

# Assessment of total and unbound cell-free heme in plasma of patients with sickle cell disease

Madhav Vissa<sup>1,2</sup>, Sandra K Larkin<sup>2</sup>, Elliott P Vichinsky<sup>1,2</sup>, Frans A Kuypers<sup>2</sup>  
and Eric Soupene<sup>2</sup>

<sup>1</sup>UCSF Benioff Children's Hospital Oakland, University of California at San Francisco, Oakland, CA, USA; <sup>2</sup>Department of Pediatrics, Division of Hematology, University of California at San Francisco, Oakland, CA, USA  
Corresponding author: Madhav Vissa. Email: madhav.vissa@ucsf.edu

## Impact Statement

This study demonstrates the application of a novel method to distinguish between protein-bound and the pathologically relevant, unbound heme in plasma in individuals living with sickle cell disease (SCD). Existing methods for heme quantification do not distinguish between these separate heme fractions. Our findings may lead to additional applications of this acetone-based assay to further clarify the pathogenic role of heme and heme scavengers in SCD and other hemolytic disorders. Furthermore, accurate quantification of heme may allow investigation of novel therapeutic agents for SCD that target hemolysis and pathways that may be activated by circulating free heme.

## Abstract

Intravascular hemolysis results in the release of cell-free hemoglobin and heme in plasma. In sickle cell disease, the fragility of the sickle red blood cell leads to chronic hemolysis, which can contribute to oxidative damage and activation of inflammatory pathways. The scavenger proteins haptoglobin and hemopexin provide pathways to remove hemoglobin and heme, respectively, from the circulation. Heme also intercalates in membranes of blood cells and endothelial cells in the vasculature and associates with other plasma components such as albumin and lipoproteins. Hemopexin has a much higher affinity and can strip heme from the other pools and detoxify plasma from cell-free circulatory heme. However, due to chronic hemolysis, hemopexin is depleted in individuals with sickle cell disease. Thus, cell-free unbound heme is expected to accumulate in plasma. We developed a methodology for the accurate quantification of the fraction of heme, which is pathologically relevant in sickle cell disease, that does not appear to be sequestered to a plasma compartment. Our data show significant variation in the concentration of unbound heme, and rather unexpectedly, the size of the unbound fraction does not correlate to the degree of hemolysis, as measured by the concentration of bound heme. Very

high heme concentrations ( $>150\mu\text{M}$ ) were obtained in some plasma with unbound concentrations that were several fold lower than in plasma with much lower hemolysis ( $<50\mu\text{M}$ ). These findings underscore the long-term effects of chronic hemolysis on the blood components and of the disruption of the essential equilibrium between release of hemoproteins/heme in the circulation and adaptive response of the scavenging/removal mechanisms. Understanding the clinical implications of this loss of response may provide insights into diagnostic and therapeutic targets in patients with sickle cell disease.

**Keywords:** Sickle cell disease, heme, hemopexin, hemolysis

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## Introduction

Heme is a complex of iron coordinated in a porphyrin ring and defines the hemoprotein family, including the essential hemoglobin (Hb). It plays a multitude of roles in biologic processes due to its ability to reversibly bind oxygen.<sup>1–4</sup> The insertion of heme into the heme-binding pockets of hemoproteins controls its reactivity, but once released, heme becomes highly cytotoxic due to the iron atom of the protoporphyrin IX ring. Due to its hydrophobic properties, cell-free heme not bound to hemoproteins readily intercalates into lipid bilayers and various cellular components, including red cell-derived microvesicles.<sup>5–9</sup> Iron acts as a Fenton's reagent to catalyze

