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RESEARCH ARTICLE

Changes in Heme Levels During Acute Vaso-occlusive Crisis in Sickle Cell Anemia

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Abstract

Objective/Background: **Sickle cell anemia (SCA) is associated with increased levels of extracellular heme, which is a key mediator of inflammation in this condition. Despite abundant evidence supporting this concept in cell and animal models, few studies addressed the association between heme levels and the development and severity of acute vaso-occlusive crises (VOC) in humans.**

Methods: **A cross-sectional study was conducted in patients with acute VOC. Total extracellular heme levels were measured in both plasma and serum at admission and after convalescence, and correlated with other clinical and laboratory markers of SCA severity.**

Results: **A total of 28 episodes of VOC in 25 patients were included. Heme levels were similar between admission and convalescence, and correlated with the difference between pre and post hemoglobin, and SCA severity estimated by a composite score of clinical and laboratory markers. Heme levels were neither associated with VOC severity nor with markers of hemostasis activation, and were similar to those reported in an independent population of SCA patients at steady state.**

Discussion: **Acute VOC are not characterized by significant increases in total extracellular heme levels. Studies measuring the fraction of free extracellular heme unbound to proteins are warranted to further refine our understanding of the role of heme in acute VOC.**

Keywords: D-dimer, Heme, Sickle cell anemia, Thromboembolism, Vaso-occlusion

1. Introduction

Intravascular hemolysis, a key component of the pathogenesis of sickle cell anemia (SCA), results in the release of hemoglobin (Hb) and heme from red blood cells, leading to the saturation of the physiological systems responsible for the sequestration of Hb and heme from the extracellular milieu based on haptoglobin and hemopexin, respectively [1]. Accordingly, higher levels of heme and decreased levels of hemopexin were demonstrated in SCA more than 50 years ago [2], and within the last 2 decades, evidence gathered mainly from animal models demonstrated that extracellular heme acts as pro-inflammatory mediator in SCA [3,4]. At the same time, several studies in cells and in mice also demonstrated that heme activates different compartments of innate immunity [5–9]. Together, these data support the concept that extracellular heme represents a key element in the pathogenesis of SCA [1,10,11].

Other hallmarks of SCA include hemostatic abnormalities, illustrated by increased levels of

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markers such as D-dimer and platelet counts, and by an increased rate of thromboembolic [12–14]. Interestingly, studies using animal models [15], or human whole blood spiked with heme [8] demonstrated that heme can activate hemostasis in a tissue factor–dependent mechanism, being potentially involved in the prothrombotic state observed in SCA.

Acute vaso-occlusive crisis (VOC) is normally triggered by conditions such as infection and dehydration, which can slow capillary transit time and exacerbate the activation of proinflammatory pathways involving neutrophils, platelets, endothelial cells, and red blood cells [1]. Whether the acute release of extracellular heme caused by increased hemolysis could also contribute to the development of VOC is yet to be determined. Higher levels of extracellular heme have been associated with the trigger of acute VOC both in animal models [3,4] and in a study in humans which associated higher free heme plasma levels with increased risk of acute chest syndrome (ACS) [16]. Here we measured total extracellular heme levels in patients with acute VOC episodes at admission and after convalescence in both serum and plasma samples, and investigated its association with clinical and laboratory markers of disease severity and with markers of hemostasis activation in SCA.

2. Materials and methods

2.1. Study population

This was a cross-sectional study that enrolled SCA patients followed at the outpatient public care center of Hematology and Hemotherapy Foundation from Amazonas State (HEMOAM; Manaus, AM, Brazil). When the study was initiated, 236 patients with SCA were registered at this center. The study was performed in accordance with the Declaration of Helsinki and approved by the institutional review board (Certificado de Apresentacao e Apreciacao Etica (CAAE): 71147817.3.0000.0009). All patients provided a written informed consent prior to any study procedure.

Inclusion criteria were admission in the emergency unit of HEMOAM in the 48 hours before enrollment due to either (a) severe acute pain crisis, defined as a typical acute pain crisis requiring at least 4 hours of parenteral analgesia; and/or (b) ACS, defined by the occurrence of new lung infiltrates (on auscultation or chest X-ray) in a patient presenting one or more of the following signs/symptoms: fever, chest pain, tachypnea, cough, or hypoxemia [17]. Exclusion criteria included hemodynamic instability, transfusion of two or more red blood cell concentrates in the same admission prior to enrollment; absence of suitable venous access for sample collection; and age < 10 years. Patients were allowed to be enrolled more than once as long as the prior VOC had completely resolved for at least 30 days. Serum heme levels from an independent population of patients with SCA followed obtained in a previous study from our center [18] were used in one of the analysis.

