 μL), d. Sterile filtered PBS 2 × 0.22 μm membrane) and aliquoted frozen platelet poor plasma were brought to room temperature. The reagents were prepared according to the manufacturer's specifications. The antibodies (Calcein AM, Anti-CD235a and Lactadherin) were also subjected to a high speed centrifugation at 20,000*g* × 5 min at room temperature to remove false positive events at analysis. After preparing fresh polypropylene tubes, 10 μL of each sample was stained with 5 μL, 10 μL, 2 μL, and 5 μL of calcein, lactadherin, anti-CD235a, and filtered sterile PBS buffer, respectively. The resultant solution was gently vortexed and incubated in the dark at 37 °C, − 5% CO_2 for 60 min initially. Thereafter, 500 μL of sterile filtered PBS was added and then incubated for the second time in the dark at 37 °C, − 5% CO_2 for 30 min. Immediately after the second incubation, the resultant solution was further diluted with 3468 μL of sterile filtered PBS to make a final dilution of 1:400 with a resultant solution totaling 4000 μL. This resultant solution was immediately analyzed on a calibrated flow cytometer, the Beckman Coutler's CytoFLEX Flow Cytometer (Model A00-1-1102), USA, using the staining buffer as negative control. Each sample was aspirated and read for 10 min by the Cytoflex machine. The RMP events were expressed per milliliter.

Other laboratory parameters

Fetal hemoglobin (HbF) was measured by high-performance liquid chromatography (HPLC) using Bio-Rad variant D10 Biosystesm USA.

Automated analyzers were used to carry out the other hematologic and biochemical analyses.

Clinical events

The patients' medical records were retrieved electronically and examined for clinical events. The number of episodes of vaso-occlusive crisis (VOC) was documented. VOC was defined as severe bone pain crisis that affects daily activities, requiring hospital visit and use of opioid analgesics and/or hospitalization. In addition, any chronic complications including leg ulcers, retinopathy, priapism, and stroke were documented. The definitions of clinical phenotypes were as previously described by Ballas et al. [20]. Tricuspid regurgitant jet velocity (TRV) obtained from Doppler echocardiography were used to categorized them into two groups. Patients with TRV < 2.5 m/s were categorized as having normal TRV, while those with TRV ≥ 2.5 m/s were categorized as having elevated TRV and considered to be at risk of pulmonary hypertension [21]. Urinary albumin-creatinine ratio (UACR) of less than 30 mg/g was taken as no albuminuria (i.e., normal), between 30 and 300 mg/g as microalbuminuria and greater than 300 mg/g was classified as macroalbuminuria [22]. Patients were also classified into two groups based on whether they had the presence or absence of proliferative SCD retinopathy as previously described [23].

Data analysis

Principal component analysis (PCA) technique was used. The SS and SB0 patients were analyzed together because of their similar disease profiles.

Suitability of data for PCA

To assess the adequacy and suitability of the data sample for PCA, the Kaiser-Meyer-Olkin measure of sample adequacy test and the Bartlett's Test of sphericity were performed. The Kaiser-Meyer-Olkin measure of sample adequacy test was 0.65, and the Bartlett's Test of sphericity was significant ($p < 0.0001$, $\chi^2 = 100.4$, $df = 3$). These indicate that the data is adequate and suitable for a valid factor analysis using the principal component analysis technique.

Derivation of hemolytic component and hemolysis assessment

Principal component analysis was used to derive a hemolytic component from lactate dehydrogenase (LDH), total bilirubin, and absolute reticulocyte count in the patients.

The hemolytic component derived from lactate dehydrogenase, total bilirubin, and absolute reticulocyte count in the patients loaded one factor with Eigenvalue value above 1.0 This factor has an eigenvalue of 1.994, a median of − 0.117831, interquartile range (− 0.288071 to 0.3979259), and predicted 66.5% shared variance among the hemolytic markers.

Derivation of regression model to predict clinical outcome

Logistic regression was done to predict the outcome of clinical events (leg ulcer, risk for pulmonary hypertension, retinopathy, stroke, VOC, osteonecrosis) as outcome variables and (RMP, heme, plasma Hb, Hb conc, LDH, reticulocyte count, total bilirubin, HbF, and RBC) as predictor variables. The predictors were selected based on literature review [5, 6, 9, 11, 14–16, 18, 24, 25].

Statistical analyses

Data were analyzed using the Statistical Package for Social Sciences software (SPSS) version 22 (IBM Corp., Armonk, NY, USA) and the GraphPad Prism version 5.0 statistical software for windows (San Diego, California, USA) using both descriptive and comparative statistics. The frequencies of variables were described and the chi-square tests or Fisher's exact tests were used to compare categorical variables as appropriate. The normal distribution of the quantitative variables was verified by the Kolmogorov-Smirnov, D'Agostino/Pearson Omnibus, and Shapiro-Wilk tests, and the significance of differences between groups was assessed using the Kruskal-Wallis analysis of variance (ANOVA) for three or more groups, and Mann-Whitney test for two groups. Bivariate correlation studies were done by Spearman's rank correlation (ρ) with the GraphPad Prism. The independent effect of hemolytic markers on clinical events was determined by logistic regression. The level of significance was set at $p < 0.05$ for all statistical tests.

Results

Biodata and biologic parameters in patients and controls

The SCD patients were made up of 51 (37%) male, while the controls were made up of 14 (36%) male. Although both the patients and controls were adults, the patients were older than the controls. There were significant differences in the levels of measured red blood cell microparticles, plasma hemoglobin, heme, haptoglobin, hemopexin, Hb concentration, and HbF between patients and controls. Similarly, there were differences in the levels of most biologic markers across the various SCD groups with the differences being more pronounced between the SS/SBeta0 and the SC patients (Table 1).

Effects of hydroxyurea on patients' parameters

None of the HbSC patients was on hydroxyurea treatment. However, 71 (78.8%) out of the 90 SS/SBeta0 patients, and four (33.0%) S-Beta+ patients were on hydroxyurea therapy.

When the effect of hydroxyurea treatment was examined on SS/SBeta0 subgroup of patients, it was observed that the levels of red blood cells microparticles, plasma hemoglobin, and heme were lower in the SS/SBeta0 patients on hydroxurea treatment compared to their counterparts not on this drug, but these values did not reach statistical significance ($p > 0.05$). However, SS/SBeta0 patients on hydroxyurea had significantly higher HbF, MCV, and hemopexin. On the contrary, they had significantly lower bilirubin, red blood count, reticulocyte counts, HbS levels, and white blood cell counts ($p < 0.05$) (Table 2).