the production of highly oxidative free radicals causing damage to proteins, cellular membrane lipids, organelles, and DNA. In these localized pools, targeted damage contributes to pathogenesis.<sup>10–15</sup> Heme also exerts toxic effects by activation of inflammation, upregulation of adhesion molecules, and recruitment of inflammatory leukocytes.<sup>9,10,16–21</sup> In blood, heme is bound to the  $\alpha$  and  $\beta$  globin chains of Hb in the red blood cell (RBC).<sup>22,23</sup> The release of Hb into plasma caused by intravascular hemolysis is common in hemolytic disorders and critical illness.<sup>15,24–31</sup> Oxidation of the heme iron from the ferrous ( $\text{Fe}^{2+}$ ) to the ferric ( $\text{Fe}^{3+}$ ) state generates met-Hb and the results in the release of unbound cell-free heme into the circulation. Released heme will partition into a variety

of pools such as the membranes of erythrocytes, leukocytes, platelets, and endothelial cells. Heme will also bind to circulating plasma proteins such as albumin, lipoproteins,  $\alpha$ -1-antitrypsin,  $\alpha$ 1-microglobulin, and scavenger proteins. Once these pools are saturated, the plasma fraction of unbound heme will increase (Figure 1). The degree to which heme releases from Hb depends on several factors including the pH of serum and availability of scavenging proteins. One such scavenging protein, hemopexin, a hepatically synthesized protein, binds heme with sufficient affinity to displace heme from the other plasma pools to neutralize its harmful effects.<sup>20,32–35</sup> In addition, haptoglobin, a scavenger protein also synthesized in the liver, binds cell-free Hb to neutralize and recycle iron.<sup>28,30,31</sup> The pool of unbound heme may play an important role in pathogenesis of hemolytic disorders, such as SCD, a condition in which hemopexin is depleted by consumption.<sup>28</sup>

The ability to distinguish and quantify the presence of unbound cell-free heme in biological samples, like plasma, is compromised by its intrinsic property to non-specifically adhere to various biological components. We adapted an approach allowing for the quantification of the two cell-free heme fractions, protein-bound and unbound, in plasma.<sup>36</sup> We employed the properties of acetone, which results in lysis of membranes and precipitation of proteins, and acid which decouples heme from protein. Acidic acetone (AA) releases heme from all sources allowing quantification of total cell-free heme present in the sample. Neutral acetone (NA) treatment, however, leaves cell-free unbound heme in solution. The bound and unbound fractions can be calculated from these two pools. In this study, we aimed to validate this methodology and apply it to plasma from individuals with SCD and correlate the measurement with other known clinical and laboratory markers of disease severity. Since the toxic effects of free heme are manifest in various pathologies, including hemolytic diseases, malaria, atherosclerosis, sepsis, and stroke, our findings suggest that this approach will allow further understanding of the role of cell-free heme in pathogenesis, as well as the roles of haptoglobin and hemopexin which serve as targets for therapeutic intervention.

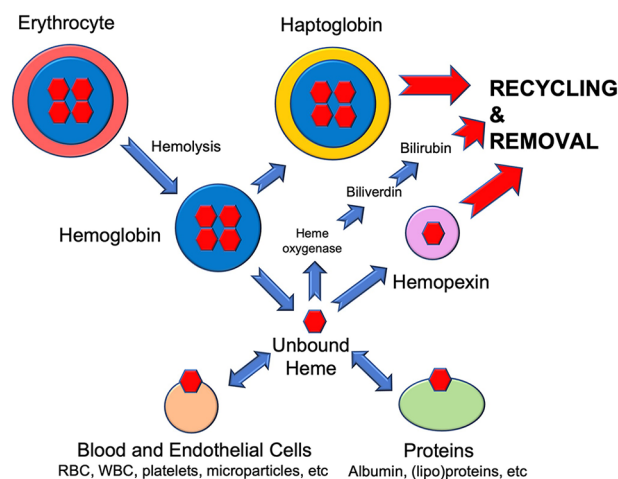
## Materials and methods

### Samples collection

Plasma was separated from whole blood, collected in EDTA (ethylenediaminetetraacetic acid) tubes, of healthy individuals and individuals with SCD at UCSF Benioff Children's Hospital Oakland after obtaining informed consent. Samples were stored at  $-80^{\circ}\text{C}$  until use. This study was approved by the UCSF institutional review board (IRB # 20-32015).