2.2. Sample collection and processing

Samples were obtained from a peripheral vein by one of the investigators, with minimal trauma. Samples were collected immediately after enrollment (admission) and after convalescence. Convalescent samples were obtained in the first outpatient visit.

For obtaining platelet-poor plasma, whole blood collected in 3.2% sodium citrate tubes was centrifuged at 1,200*g* for 15 minutes at 22 °C. For serum, non-anticoagulated blood was allowed to clot at 22 °C for 30 minutes, and then centrifuged at 800*g* for 10 minutes at 4 °C. Serum and plasma aliquots were stored at −80 °C until analysis. Samples for hematological analyses were collected in EDTA tubes and immediately sent for analysis.

2.3. Clinical and laboratory data

Demographic and clinical data, including the clinical course and outcome of VOC were obtained from the medical records of HEMOAM. For a subgroup of patients (16/25), a validated SCA severity score [19] was available from a recent publication from our group and was included in our analysis [20]. Heme levels were measured in plasma and in serum, using a colorimetric assay (Quantichrom, BioAssay Systems, Hayward, CA, USA), which measures heme bound to albumin and other proteins (i.e., total heme). The rationale for measuring heme in serum was to investigate whether total heme in serum could be a more reliable proxy of free heme than total heme in plasma, which is normally reported in SCA literature. D-dimer levels were measured in platelet-poor plasma with an automated coagulometer (ACL AcuStar, Instrumentation Laboratory, USA) using an immunoturbidimetric assay (HemosIL, AcuStar D-Dimer test, Instrumentation Laboratory, USA). Reticulocyte and complete blood counts were performed in an automated hematology analyzer (Advia 2120, Siemens Healthcare, Germany).

2.4. Statistical analysis

The comparison between admission and convalescent samples was performed using paired *t* test or Wilcoxon test. For unpaired variables, comparisons were made using the *t* or Mann–Whitney *U* test. Correlations were assessed by the Pearson or Spearman tests. All statistical analyses were performed using the GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, CA, USA). A minimal sample size of 16 patients was defined to detect differences of 1 SD between admission and convalescent samples, with a statistical power of 0.8. A p value <0.05 was considered as statistically significant.

Table 1. Demographic and Clinical Characteristics of the Study Population.

Demographic and clinical characteristics	$n = 25$
Age, years	24 (10–50)
Sex, male/female	13/12
SCA genotype	
HbSS	25 (100)
Use of hydroxyurea	25 (100)
Regular transfusion therapy	None
History of stroke	6 (24)
Frequency of VOC in the previous 12 months	5 (2–16)
SCA severity score[a]	0.67 (0.13–0.97)

Note. Data are presented as *n* (%) or median (range). SCA = Sickle cell anemia; VOC = vaso-occlusive crisis.
[a] Data available for 16 patients.

Table 2. Clinical Presentation of Acute Vaso-Occlusive Crisis.

Clinical presentation	$n = 28$
Pain	24[a] (86)
Acute chest syndrome	7[a] (25)
Fever	6 (21)
Hospital length of stay, days	4 (2–23)
Treatment strategies during vaso-occlusive crisis	
Analgesia with opioids	
Antibiotics	28 (100)
Transfusions (any)	5 (18)
Exchange transfusion	7 (25)
Oxygen therapy	7 (25)
Mechanical ventilation	10 (36)
Vasoactive drugs	None

Note. Data are presented as *n* (%) or median (range).
[a] Twenty four episodes presented as pain crisis, of which three evolved to acute chest syndrome; four episodes were characterized as acute chest syndrome from the time of admission.

3. Results

Between August 2018 and March 2019, 37 admissions due to severe acute pain crisis or ACS were recorded. Nine admissions were excluded from the study due to: refusal to participate ($n = 3$), two or more transfusions before enrollment ($n = 2$), absence of venous access ($n = 2$), and age <10 years ($n = 2$). In total, 28 episodes of VOC were included in 25 patients. Demographic and clinical characteristics of the study population are shown in Table 1. The clinical characteristics of the VOC are shown in Table 2. Blood samples were obtained from all patients at admission, and from 20 patients at convalescence. The hematological variables measured during acute VOC are shown in Table 3. Most hematological parameters differed when these two time points were compared.