Correlational studies of measured red blood cell microparticles in the patients

The correlations of RMP with other biologic markers are shown in Fig. 1. RMP had positive correlations with heme ($r = 0.64$, $p < 0.0001$, Fig. 1a), LDH ($r = 0.57$, $p < 0.0001$, Fig. 1b), plasma hemoglobin ($r = 0.54$, $p < 0.0001$), Fig. 1c), total bilirubin ($r = 0.46$, $p < 0.0001$, Fig. 1d), and reticulocyte count ($r = 0.44$, $p < 0.0001$, Fig. 1e). It also showed a modest

Table 1 The summary of participants and parameters

Parameters	a. SS/SBeta0 ($N = 90$) Median (interquartile range)	b. SBeta+ ($N = 12$) Median (interquartile range)	p value (a vs b)	c. SC ($N = 36$) Median (interquartile range)	p value (a vs c)	d. AA ($N = 39$) Median (interquartile range)	p value (a vs b vs c vs d)
Age in years	41 (33.7–60)	39 (29–48.8)	0.6891	45 (37–54)	0.061[a]	35 (28–46)	*0.0134*1*
No. on hydroxyurea	71 (78.9%)	4 (33.3%)		0 (0%)		NA	
Red blood cell microparticles (events/ML)	200,000.0 (80,000.0–490,000.0)	40,000.0 (10000–70,000)	*0.0002*[a]	80,000.0 (40,000.0 - 200000.0)	*0.0007*[a]	0.0 (0.070–4000.0)	*< 0.0001*2*
Plasma hemoglobin (mg/dL)	84.7 (61.6–113.7)	58.7 (40.0–79.0)	*0.0164*[a]	63.5 (45.3–78. 5)	*0.0002*[a]	42.0 (34–51)	*< 0.0001*3*
Haptoglobin (ng/mL)	1937.0 (1239.0–3092.0)	5417.0 (1644.0–11,674.0)	*0.035*[a]	2832.0 (1503.0–7694.0)	*0.027*[a]	986,000.0 (860,700.0–1,197,000.0)	*< 0.0001*4*
Heme (μM)	55.6 (43.1–81.9)	45.4 (24.2–83.4)	0.182[a]	42.4 (34.5–54.8)	*0.0023*[a]	28.5 (26–42.7)	*< 0.0001*5*
Hemopexin (μg/mL)	562.6 (241.0–916.7)	704.0 (174.0–1322.0)	0.659[a]	761.0 (356.0–1188.0)	0.114[a]	989.0 (6933.1–71,150.0)	*< 0.0001*6*
HbF (%)	13.1 (6.3–18.6)	11.7 (2.5–22.8)	0.460[a]	0.8 (0.4–1.3)	*< 0.0001*[a]	0.2 (0.2–0.4)	*< 0.0001*7*
Hb Conc (g/dL)	8.5 (7.7–9.4)	9.8 (8.8–10.5)	*0.0007*[a]	11.3 (10.1–12.3)	*< 0.0001*[a]	13.5 (13–14.7)	*< 0.0001*8*
Reticulocyte count ($\times 10^9$/L)	258.0 (182.0–349.0)	236.0 (153.0–331)	0.689[a]	207.0 (167.0–312,0)	0.1319[a]	NA	0.3304 *
Total bilirubin (mg/dL)	1.7 (1.3–2.8)	1.2 (0.7–2.1)	0.031[a]	1.1 (0.8–1.5)	*< 0.0001*[a]	NA	*< 0.0001*9*
LDH (IU)	383.5 (296–517)	199.0 (181.0–338.0)	*0.0002*[a]	237 (214–346)	*0.0001*[a]	NA	*< 0.0001*10*
RBC count ($\times 10^{12}$/L)	2.4 (2.0–2.8)	4.0 (3.2–4.6)	*< 0.0001*[a]	3.9 (3.4–4.6)	*< 0.0001*[a]	4.8 (4.0–5.0)	*< 0.0001*10*

Significant p values are indicated in italic fonts. Kruskal-Wallis test with Dunn's multiple comparison post hoc tests with differences in *1 = SC vs AA only; *2 = (SS/SBeta0 vs SBeta+), (SS vs SC), (SS/SBeta0 vs AA), (SC vs AA); *3 = (SC vs AA), (SS/SBeta0 vs AA), (SS/SBeta0 vs SC); *4 = (SS/SBeta0 vs AA), (Sbeta+ vs AA), (SC vs AA); *5 = (SS/SBeta0 vs SC), (SS/SBeta0 vs AA), (SC vs AA); *6 = (SS/SBeta0 vs AA), (SC vs AA); *7 = (SS/SBeta0 vs AA), (SS/SBeta0 vs SC), (SBeta+ vs SC), (SBeta + vs AA), (SC vs AA); *8 = (SS/SBeta0 vs SC), (SS/SBeta0 vs AA), (SBeta+ vs AA), (SC vs AA); *9 = SS/SBeta0 vs SC only; *10 = (SS/SBeta0 vs SBeta+), (SS/SBeta0 vs SC)

NA not applicable, *RMP* red blood cell microparticle, *HbF* fetal hemoglobin, *RBC* red blood cell, *Hb* hemoglobin concentration, *WBC* white blood cell count, *LDH* lactate dehydrogenase, *SS* sickle cell anemia, *SBeta+* sickle β^+-thalassemia, *SBeta0* sickle β^0-thalassemia, *SC* HbSC disease, *AA* healthy HbAA controls

[a] Mann-Whitney test

Table 2 Effects of hydroxyurea treatment on biologic markers of SS/SB0 patients

Parameters	Hydroxyurea ($N = 71$) Yes	Hydroxyurea ($N = 19$) No	p values
RMP (events/mL)	160,000.0 (80,000.0–440,000.0)	400,000.0 (120,000.0–1,570,000.0)	0.09
Plasma Hb (mg/dL)	83.9 (60.7–106.9)	98.4 (66.4–145.8)	0.07
Haptoglobin (ng/mL)	2001 (1403–3064)	1296 (950–3525)	0.200
Heme (μM)	53.3 (43–81)	64.4 (48–88.4)	0.222
Hemopexin (μg/mL)	638 (313–971)	260 (196–369)	*0.004*
HbF (%)	14.9 (8.2–19)	8.9 (4.5–14)	*0.033*
HbS (%)	76.3 (67.8–81.2)	83.8 (70–88)	*0.04*
RBC (million cells/μL)	2.3 (1.9–2.7)	2.7 (2.4–3.4)	*0.0013*
MCV (fL)	121.0 (101–131)	95 (83–104)	*< 0.0001*
Total bilirubin (mg/dL)	1.7 (1.2–2.6)	2.8 (1.4–4.1)	*0.04*
Hb (g/dL)	8.6 (7.7–9.4)	8.5 (7.7–8.8)	0.460
Reticulocyte ($\times 10^9$/L)	224 (180–331)	340 (229–450)	*0.015*
LDH (IU)	383 (295–503)	396 (315–595)	0.397
Platelet ($\times 10^3$/μL)	338 (265–423)	372 (294–423)	0.352
WBC ($\times 10^3$/μL)	5.8 (4.4–8.0)	10.6 (9–12)	*< 0.0001*