### Acetone solutions

AA was prepared by mixing acetone with 3M HCl at a 9:1 (v/v) ratio. NA was prepared by buffering acetone with HBS (HEPES-buffered saline; 145 mM NaCl, 20 mM HEPES pH 7.0) at a 9:1 (v/v) ratio. Solutions were kept cold at  $-20^{\circ}\text{C}$ .



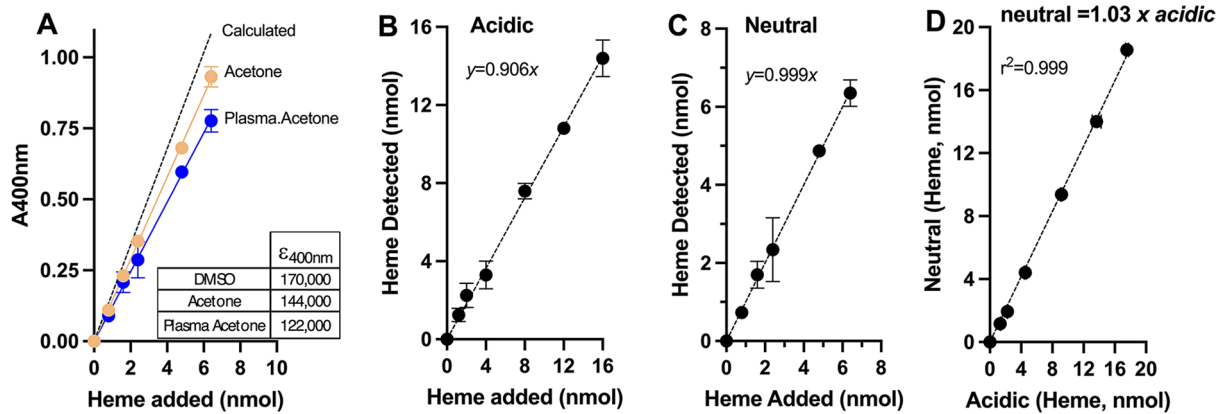
**Figure 1.** Heme pools in blood. Most of the approximately 8 mM of heme present in blood is bound to hemoglobin in red blood cells. Heme released from hemoglobin will disperse into a variety of hydrophobic pools including cell membranes and plasma proteins. Cell-free hemoglobin, released by hemolysis, is bound by haptoglobin to be recycled. These pools are in equilibrium with each other based on their abundance and binding capacity. Hemopexin with a high affinity for heme will remove heme from these pools for recycling. Heme is also metabolized by heme-oxygenase-1 into biliverdin, which is converted to bilirubin by biliverdin reductase.

### Hemin, bilirubin, Hb preparation

A 10-mM hemin (ferric chloride heme) stock solution was prepared by dissolving 65 mg of bovine hemin (Sigma H9039) in 10 mL of 0.1 mM freshly made NaOH with constant stirring for 1 h at room temperature. The solution was then neutralized with 1N HCl and stored protected from light at  $4^{\circ}\text{C}$ . The solution was diluted to 0.1 mM immediately before use (see below). Human Hb (Sigma H7379) was dissolved in water at 0.2 mM and bilirubin (Sigma B4126) was dissolved in chloroform at 1 mM.