Since we aimed to investigate the kinetics of heme levels during VOC not only in plasma, which is normally used in studies measuring heme, but also in serum samples, we first assessed the correlation between total heme in these two biological matrixes. As shown in Fig. 1, a strong correlation (Rs = 0.77) was observed between these two methods (Fig. 1A), with heme levels in serum being on average 26.3636.68 M higher than in plasma (Fig. 1B). Next, we went on to compare admission and convalesce total heme levels in both types of sample. As shown in Fig. 2, no difference could be observed in both plasma and serum samples.

Table 3. Hematological Parameters During Acute Vaso-Occlusive Crisis.

	Admission ($n = 28$)	Convalescence ($n = 20$)	p [a]
Hemoglobin, g/dL	7.5 (5.2–11.0)	8.4 (6.6–10.6)	0.008
Mean corpuscular volume, fL	91.1 (64.5–122.9)	91.1 (70.6–106.3)	0.736
Red blood cell distribution width, %	21.2 (16.4–26.4)	20.3 (16.1–28.1)	0.585
Platelet count, $\times 10^9$/L	417.5 (154.0–911.0)	471.0 (276.0–1,060.0)	0.048
Mean platelet volume, fL	9.2 (6.4–10.9)	10.0 (6.6–12.9)	< 0.0001
Leukocytes, $\times 10^9$/L	11.1 (3.0–26.51)	9.2 (2.2–15.9)	0.008
Neutrophils, $\times 10^9$/L	7.1 (0.8–13.5)	4.9 (0.9–11.6)	0.001
Lymphocytes, $\times 10^9$/L	3.1 (0.9–4.8)	3.0 (0.7–6.8)	0.038
Monocytes, $\times 10^9$/L	0.7 (0.2–1.7)	0.6 (0.2–1.2)	0.002
Reticulocytes, $\times 10^9$/L	260.0 (11.9–706.0)	231.0 (22.5–432.0)	0.272
Reticulocytes, %	9.6 (0.5–25.0)	7.2 (2.1–19.4)	0.617
D-dimer	2,383 (529–11,862)	1,835 (635–14,186)	0.841

Note. Data are presented as median (range).
[a] Paired *t* test.

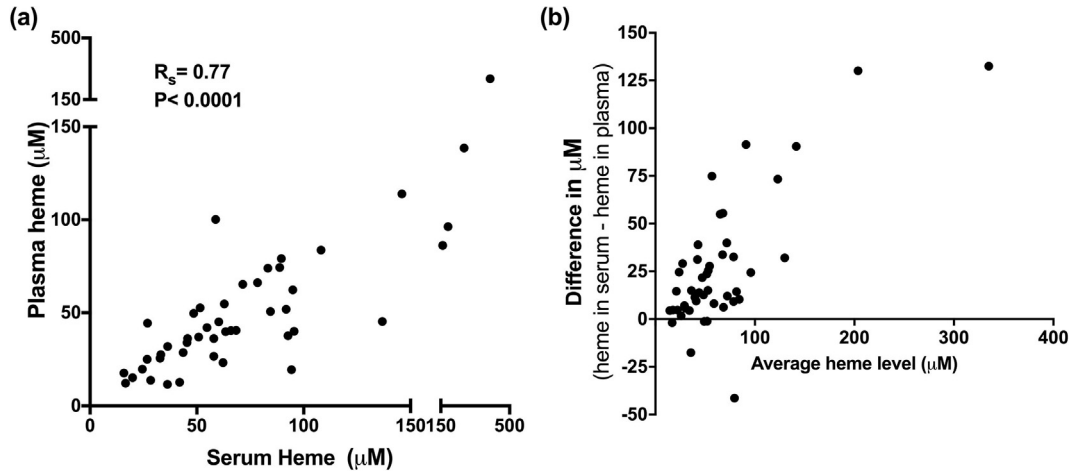

Fig. 1. (A) Correlation between total heme levels in plasma and serum showing a strong correlation (Spearman correlation coefficient). (B) Bland–Altman plot depicting the difference between serum and plasma heme levels according to the average between these two measurements, showing a positive bias of 26.36 in serum compared with plasma.