Significant p values are in italic fonts, Test statistics = Mann-Whitney test

RMP red blood cell microparticle, *HbF* fetal hemoglobin, *RBC* red blood cell, *Hb* hemoglobin concentration, *HbS* hemoglobin S, *MCV* mean corpuscular volume, *WBC* white blood cell count, *LDH* lactate dehydrogenase

significant positive correlation with TRV ($r = 0.36$, $p < 0.0001$, Fig. 1f). On the contrary, a negative correlation was observed between RMP and HbF ($r = -0.4$, $p = 0.0001$, Fig. 1g), hemopexin ($r = -0.40$, $p < 0.0001$, Fig. 1h, red blood cells count ($r = -0.37$, $p < 0.0001$, Fig. 1i) and Hb concentration ($r = -0.33$, $p = 0.0001$, Fig. 1j). Also, the red blood cell microparticles of the patients showed a negative correlation with the haptoglobin ($r = -0.27$, $p = 0.002$), Fig. 1k). The plasma hemoglobin, heme, and TRV showed significant correlations with other hemolysis markers. These are shown in the supplement materials.

Clinical events among the patients

Regarding the acute symptoms or complications of SCD within the last 12 months, 48 (34.8%) had history of VOC. In general, lifelong, cumulative, and long-term complications were more common among patients with HbSS and SBeta0 genotypes. However, the occurrence of proliferative retinopathy was more among HbSC patients (Supplement Table 1).

Distribution of laboratory parameters and clinical events by hemolytic component category

There were significant differences in the laboratory parameters across the different hemolytic quartiles among the SCD patients, and the occurrence of leg ulcer, stroke, and osteonecrosis was also differentiated by hemolytic quartile. These differences were highest between the lowest and highest hemolytic component quartiles (Table 3). Also, similar trends were observed among the SS/SBeta0 patient subgroup ($n = 90$) with respect to laboratory parameters but to a lesser extent in respect of clinical events. Although more SS/SBeta0 patients with leg ulcer belonged to higher hemolytic component group, this did not attain statistical significance (Table 4).

Relationships between clinical events and biologic parameters of patients with sickle leg ulcer

The red blood cell microparticles and heme were significantly higher in SS/SB0 patients who had sickle leg ulcer ($p < 0.05$) (Table 5).

Elevated TRV and risk of pulmonary hypertension

The red blood cell microparticles and LDH were significantly higher in SS/SB0 patients who had elevated tricuspid regurgitation velocity (TRV), while their hemoglobin concentration and RBC counts were significantly lower ($p < 0.05$) (Table 6).

Painful crisis (VOC)

There was no association between RMP and occurrence of VOC among the SS/SB0 group of patients in the preceding 1 year before the study. However, lower HbF was associated with the occurrence of VOC among the patients (Supplement Table 2).

Other clinical events

None of the other clinical events was associated with RMP.