### Detection and quantification

To accurately quantify heme extracted from plasma in either NA or AA solutions (see "Results" section), two heme standard curves were established in NA and in AA extracts obtained from a control plasma sample. Control plasma consisted of pooled plasma samples from individuals without SCD. Small volumes of plasma were aliquoted and kept frozen until use. Hemin was then added to the extracts in a 200  $\mu\text{L}$  final volume at the concentrations indicated in Figure 2. These samples were then treated as for the experimental samples (see below). Experiments were performed without addition of heme, and the absorbance values at 400 nm were subtracted from the values obtained with the spiked heme samples. The corrected  $A_{400\text{nm}}$  values were then plotted as a function of the concentration of heme in the samples. To establish an assay using sickle cell patient samples, the minimum volume of plasma sufficient to reliably allow quantification of heme over a wide range of samples was determined. Routinely, 40 and 160  $\mu\text{L}$  of plasma were used for AA and NA extraction, respectively. Five volumes of AA or NA (chilled at  $-20^{\circ}\text{C}$ ) were added to plasma. After mixing by vortex for 5 s and incubation at  $4^{\circ}\text{C}$  for 60 min, the proteins were removed by centrifugation at  $4^{\circ}\text{C}$  and 16,000g for



**Figure 2.** Absorbance of heme under various extraction conditions. Panel A: Heme was added to neutral acetone (orange circles) and to a plasma extracted with neutral acetone (blue circles). Extracts were then dried, resuspended in DMSO, and measured at 400 nm, as described in the Methods section. The plot of the absorbance values (calculated with the molar extinction coefficient value,  $\epsilon_{400nm}$  of  $170,000\text{ M}^{-1}\text{ cm}^{-1}$  at 400 nm) of heme dissolved in DMSO<sup>40,41</sup> is shown as a dashed line. The measured  $\epsilon_{400nm}$  values are presented in the table. Panels B and C: Recovery of heme added to acidic and neutral acetone extracts of plasma. Heme amounts were calculated using the  $\epsilon_{400nm}$  values obtained from standard curves performed under acidic or neutral conditions. The efficiencies of the extraction under acidic (Panel B) and neutral (Panel C) conditions are indicated in each of the panel as the linear relation of heme detected to heme added. Panel D: Values obtained in Panel C were plotted relative to the values obtained in Panel B. Quantification of heme under neutral and acidic condition shows a near perfect equivalence of 1. In all panels, the error bars represent the standard deviations of values obtained from three measurements.

25 min. The supernatants were collected and dried at room temperature with a SpeedVac centrifuge (Savant SpeedVac plus). The heme present in the pellet was resuspended in 100  $\mu\text{L}$  dimethyl sulfoxide (DMSO) and solubilized for 20 min in a sonicator bath (Fisher Scientific Ultrasonic FS-9). Heme measurements were performed at 400 nm with a microplate reader (BioTek Synergy Neo2). To measure heme extraction from Hb, five volumes of AA or NA (chilled at  $-20^{\circ}\text{C}$ ) were added to increasing concentrations of Hb (0–50  $\mu\text{M}$ ) made in HBS buffer and treated with the same protocol as above to measure absorbance. Likewise, bilirubin dissolved in chloroform was spiked in plasma at concentrations of 0–250 M for extraction in AA and NA as above.