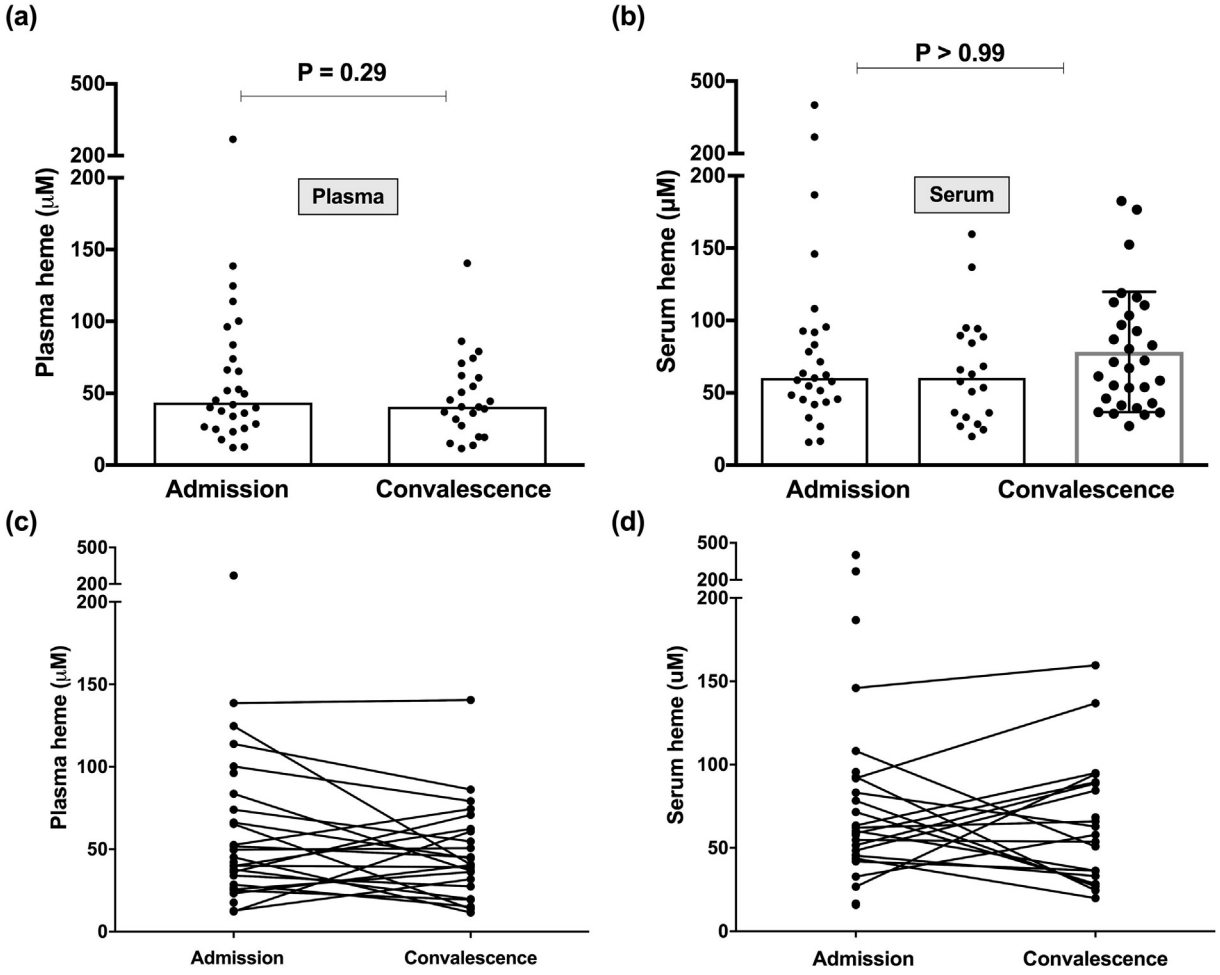

Fig. 2. Total heme levels measured in plasma (A and C) and serum (B and D) of sickle cell anemia patients at admission (n = 28) and convalescence (n = 20). In the upper panels (A and B), dot plots and median values of all patients are presented. In the lower panels (C and D), the same data are presented, depicting the change in admission and convalesce heme levels per individual patient. Comparisons were performed with the Wilcoxon test.

We then explored the correlations between heme levels in plasma and serum with clinical and laboratory parameters associated with VOC severity, and with activation of inflammation and hemostasis (Table 4). No correlation was observed between heme levels in plasma or serum with D-dimer or platelets, nor with clinical proxies of VOC severity such as hospital length of stay (LOS) and days of opioid use. The only laboratory correlation was between heme and delta Hb. Interestingly, we also observed a trend towards a positive correlation between plasma heme levels and SCA severity score.