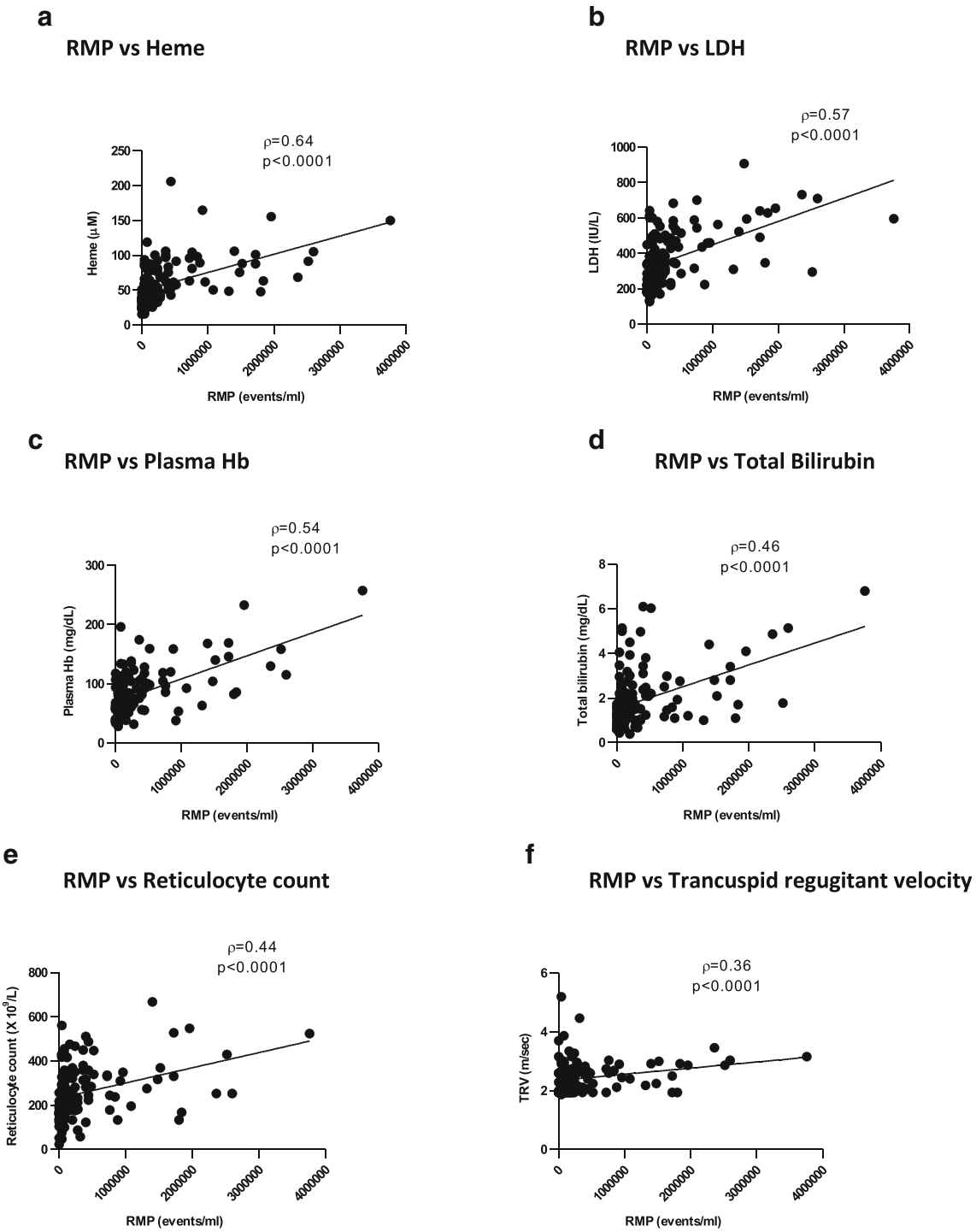

Fig. 1 Red blood cell microparticles correlational studies RMP- red blood cell microparticles, LDH- lactate dehydrogenase, TRV- trancuspid regugitant velocity, HbF- fetal hemoglobin, RBC- red blood cells

Regression analysis model to predict clinical outcomes

Out of the six models to predict different clinical outcomes (leg ulcer, risk for pulmonary hypertension, retinopathy, stroke, VOC, osteonecrosis), the predictor variables significantly predicted only three outcome variables/clinical events (leg ulcer, retinopathy, and osteonecrosis). Leg ulcer was significantly associated with heme ($p = 0.009$) and RBC ($p = 0.03$), retinopathy (HbF, $p = 0.004$), and osteonecrosis (RBC, $p = 0.028$) (Table 7). None of the clinical outcome was predicted by RMP on multiple regression analysis.

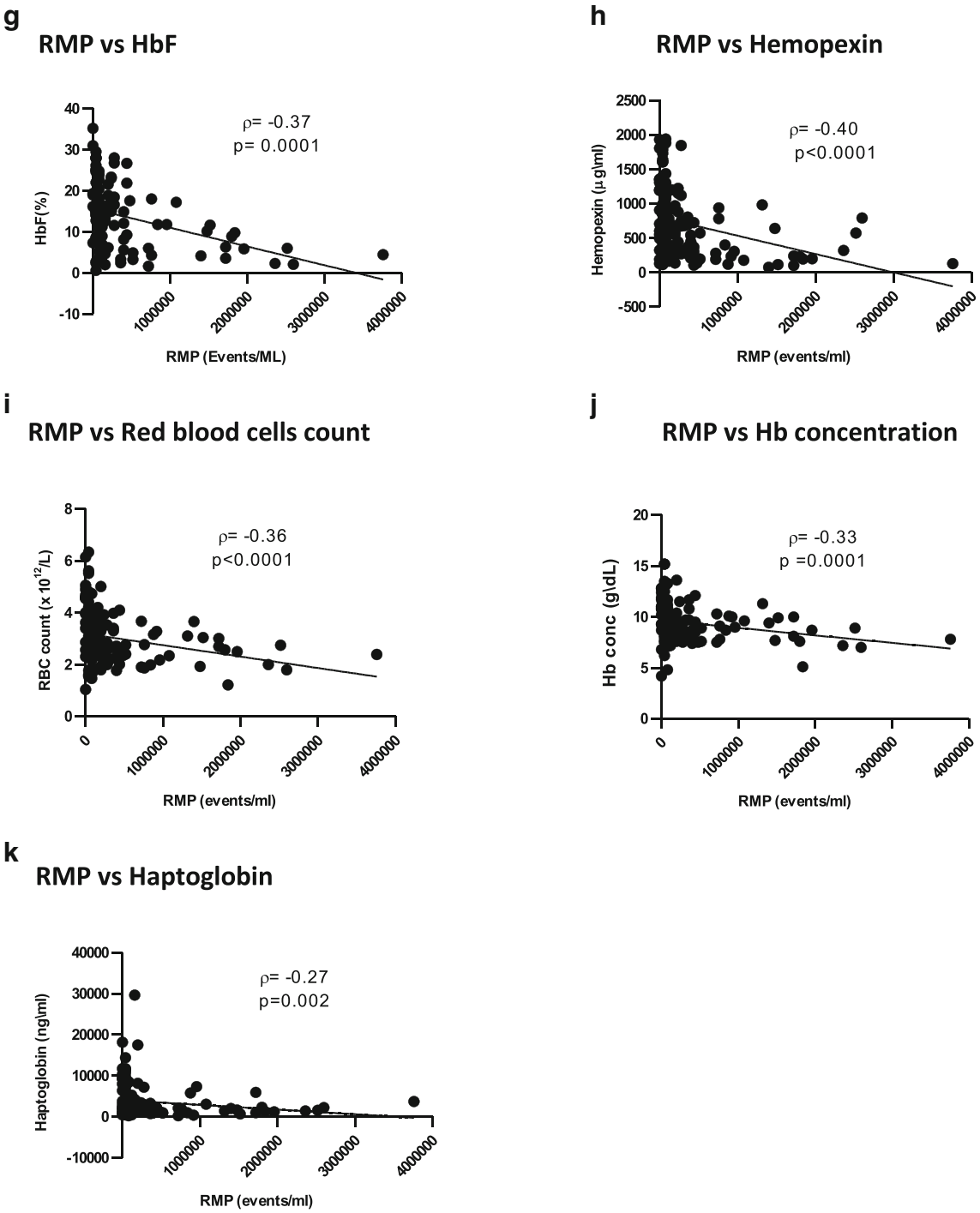

Fig. 1 (continued)

Discussion

Due to the impaired feasibility to measure hemolysis directly in most clinical settings, clinicians use indirect or surrogate markers to characterize intravascular hemolysis in SCD [5, 6, 9, 24]. There is a suggestion of a relationship between hemolysis markers (LDH, reticulocyte count, Hb concentration, total bilirubin, plasma Hb, and Heme), and SCD manifestations (leg ulcer, elevated TRV, pulmonary hypertension, stroke, priapism, oxygen saturation, and mortality) [5, 6, 9, 24]. However, there are some controversies concerning the degree of intravascular hemolysis and its contributions to endothelial lesions seen in these patients [6, 7, 13, 26]. For example, Quinn et al. [7] found that some markers of hemolysis (LDH, AST, plasma Hb, and bilirubin) do not correlate with directly measured RBC survival. They also found that

Table 3 Distribution of laboratory parameters and clinical events by hemolytic component quartiles among all patients (results are in median, interquartile range) unless otherwise indicated.