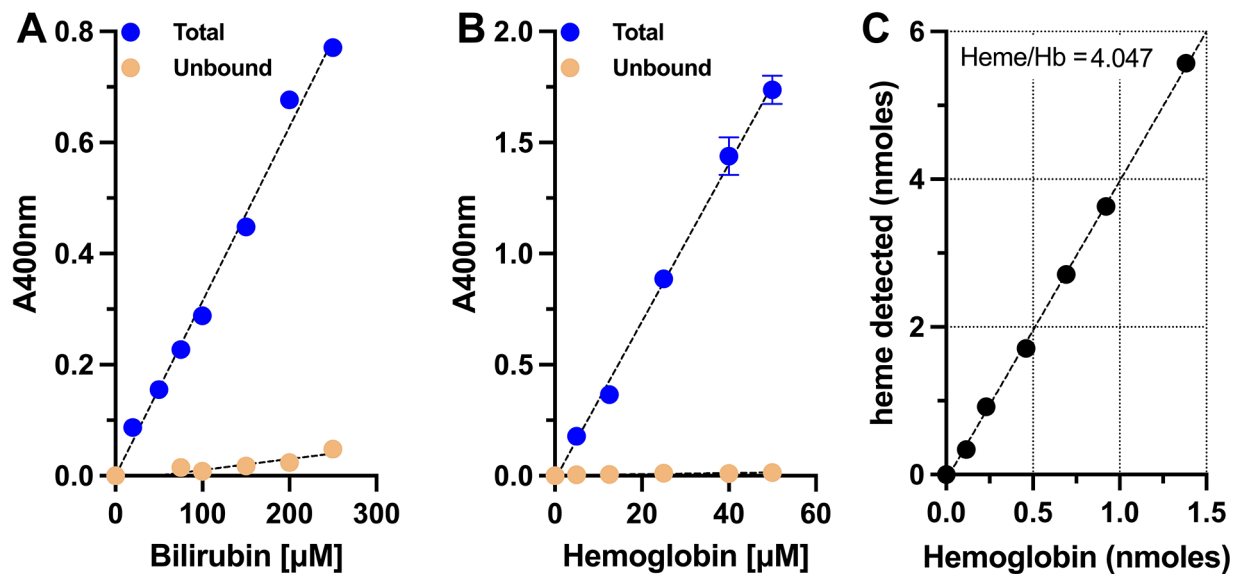
Hemopexin in human plasma was determined by ELISA (enzyme-linked immunosorbent assay) according to the manufacturer instructions (KT-502; Kamiya Biomedical Company). Complete blood counts including reticulocyte counts were performed on an Advia 2120 (Siemens). Fetal hemoglobin (HbF) was quantified using high-performance liquid chromatography (HPLC) and HbF containing erythrocytes were measured using flow cytometry (Becton Dickinson, Fortessa). All heme and hemopexin measurements were made in triplicate. Calculations and statistical analysis (*t* test or two-way analysis of variance [ANOVA], as appropriate) were performed with GraphPad Prism 9.

## Results

### Cell-free bound and unbound heme in plasma

The detection of a putative fraction of unbound heme in human plasma required both the removal of bound heme and the need to avoid depletion of the free form by techniques such as dialysis or filtration as well as its release from the bound pool under experimental conditions. Removal of bound heme by acetone precipitation of hemoproteins under neutral condition followed by the measurement of heme present in the acetone supernatant provided an efficient

and sensitive quantification of “unbound” heme in plasma. Under an acidic condition<sup>37–39</sup> heme is released into the acetone environment and the denatured proteins, including the abundant globin chains, precipitate and are removed by centrifugation, resulting in the detection of the total heme content present in plasma. Both the NA and AA heme extracts were dried down and resuspended in a small volume of the solvent DMSO, allowing increased detection sensitivity. The fraction of bound heme can be calculated from the values of total and unbound heme. Heme extracted from various environments and methods is often quantified from the Soret band absorbance ( $\lambda \approx 400\text{--}420\text{ nm}$ ) using a single molar extinction coefficient value of  $\approx 170,000\text{ M}^{-1}\text{ cm}^{-1}$  which has been established with hemin dissolved in DMSO.<sup>40,41</sup> However, the heme absorption spectrum is greatly influenced by various parameters, such as pH, water, and oligomerization.<sup>40,41</sup> Although heme extracted under different conditions was dried and suspended in DMSO for measurements, we found significant differences in absorbance after recovery from acetone and acetone extract of plasma (Figure 2, panel A). The absorbance of heme in DMSO was also different when recovered from NA and AA. Thus, calibration standard curves were established under those two conditions to allow accurate quantification of extracted heme from the  $A_{400nm}$  values (Figure 2, panels B and C). The efficiency of heme recovery from plasma under acidic condition was 90% (Figure 2(B)) and 99% under neutral condition (Figure 2(C)). Overall, there was only a 3% difference in heme quantification under these two conditions (Figure 2(D)). Using a small volume of 100  $\mu\text{L}$  plasma, a reliable experimental detection limit of  $\geq 4\text{ }\mu\text{M}$  heme was achieved in both conditions and the procedure was reliable over a 10-fold concentration range, which eliminates the need of adjusting the volume of plasma for atypical samples with extremely low or high heme content. It should be noted that the plasma-acetone extracts are dried down and resuspended before analysis. Hence, the volume of plasma can be increased to allow detection of heme at even lower levels ( $<4\text{ }\mu\text{M}$ ).