Next we investigated whether plasma heme levels at admission were associated with clinical parameters of VOC severity (LOS and days of opioid use; Fig. 3A and 3B) and of SCA severity (Fig. 3C). As shown in Fig. 3, heme levels were not associated with any of these presentations. We also compared plasma heme levels at admission between patients with ACS or acute pain crisis. As shown in Fig. 4, no difference could be observed. Similar data were obtained with serum heme (data not shown).

Finally, we compared the kinetics of total plasma heme in plasma between patients whose convalescence sample were obtained in the first 33 days after discharge compared with the remaining patients. This time point corresponds to the median time from discharge to convalescent sample collection (median 33 days, range 7–133). As shown in Fig. 5A and 5B, for patients in the former group (early convalescence) a clear decrease in total heme levels could be observed from admission to convalescence, while for the remaining patients, no significant changes were observed. When heme levels at admission were compared with values at each of these time points (Fig. 5C), no difference could be observed. We also compared serum heme levels at each of these time points with those from a population of SCA patients at steady state described in a different study from our group (submitted for publication), and no significant difference could be observed (Fig. 5D).

4. Discussion

In the past two decades, robust data in cells and animal models were published supporting the concept that extracellular heme is a key mediator of inflammation in SCA, and a potential activator of hemostasis in hemolytic anemias [1,10]. However, limited data are available associating circulating heme levels with clinical and laboratory markers of severity and/or inflammatory activity in SCA. Using a population of SCA patients, we explored the kinetics of heme levels from admission to convalescence of VOC, as well as the associations of heme levels with relevant clinical and laboratory features of SCA. The main result of our study was that acute VOC was not preceded by a peak in levels of total extracellular heme in either plasma or serum, and that this parameter was not associated with most of the relevant clinical and laboratory markers of acute VOC severity and hemostatic activation.

Despite robust evidences associating extracellular heme with innate immune activation and with the development of acute manifestations of SCA in animal models, it has not been yet demonstrated that fluctuations in heme levels are involved in the development of VOC in SCA patients. In fact, while several studies confirmed that levels of heme in plasma of SCA patients are higher than in healthy individuals [2,21], as far as we are aware only one report associated heme levels with increased risk of VOC in SCA [16], and no previous study has evaluated fluctuations in levels of circulating heme during acute VOC.

Our initial hypothesis was that heme levels would be higher at admission when compared with convalescence, thus supporting the concept that acute VOC would be characterized by peaks in the release of heme to the extracellular space which

Table 4. Correlation of Heme Levels Measured at Admission with Clinical and Laboratory Parameters.

Variables	Plasma heme		Serum heme	
	R_s coefficient (95% CI)	p	R_s coefficient (95% CI)	p
D-dimer	0.09 (−0.29–0.46)	0.61	−0.11 (−0.47–0.26)	0.54
Hemoglobin	0.19 (−0.19–0.54)	0.31	−0.09 (−0.45–0.28)	0.62
Mean corpuscular volume	0.06 (−0.32–0.44)	0.72	0.16 (−0.22–0.50)	0.39
Red blood cell distribution width	0.07 (−0.32–0.44)	0.71	0.08 (−0.29–0.44)	0.67
Platelet count	−0.02 (−0.40–0.35)	0.88	−0.27 (−0.58–0.10)	0.15
Mean platelet volume	0.24 (−0.15–0.57)	0.21	0.27 (−0.11–0.58)	0.16
Leukocytes	0.07 (−0.31–0.44)	0.71	−0.01 (−0.38–0.36)	0.95
Neutrophils	−0.009 (−0.39–0.37)	0.96	−0.01 (−0.38–0.36)	0.94
Monocytes	0.15 (−0.24–0.50)	0.43	0.04 (−0.33–0.41)	0.80

Note. CI = confidence interval.