Parameters	**Quartile 4 (Q4 = highest hemolytic quartile) *N* = 34**	**Quartile 3 (Q3) *N* = 35**	**Quartile 2 (Q2) *N* = 35**	**Quartile 1 (Q1 = lowest hemolytic quartile) *N* = 34**	**P1 = Q4 vs (Q3 to Q1)**	**P2 = Q4 vs Q1**	**P3 = Q1 vs Q2 vs Q3 vs Q4**
Age	36 (32–40)	47 (42–53)	39 (31–50)	46 (38–54)	*0.0002*[a]	*0.0011*[a]	*< 0.0001*1*
Sex							
M	13	18	11	8			0.08
F	21	17	24	26			
Genotype							
SS/Sβ0	29 (85.4%)	26 (74.3%)	24 (68.6%)	11 (32.4%)	*0.015*[a]	*< 0.0001*[a]	*0.0003*
$Sβ^+$	2 (5.8%)	3 (8.6%)	1 (2.9%)	6 (17.6%)			
SC	3 (8.8%)	6 (17.1%)	10 (28.6%)	17 (50%)			
Red blood cell microparticles (events/ML)	400,000 (110000–1,490,000)	160,000 (80000–440,000)	80,000 (40000–240,000)	40,000 (0–80,000)	*< 0.0001*[a]	*< 0.0001*[a]	*< 0.0001*2*
Plasma hemoglobin (mg/dL)	105.0 (82–142)	83.9 (56.8–104)	70.7 (59.5–84.0)	56.7 (36.6–68.5)	*< 0.0001*[a]	*< 0.0001*[a]	*< 0.0001*2*
Haptoglobin (ng/mL)	1625 (974–2699)	1782 (1237–3048)	2359 (1627–3797)	3750 (1661–937.8)	*0.0064*[a]	*0.0015*[a]	*0.0016*3*
Heme (μM)	77.3 (51.3–97.1)	61.7 (44.3–85.0)	46.3 (37.0–58.8)	37.2 (25.8–49.1)	*< 0.0001*[a]	*< 0.0001*[a]	*< 0.0001*4*
Hemopexin (μg/mL)	304.4 (195–663)	546.8 (262.3–729.0)	848.6 (320–1072)	850 (461–1375)	*0.0015*[a]	*0.0021*[a]	*0.0012*5*
HbF (%)	6.3 (3.4–1.9)	11.6 (3.3–17.2)	12.6 (1.5–17.2)	5.2 (0.8–24.1)	0.410[a]	0.7312[a]	0.7045
Total bilirubin (mg/dL)	3.2 (24–49)	1.8 (1.5–2.1)	1.3 (1.0–1.5)	0.8 (0.65–1.7)	*< 0.0001*[a]	*< 0.0001*[a]	*< 0.0001*6*
Hb Conc (g/dL)	8.7 (7.7–9.7)	9.0 (8.5–10.6)	8.9 (8.0–10.8)	10.10 (9.2–11.0)	*0.011*[a]	*0.0029*[a]	*0.0021*7*
Reticulocyte count ($\times 10^9$/L)	393.3 (305.3–479)	285 (195–342)	217 (182–264)	148.5 (113–194.5)	*< 0.0001*[a]	*< 0.0001*[a]	*< 0.0001*8*
LDH (IU)	532 (476–641)	379 (324–471)	315 (247–348)	214 (180–258)	*< 0.0001*[a]	*< 0.0001*[a]	*< 0.0001*8*
Parameters	**Quartile 4 (Q4) *N* = 34 *n* (%)**	**Quartile 3 (Q3) *N* = 35 *n* (%)**	**Quartile 2 (Q2) *N* = 35 *n* (%)**	**Quartile 1 (Q1) *N* = 34 *n* (%)**	**P1 = Q4 vs (Q3 to Q1)**	**P2 = Q1 vs Q4**	**P3 = Q1 vs Q2 vs Q3 vs Q4**
No on hydroxyurea	19 (55.9)	23 (65.7)	20 (57.1)	13 (38.2)	0.212	0.816	*0.04 ($\chi^2 = 8.141$, $df = 3$)*
History of VOC in the past 1 year (yes/no)	14 (4.1)	15 (42.8)	10 (28.6)	9 (25.7)	0.4097	0.305	0.3547 ($\chi^2 = 3.249$, $df = 3$)
VOC rate, i.e., no. of severe bone pain episodes in the past 1 year	0 (0–11)	0 (0–1)	0 (0–1)	0 (0–1)	0.240	0.1275	0.3246
Acute chest syndrome (yes/no)	3 (8.8)	5 (14.3)	4 (11.4)	2 (5.9)	1.000	1.000	0.690 ($\chi^2 = 1.464$, $df = 3$)
Leg ulcer (yes/no)	6 (17.6)	5 (14.3)	6 (17.1)	0 (0)	0.365	*0.024*	0.08 ($\chi^2 = 6.550$, $df = 3$)
Stroke (yes/no)	0 (0)	4 (11.4)	4 (11.4)	4 (11.7)	*0.038*	0.113	0.230 ($\chi^2 = 4.300$, $df = 3$)
Retinopathy (yes/no)	6 (17.6)	8 (22.8)	9 (25.7)	10 (29.4)	0.365	0.3917	0.7096 ($\chi^2 = 1.383$, $df = 3$)
Osteonecrosis (yes/no)	3 (8.8)	13 (37.1)	3 (8.6)	13 (38.2)	*0.0382*	*0.0087*	*0.001 ($\chi^2 = 16.29$, $df = 3$)*
	11 (39.3)	17 (48.6)	13 (39.4)	11 (33.3)	0.543	0.789	0.569 ($\chi^2 = 2.015$, $df = 3$)

Table 3 (continued)

Risk of pulmonary hypertension (TRV ≥ 2.5 m/s) (yes/no)	

Significant *p* values are indicated in italic fonts. Kruskal-Wallis test with Dunn's multiple comparison post hoc tests with differences in *1 = (Q4 vs Q3), (Q4 vs Q1), (Q3 vs Q2); *2 = (Q4 vs Q2), (Q4 vs Q1), (Q3 vs Q1); *3 = (Q4 vs Q1), (Q3 vs Q1); *4 = (Q4 vs Q2), (Q4 vs Q1), (Q3 vs Q2), (Q3 vs Q1); *5 = (Q4 vs Q2), (Q4 vs Q1); *6 = (Q4 vs Q3), (Q4 vs Q2), (Q4 vs Q1), (Q3 vs Q2), (Q3 vs Q1), (Q2 vs Q1); *7 = (Q4 vs Q1); *8 = (Q4 vs Q3), (Q4 vs Q2), (Q4 vs Q1), (Q3 vs Q1), (Q2 vs Q1)