**Figure 3.** Analysis of extraction procedure on bilirubin and hemoglobin. Panel A: Recovery of bilirubin added to plasma under acidic (total) and neutral (unbound) acetone extraction conditions, as described in Figure 2(B) and (C). Note that at concentrations up to 100  $\mu\text{M}$ , no unbound bilirubin could be detected. At higher concentrations, bilirubin appeared to saturate the binding capacity of serum albumin and could be detected by extraction under neutral conditions. Panel B: Recovery of heme from hemoglobin under AA and NA extraction conditions. Extraction under neutral conditions could not release heme, whereas acidification resulted in the release of heme. Panel C: As presented in Figure 2(B), a standard curve was established under acidic condition to convert the absorbance values shown in panel B to the amount of heme extracted from hemoglobin. Heme values are presented plotted relative to the amount of hemoglobin extracted and established that four heme groups were recovered per molecule of Hb.

### Detection of major heme source in plasma

As the result of intravascular hemolysis, cell-free Hb is a major source of heme in plasma. Bilirubin, a catabolic breakdown product of heme, is present in plasma samples from patients with chronic hemolysis. Heme and unconjugated bilirubin are tightly bound to globin chains and to serum albumin, respectively, and should not be detectable under the NA extraction condition. Increased concentrations of Hb and bilirubin were added to plasma and the results of the extraction under acidic and neutral conditions are presented in Figure 3. As expected, acidification resulted in the quantitative extraction of heme from Hb and of bilirubin from albumin. Heme quantification performed as described in Figure 2, resulted in the detection of four heme moieties per Hb. However, under neutral condition heme and bilirubin could only be detected when very high concentrations were added to plasma. These findings confirmed that heme and heme products bound to plasma hemoproteins remain bound under neutral condition and that detection under this condition represents the cell-free unbound fraction present in plasma. The efficient dissociation obtained under acidic condition confirmed that this condition provides the total amount of cell-free heme.

### Detection of unbound heme in plasma

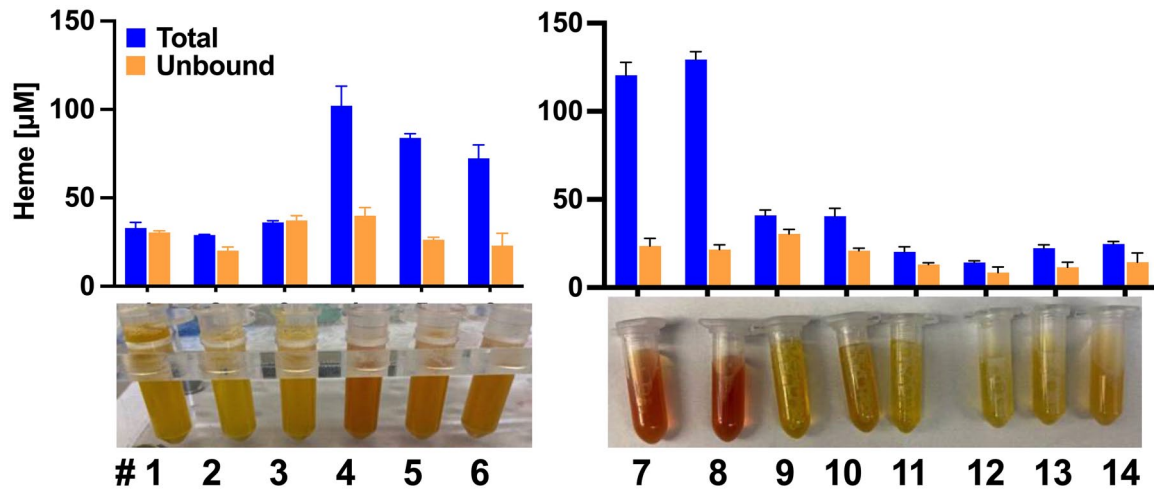
Analysis of a cohort of plasma samples from blood collected from individuals with SCD established that a wide range of total and unbound heme was detected after acetone extraction (Figure 4). The presence of a high amount of cell-free Hb was noticeable in some samples with very high total heme content ( $>100 \mu\text{M}$ ), as indicated by the reddish color of the plasma. However, hemolysis did not bias the analysis since