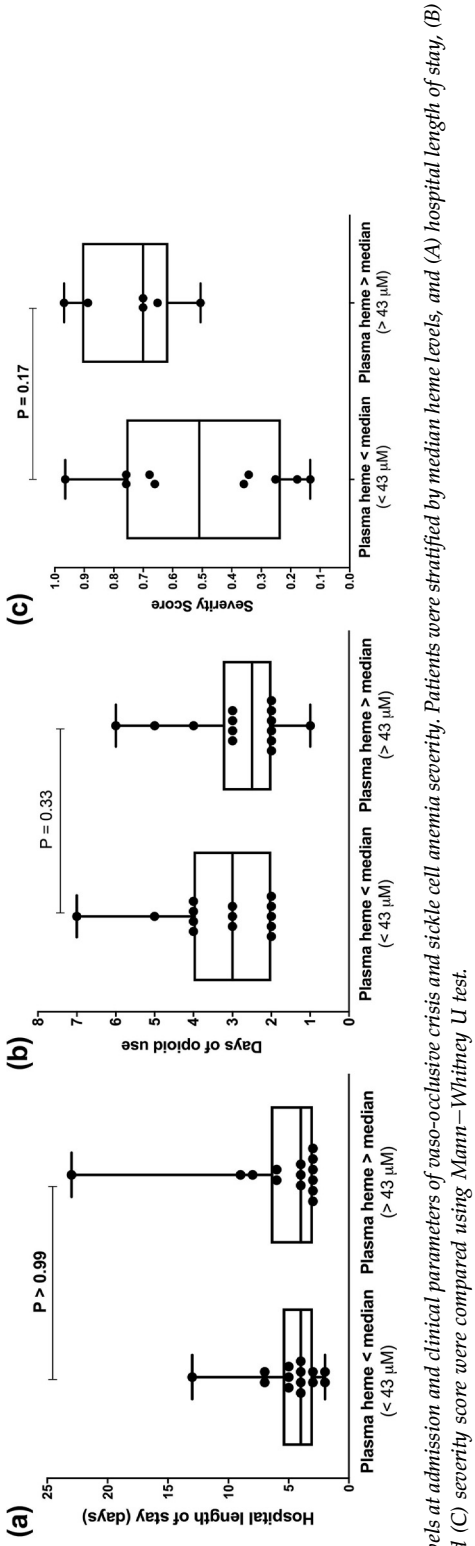

Fig. 3. Plasma heme levels at admission and clinical parameters of vaso-occlusive crisis and sickle cell anemia severity. Patients were stratified by median heme levels, and (A) hospital length of stay, (B) days of opioid use, and (C) severity score were compared using Mann–Whitney U test.

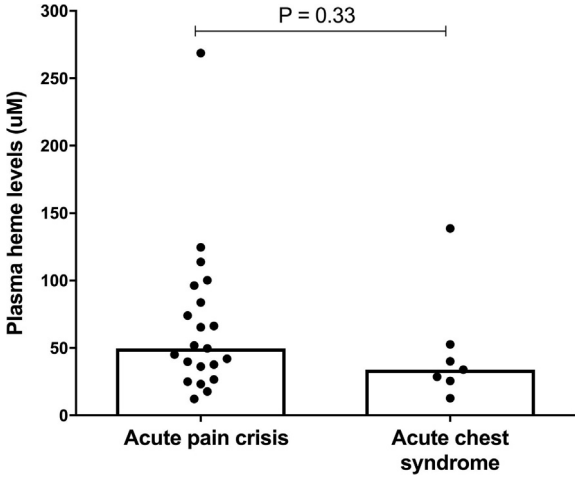

Fig. 4. Plasma heme levels in patients presenting with acute chest syndrome (n = 7) or acute pain crisis (n = 21). Mann–Whitney U test.

could contribute to its pathogenesis, in accordance to what has been described in animal models of SCA. However, although heme levels were higher at admission when compared with convalescent samples obtained in the first month after VOC, this did not hold true when all convalescent samples were included in the analysis. Of note, while most previous studies measured heme in plasma, we confirmed these negative results in both serum and plasma. Moreover, we also compared serum heme levels at admission with those described in an independent population of SCA patients at steady state (from another study from our center), and could not demonstrate any significant difference, further corroborating that peaks in total extracellular heme levels are not required for the development of acute VOC. This result is in accordance with a previous study that measured heme using the same method in children with SCA during VOC and in steady state, and that also reported no significant differences between these time points [22]. By measuring heme levels in convalescent samples, our study provides additional information about the kinetics of extracellular heme levels in the course of acute VOC.