RMP red blood cell microparticle, *HbF* fetal hemoglobin, *RBC* red blood cell, *Hb* hemoglobin concentration, *WBC* white blood cell count, *LDH* lactate dehydrogenase, *TRV* tricuspid regurgitation velocity, *VOC* vaso-occlusive crisis, *Q4* highest hemolytic quartile, *Q3* second highest hemolytic quartile, *Q2* penultimate lowest hemolytic quartile, *Q1* lowest hemolytic quartile

[a] Mann-Whitney Test

reticulocyte count had the best correlation with RBC survival, while only 70% of the variation in RBC survival was reflected in the reticulocyte count [7]. Furthermore, some authors [6, 7, 13] have hinted that not all the elevation of serum LDH in SCD patients is derived from hemolysis as some is also released from tissue damage in the patients. Nevertheless, LDH levels have been found to correlate well with more direct markers of hemolysis in both disease state and physiologic human studies [6]. Despite these observations, several studies [5, 6, 9, 11, 24] have linked markers of hemolysis with SCD complications/manifestations. Therefore, a focus on the roles of other potential carriers of hemoglobin and heme such as the RMP is desirable and has been suggested [14–18].

An important relevance of this study is the finding of joint associations between RMP and a consortium of many traditional markers of intravascular hemolysis and red blood cells products. We are not aware of any previous study that has jointly examined the relationships between the RMP and these hemolysis markers (plasma hemoglobin, heme, haptoglobin, and hemopexin), and validate their joint relationships with other previously established traditional markers of hemolysis as done in this study. Given that multiple components contribute to the pathophysiology and downstream events in SCD, this study further sheds light on the contributions of RMP to hemolysis and possible endothelial cell lesions in SCD which are both consequences of the HbS polymerization. Similar to the findings in this study, Beers et al. [27] found that RMP correlated positively with plasma hemoglobin, and LDH, while Setty and colleagues [28] found that RMP positively correlated with reticulocyte count of SCD patients. These suggest association of RMP with hemolysis.

Although our SS/SBeta0 cohorts with hydroxyurea treatment had relatively lower RMP, plasma hemoglobin, and heme, compared to the patients without hydroxyurea treatment, these did not reach statistical significance. The lack of a significant reduction with hydroxyurea treatment with respect to the RMP is not clear to us. We suspect that this could be due to the fact that only a very small number of patients in this study were not treated with hydroxyurea thus making comparison difficult. Nonetheless, a previous study by Gerotziafas et al. [29], studies by Nebor et al. in France [30], and Piccin et al. in Italy [31] all found that hydroxyurea treatment reduced the levels of RMP in their SCD patients. On the contrary, another study from Brazil by Brunetta et al. [32] found higher RMP levels in their SCD patients treated with hydroxyurea. These observations raise the need for more studies to unravel how hydroxyurea treatment affects RMP in SCD patients.

The findings of higher RMPs in SS/SB0 subgroup of patients compared to HbSC patients (Table 1) have been previously reported by Garnier et al. [18], and this perhaps is not unexpected given that SS/SB0 patients are known for more disease severity and hemolysis [1–4]. Also, the observation

Table 4 Distribution of laboratory parameters and clinical events by hemolytic component quartiles among SS/SBETA0 patients (results are in median, interquartile range) unless otherwise indicated

Parameters	Quartile 4 (Q4 = highest hemolytic quartile) *N* = 29	Quartile 3 (Q3) *N* = 26	Quartile 2 (Q2) *N* = 24	Quartile 1 (Q1 = lowest hemolytic quartile) *N* = 11	P1 = Q4 vs (Q3 to Q1)	P2 = Q1 vs Q4	P3 = Q1 vs Q2 vs Q3 vs Q4
Red blood cell microparticles (events/ML)	440,000 (200000–1,620,000)	220,000 (80,000–490,000)	100,000 (50000–240,000)	40,000 (40000–120,000)	*< 0.0001*[a]	*< 0.0001*[a]	*< 0.0001*1*
Plasma hemoglobin (mg/dL)	113 (86.0–147.0)	89.4 (60.6–111.0)	72.5 (59.8–83.9)	57 (32.2–97.6)	*< 0.0001*[a]	*0.0008*[a]	*< 0.0001*2*
Haptoglobin (ng/mL)	1497.0 (972.0–2234.0)	1597.0 (1178.0–2930.0)	2288 (11,859.0–3708.0)	2515.0 (1464–7196.0)	*0.036*[a]	*0.0008*[a]	*0.022*3*
Heme (μM)	81.0 (52.0–101.0)	63.9 (50.0–85.4)	44.6 (35.0–57.0)	35 (23.5–87.8)	*0.0001*[a]	*< 0.0001*[a]	*< 0.0001*4*
Hemopexin (μg/mL)	279.0 (194.0–564.0)	566.0 (256 .0–740.0)	877.0 (296.0–1183.0)	1114.0 (606.0–1607.0)	*0.0002*[a]	*0.0003*[a]	*0.0001*2*
HbF (%)	7.0 (4.2–11.9)	12.6 (5.9–18.0)	15.5 (12.0–21.3)	24.6 (18.0–35.0)	*< 0.0001*[a]	*< 0.0001*[a]	*< 0.0001*1*
Hb Conc (g/dL)	8.3 (7.6–9.4)	8.9 (8.2–10.8)	8.5 (7.7–9.3)	7.6 (7.5–9.8)	0.476[a]	0.55[a]	0.384
Total bilirubin (mg/dL)	3.8 (2.8–4.9)	1.7 (1.4–2.1)	1.4 (1.1–1.6)	0.9 (0.7–1.3)	*< 0.0001*[a]	*< 0.0001*[a]	*< 0.0001*5*
Reticulocyte count ($\times 10^9$/L)	369.0 (269.0–471.0)	253.0 (181.0–343.0)	208.0 (181.0–226.0)	104.0 (96.0–161.0)	*< 0.0001*[a]	*< 0.0001*[a]	*< 0.0001*5*
LDH (IU)	533.0 (483.0–648.0)	413.0 (322.0–498.0)	326 (296.0–381.0)	258.0 (210.0–286.0)	*< 0.0001*[a]	*< 0.0001*[a]	*< 0.0001*5*
Parameters	**Quartile 4 (Q4) *N* = 29 *n* (%)**	**Quartile 3 (Q3) *N* = 26 *n* (%)**	**Quartile 2 (Q2) *N* = 24 *n* (%)**	**Quartile 1 (Q1) *N* = 11 *n* (%)**	**P1 = Q4 vs (Q3 to Q1)**	**P2 = Q1 vs Q4**	**P3 = Q1 vs Q2 vs Q3 vs Q4**
No on hydroxyurea	19 (65.5)	22 (84.6)	20 (83.3)	10 (90.9)	0.051	0.232	0.18
History of VOC in the past 1 year (yes/no)	12 (41.4)	13 (50.0)	8 (33.3)	2 (18.2)	0.818	0.2698	0.2930 ($\chi^2 = 3.72$, $df = 3$)
VOC rate, i.e., no. of severe bone pain episodes in the last 1 year	0 (0–1)	0.5 (0–1)	0 (0–1)	0 (0–1)	0.632	0.2763	0.474
Acute chest syndrome (yes/no)	3 (10.3)	5 (19.2)	4 (16.7)	1 (9.1)	0.536	1.000	0.7464 ($\chi^2 = 1.227$, $df = 3$)
Leg ulcer (yes/no)	6 (20.7)	5 (19.2)	5 (20.8)	0 (0)	0.768	0.1623	0.433 ($\chi^2 = 2.737$, $df = 3$)
Stroke (yes/no)	0 (0)	4 (15.4)	4 (16.7)	2 (18.1)	*0.027*	0.07	0.1440 ($\chi^2 = 5.413$, $df = 3$)
Retinopathy (yes/no)	4 (13.8)	4 (15.4)	2 (8.3)	0 (0)	0.721	0.5602	0.521 ($\chi^2 = 2.254$. $df = 3$)
Osteonecrosis (yes/no)	2 (6.9)	7 (2.7)	3 (12.5)	3 (27.3)	0.130	0.117	0.1609 ($\chi^2 = 5.153$, $df = 3$)
	12 (44.8)	16 (61.5)	11 (45.8)	6 (54.5)	0.367	0.4977	0.4745 ($\chi^2 = 2.504$, $df = 3$)