Similarly, in contrast to our initial hypothesis, no consistent association could be observed between total heme levels in serum or in plasma with several relevant clinical and laboratory markers of SCA severity, or of hemostasis such as hospital LOS, days of opioid use, development of ACS, neutrophil and platelet count, or D-dimer. Of note, D-dimer was selected because it remains as the only single laboratory marker of thrombin generation capable to identify patients with increased risk of venous thromboembolism recurrence in several large

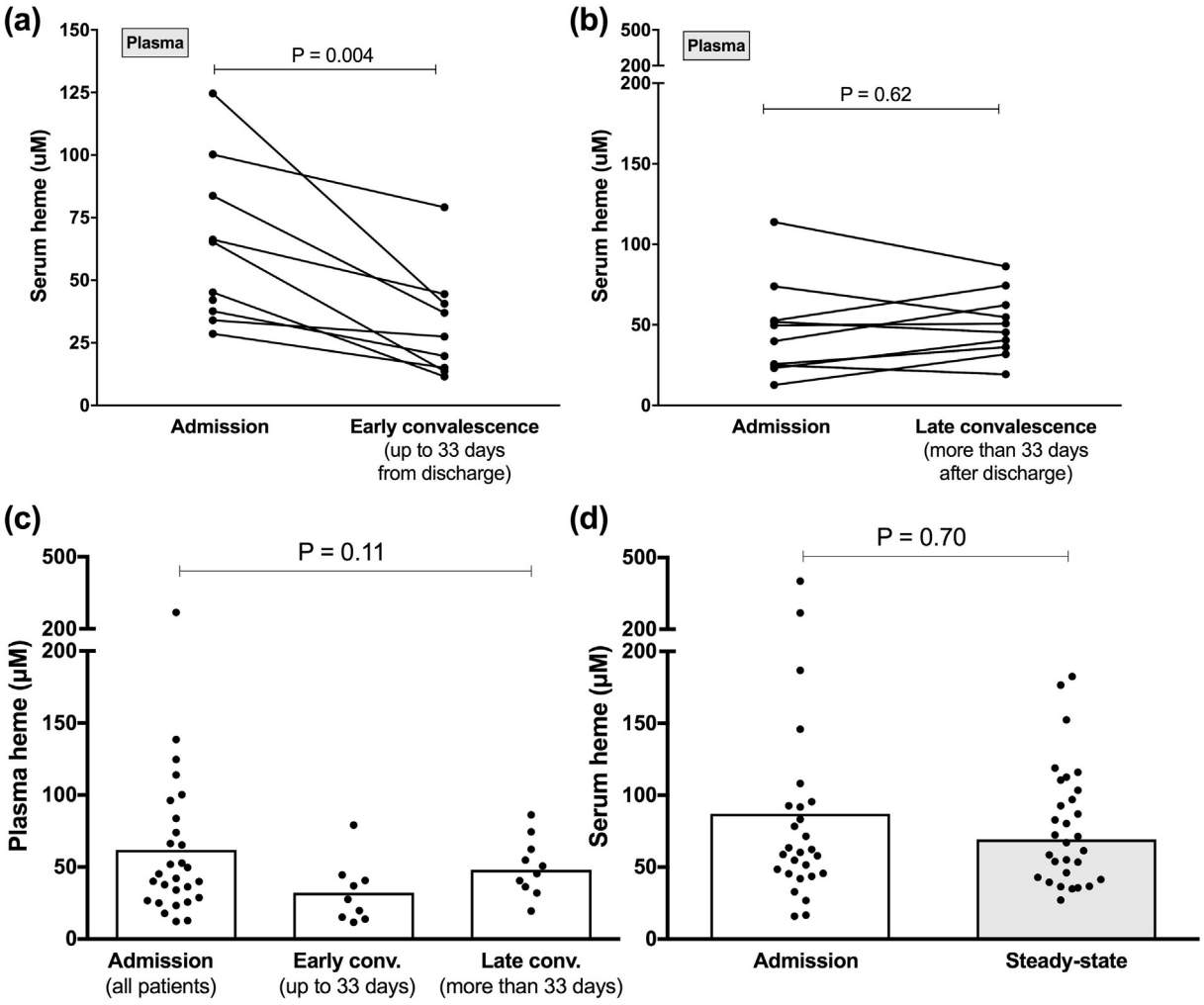

Fig. 5. Total heme levels measured in the plasma of sickle cell anemia (SCA) patients at admission and convalescence (n = 20 paired samples). (A) Data from patients whose convalescence samples were obtained in the first 33 days after discharge (median) and (B) data from the remaining patients (more than 33 days from discharge). Comparisons were performed with Wilcoxon test. (C) Heme values at admission are compared with those obtained at early and late time points in convalescent patients (Kruskal–Wallis test). (D) Serum heme levels at admission for vaso-occlusive crisis (n = 27) and in an independent population of SCA patients in steady state (n = 30; Kruskal–Wallis test). Note. Conv. = convalescence.

population studies [23], performing better than biomarkers such as thrombin generation, whose association with clinically relevant hypercoagulability in large populations with different diagnosis is still disputable [24]. The only consistent correlation of heme levels in both serum and plasma was that with delta Hb, which is a proxy of hemolysis. Interestingly, serum heme levels were also correlated with the SCA severity score, which is a composite score of encompassing laboratory and clinical markers of SCA severity. To the best of our knowledge, this is a novel result which supports the role of heme in the pathogenesis of inflammation in SCA.

There are at least two potential explanations for our negative findings. First, the method used to measure extracellular heme in our study does not separate heme bound to proteins (albumin, hemopexin, or free Hb) from free unbound heme, which is the active form of heme. This is a major caveat of studies that measured heme in patients, which normally use methods similar to ours. While the fact that we also measured heme in serum allowed us to both confirm our results, and to first describe the association of total heme in serum and plasma, it does not provide any additional information about free heme. Since unbound free heme is the molecule responsible for direct and indirect toxicity to cells and tissues, there have been recent attempts to address this caveat, which was recently explored in a study that confirmed that colorimetric methods are not capable to separate heme bound to Hb from heme bound to proteins such as albumin and

hemopexin, and that physical methods aimed to separate free heme from protein-bound heme using filters can lead to underestimation of heme levels [25]. While two groups have recently described methods capable to measure heme that is not bound to proteins [26,27], as far as we are aware these methods have not yet been applied to investigate the association of heme levels with clinical and laboratory characteristics of SCA in humans. A second explanation for our negative findings involve the heterogeneous effect of heme on tissues, in that the direct and indirect (immune-mediated) toxicity of heme are counterbalanced by its beneficial effect of upregulating cytoprotective pathways involving NRF2 and heme-oxygenase (HMOX1). Accordingly, heme infusions have been shown to decrease inflammation in animal models of atherosclerosis, sepsis, and intracerebral hemorrhage by mechanisms dependent on the expression of HMOX1 [28,29], and hematin infusions in humans also support a heterogeneous effect of heme in inflammation [30]. From this perspective, the failure to show an association between heme levels and disease severity and inflammation could be at least in part due to the fact that heme also activates anti-inflammatory and cytoprotective pathways during VOC. Until an assay that measures free heme is accessible, a precise understanding of these issues will remain speculative.

In addition to the methodological caveat described above, and that is characteristic of the vast majority of studies aimed to investigate the association between heme and inflammation in humans, our study has additional limitations that need to be acknowledged. First, the large variation in the interval between admission and convalescent samples may have masked a more evident difference between admission and convalescence samples, which would support the hypothesis that acute VOC would be preceded by peaks in heme levels, as in our initial hypothesis. However, since admission levels were observed in half of the patients, as well as in an independent population of steady state patients, we believe that our results are sufficient to exclude that acute VOC is preceded by major increases in circulating levels of total heme, thus supporting our main conclusion. Second, we acknowledge that our panel of markers of inflammatory and hemostasis activation markers could have included additional markers commonly associated with endothelial activation. However, although relatively short, our panel includes markers whose biological relevance as prognostic factor of SCA or hypercoagulability are consolidated in larger clinical studies, as is the case for D-dimer and platelet counts, both associated with robust thrombotic clinical outcomes in other contexts.

In conclusion, the kinetics of total extracellular heme levels in plasma and serum in SCA patients during VOC supports that brisk increases in total heme levels are not required to trigger these events. Total extracellular heme levels are associated with SCA severity as measured by a composite score of clinical and laboratory markers, but not with clinical and laboratory characteristics of acute VOC. Finally, our results also emphasize the importance of refining methods to measure free heme that is unbound to proteins in complex biological matrices.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authors' contributions

E.C.C.: handled ethics approval, enrolled patients, collected and processed samples, analyzed data, and drafted the manuscript; P.V.S.N.: enrolled patients, collected and processed samples, and analyzed data; B.W.H., F.C., and F.L.: performed heme and D-dimer assays and analyzed data; C.X.A and P.C.: provided care for patients and contributed to recruitment; N.P.G.: provided data from steady state patients; A.M.: collaborated with laboratory infrastructure and reagents; E.V.D.P.: designed the study, analyzed data, and drafted the manuscript; N.A.F. designed the study and analyzed data. All authors revised and approved all submitted versions of the manuscript.

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