Table 4 (continued)

Risk of pulomary hypertension, i.e., TRV ≥ 2.5 m/s (yes/no)					

Significant *p* values are indicated in italic fonts. Kruskal-Wallis Test with Dunn's multiple comparison post-hoc tests with differences in *1 = (Q4 vs Q1), (Q4 vs Q2), (Q3 vs Q1); *2 = (Q4 vs Q2), (Q4 vs Q1); *3 = (Q4 vs Q1); *4 = (Q4 vs Q2), (Q4 vs Q1), (Q3 vs Q2), (Q3 vs Q1); *5 = (Q4 vs Q3), (Q4 vs Q2), (Q4 vs Q1), (Q3 vs Q1)

RMP red blood cell microparticle, *HbF* fetal hemoglobin, *RBC* red blood cell, *Hb* hemoglobin concentration, *WBC* white blood cell count, *LDH* lactate dehydrogenase, *TRV* tricuspid regurgitation velocity, *VOC* vaso-occlusive crisis, *Q4* highest hemolytic quartile, *Q3* second highest hemolytic quartile, *Q2* penultimate lowest hemolytic quartile, *Q1* lowest hemolytic quartile

[a] Mann-Whitney Test

that the SCD patients had higher RMPs relative to the control group is in keeping with literature data suggestions [14–16].

Regarding the clinical events, our findings of higher levels of both RMP and heme in patients with sickle leg ulcer could be a further confirmation of their roles as factors complementary to hemolysis-induced SCD lesions given that previous studies have associated leg ulcer with SCD hemolysis subphenotype [5, 6, 9, 11, 25]. Although not sustained at multivariate level (probably because of size effect), nevertheless, the association of leg ulcer also with heme is a further confirmation of the possible contributions of both products to SCD lesions among the study participants. We suspect that both RMP and heme perhaps mediate and promote leg ulceration through their oxidative injuries and possible NO depletion upon their release as demonstrated by Camus and colleagues [17]. The observations by Camus and colleagues were also earlier corroborated by Liu et al. [33], as they demonstrated that, through a concentration-dependent gradient, RMPs are capable of entering significantly into the free zone of vasculature from where they can interact with endothelial cell lining of blood vessels and mediate NO depletion thereby causing vascular damages and other injuries. Drawing from these, it is therefore possible that the association of both the RMP and heme with leg ulcer among the patients in this study could be as a result of injuries from NO depletion, oxidative stress, and other mediatory molecules causing both vascular and cellular damages to these patients. This further points to the possible contributions of RMP to the pathophysiology of SCD through hemolysis and possible endothelial cell lesions, both of which are consequences of the HbS. This is more so, given that the sickle leg ulcer phenotype has been closely linked to both hemolysis and vasculopathy among other mechanisms in SCD [5, 9, 11, 24, 25].

In addition, patients with the sickle leg ulcer phenotype significantly belonged to the SCD patients with higher hemolytic quartile (Table 3) further reinforcing findings by previous authors that leg ulcer is associated with higher hemolysis and worsening anemia in patients with SCD [5, 11, 24, 25]. To the best of our knowledge, this study is the first study to report any joint relationships between leg ulcer and both RMP and heme in patients with SCD. Hence, this study has raised the possibility of including both RMP and heme as possible markers of leg ulcer phenotype in patients with SCD.

The observed correlations between TRV and RMP and other surrogate hemolysis markers suggest that TRV may be related to RMP and hemolysis as previously observed [6, 9, 34]. Also, the findings of worsening anemia, higher RMP, and LDH in our patients with elevated TRV are in conformity with previous reports that have suggested that TRV is associated with this vasculopathic phenotype of SCD [6, 35, 36]. TRV is a marker of pulmonary vascular disease and possibly myocardiac stress [21, 37], and several studies have shown that SCD patients with raised TRV are at risk of developing

Table 5 Associations between leg ulcer and biologic markers among SS/SB0 patients

Parameters	Leg ulcer Yes ($N = 17$)	Leg ulcer No ($N = 73$)	p values
RMP (events/mL)	440,000.0 (180,000.0–900,000.0)	120,000 (80,000.0–400,000.0)	*0.028*
Plasma Hb (mg/dL)	86.0 (66.3–118.0)	84 (60.4–112.0)	0.699
Haptoglobin (ng/mL)	178.0 (1167–3056)	2377 (1467–3662)	0.21
Heme (μM)	81.3 (50–105)	51.4 (43–78)	*0.014*
Hemopexin (μg/mL)	400 (246.7–784)	572 (238.5–953.5)	0.49
HbF (%)	12.6 (6.1–20.6)	13.4 (6.6–18.7)	0.84
HbS (%)	80.2 (72.5–87)	76.3 (66.7–82)	0.108
RBC (million cells/μL)	2.3 (1.9–2.6)	2.4 (3.1–2.9)	0.19
Total bilirubin (mg/dL)	1.7 (1.2–3.3)	1.7 (1.2–2.8)	0.8
Hb (g/dL)	8.5 (7.8–9.2)	8.6 (7.6–9.4)	0.768
Reticulocyte ($\times 10^9$/L)	245.2 (201.5–359.0)	232.9 (172–349)	0.340
LDH (IU)	4.37 (333.0–567.0)	372.0 (295–507.0)	0.21
Platelet ($\times 10^3$/μL)	330 (283–395.0)	346.0 (260–424)	0.94
WBC ($\times 10^3$/μL)	6.4 (5–8.5)	7.2 (4.9–9.2)	0.55

Significant p values are in italic fonts, Test statistics = Mann-Whitney test

RMP red blood cell microparticle, *HbF* fetal hemoglobin, *RBC* red blood cell, *Hb* hemoglobin concentration, *HbS* hemoglobin S, *WBC* white blood cell count, *LDH* lactate dehydrogenase

pulmonary hypertension [6, 35–39]. The relationships between RMP and TRV in this study have further exposed the possibility of using the RMP as a biomarker for identifying high-risk SCD patients that may need further evaluation for some vascular complications of the disease. This is more apt given that pulmonary hypertension has been associated with increased risk of morbidities and deaths among SCD patients [6, 36–38]. Therefore, this study further highlights the possible contributions of RMP to the clinical outcome of patients with SCD if used as a surrogate marker for vascular complications of the disease.

The lack of any relationship between RMP and VOC in this study contrasts with a previous report by Tantawy and colleagues [40] where higher MPs were associated with both VOC and elevated TRV (risk of pulmonary hypertension). The differences in the age of participants in this study and that

Table 6 Associations between elevated TRV (risk for pulmonary hypertension) and biologic markers among SS/SB0 patients

Parameters	Elevated TRV (at risk of pulmonary hypertension) ($N = 44$)	Normal TRV (not at risk of pulmonary hypertension) ($N = 46$)	p values
RMP (events/mL)	240,000.0 (80,000.0–7470,000.0)	80,000 (40,000–280,000.0)	*0.011*
Plasma Hb (mg/dL)	84 (57–116)	82 (59–104)	0.855
Haptoglobin (ng/mL)	1634.0 (1245.0–2880)	2165 (1065–4942)	0.355
Heme (μM)	54.0 (43–82.9)	50.8 (40–76)	0.44
Hemopexin (μg/mL)	543 (232–932.0)	582 (304.0–931)	0.396
HbF (%)	13 (6.4–20)	13.4 (6.3–19)	0.84
HbS (%)	77.3 (68–82)	76 (65–82.0)	0.509
RBC (million cells/μL)	2.4 (2–2.7)	2.7 (2.3–3.3)	*0.003*
Total bilirubin (mg/dL)	1.6 (1.3–2.6)	1.6 (1.0–2.7)	0.43
Hb (g/dL)	8.5 (7.7–9.1)	9.1 (7.9–10.2)	*0.025*
Reticulocyte ($\times 10^9$/L)	242 (194–347.0)	236.0 (164–329.0)	0.542
LDH (IU)	384.0 (295.0–550.0)	324 (258–451)	*0.021*
Platelet ($\times 10^3$/μL)	327.0 (261–417.0)	353.0 (260–424)	0.410
WBC ($\times 10^3$/μL)	6.6 (4.5–8.5)	8.2 (5.5–10.6)	0.08

Significant p values are in italic fonts, Test statistics = Mann-Whitney test

RMP red blood cell microparticle, *HbF* fetal hemoglobin, *RBC* red blood cell count, *Hb* hemoglobin concentration, *HbS* hemoglobin S, *WBC* white blood cell count, *LDH* lactate dehydrogenase, *TRV* tricuspid regurgitation velocity

Table 7 Logistic regression models for clinical events

Model	Hosmer and Lemeshow Test χ^2, *df*, (*p* value)	Cox and Snell *R* square (%)	Nagelkerke *R* square (%)	*p* value significance	Odds ratio	95% confidence interval	
						Lower	Upper
Leg ulcer	10.89, 8, (0.21)	15.5	29.4				
Heme				*0.009*	1.03	1.01	1.06
RBC				*0.03*	0.13	0.02	0.77
Retinopathy	8.24, 8, (0.411)	21.5	32.2	*0.004*	0.89	0.81	0.96
HbF							
Osteonecrosis	8.99, 8, (0.343)	12.4	18.7				
RBC				*0.028*	2.49	1.104	5.63

RBC red blood cell count, *Hb* hemoglobin concentration, *HbF* fetal hemoglobin

by Tantawy and colleagues may account for this observation. While they study younger SCD population, our study participants were relatively older. However, we are not aware of any study that has described age-related differences in the RMP levels of SCD patients.

The lack of associations of RMPs with the described phenotypes on multivariate analysis may be due to the size of this study and by extension, the small numbers of the various phenotypes. Furthermore, our finding is in conformity with a previous study by Garnier and colleagues [18] where no relationship was also found thus raising the need for further studies to unmask the contributions of RMPs to clinical events in SCD.

In conclusion, although RMP was not included in the original models described as markers of the SCD hemolysis and its vasculopathy-associated sub-phenotype [6, 39], findings from this study and other emerging evidences [9, 14–18, 33, 40] suggest that RMP alongside its trapped heme may constitute important mediators of some SCD pathophysiology/complications and therapies targeting them could perhaps be another strategy to combat SCD complications.

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Authors' contributions All authors contributed to critical aspects of the study. OSO, AA, and FFC conceived and designed the study. OSO wrote the paper, collected and analyzed the data, and performed the experiments. CL, CFP, and ALL performed the experiments. KYF, STS, and FFC managed the patients. FFC supervised the study. All authors participated in reviewing the manuscript for important intellectual contents and agreed to the final version.

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Compliance with ethical standards

The study was conducted according to the international standards of biomedical and human research. The study was approved by the institutional review board of UNICAMP no. CAAE 54031115.9.0000.5404, and written informed consent was obtained from all participants before being included in the study.

Competing interests The authors declare that they have no conflict of interest.

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