some plasma samples with no significant visible hemolysis had higher levels of unbound heme than the samples with higher hemolysis. The finding that the lysis of the RBCs and release of Hb in plasma were not predictive of the amount of unbound heme indicated that differences in plasma-binding capacity appeared to be a major critical factor influencing the partitioning of heme and its release into the circulation.

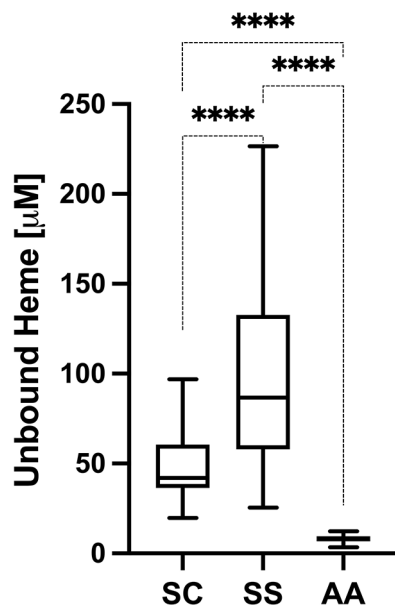
The heme measurement was stratified by beta globin genotype and other hematologic biomarkers of disease severity such as reticulocytosis and HbF production. Overall, plasma of the homozygous HbSS cohort, assumed to have a more severe phenotype than heterozygous HbSC on average,<sup>42</sup> had significantly increased plasma unbound heme compared with HbSC and HbAA (normal Hb) samples (Figure 5). In addition, samples from HbSS patients with high reticulocytosis, indicative of shorter RBC life span, also had increased plasma heme content compared with HbSS patients with high antisickling HbF, which is protective in SCD (Figure 6(A) and (C)). Total and unbound heme were also increased in patients with anemia as indicated by their lower RBC counts (Figure 6(B) and (D)). As confirmation of those findings, a significant trend of the accumulation of plasma heme as a function of increased erythropoiesis was observed (Figure 7).

The protective effect of HbF on RBC heme release was also analyzed in the cohort of HbSS patients. Heme content was 40% lower in patients with HbF levels of  $>15\%$  compared with patients with HbF  $<15\%$  (Figure 7(B)). Even in patients with severe anemia, RBC count  $<3 \times 10^6$  cells/ $\mu\text{L}$ , the heme levels were lower when HbF levels were increased (Figure 7(C)).

The levels of unbound heme correlated well with the percent of unbound heme calculated relative to total heme



**Figure 4.** Quantification of heme in plasma of sickle cell disease patients. The bar graphs represent the concentration of heme detected under acidic (total) and neutral (unbound) extraction conditions in the plasma of several SCD patients. Pictures of the plasma samples from each patient taken before extraction are shown below their respective total/unbound bars on the graphs. As indicated by the reddish color of the plasma, a high level of hemoglobin was noticeable in some samples (#4, 7, 8). Very high levels of total heme were detected in those samples, but it did not result in higher levels of unbound heme. Some samples (#3, 9) with lower total heme and no visible hemolysis had higher level of unbound heme than in the samples with high cell-free Hb (#4, 7, 8). The error bars represent the standard deviations of values obtained from three measurements.



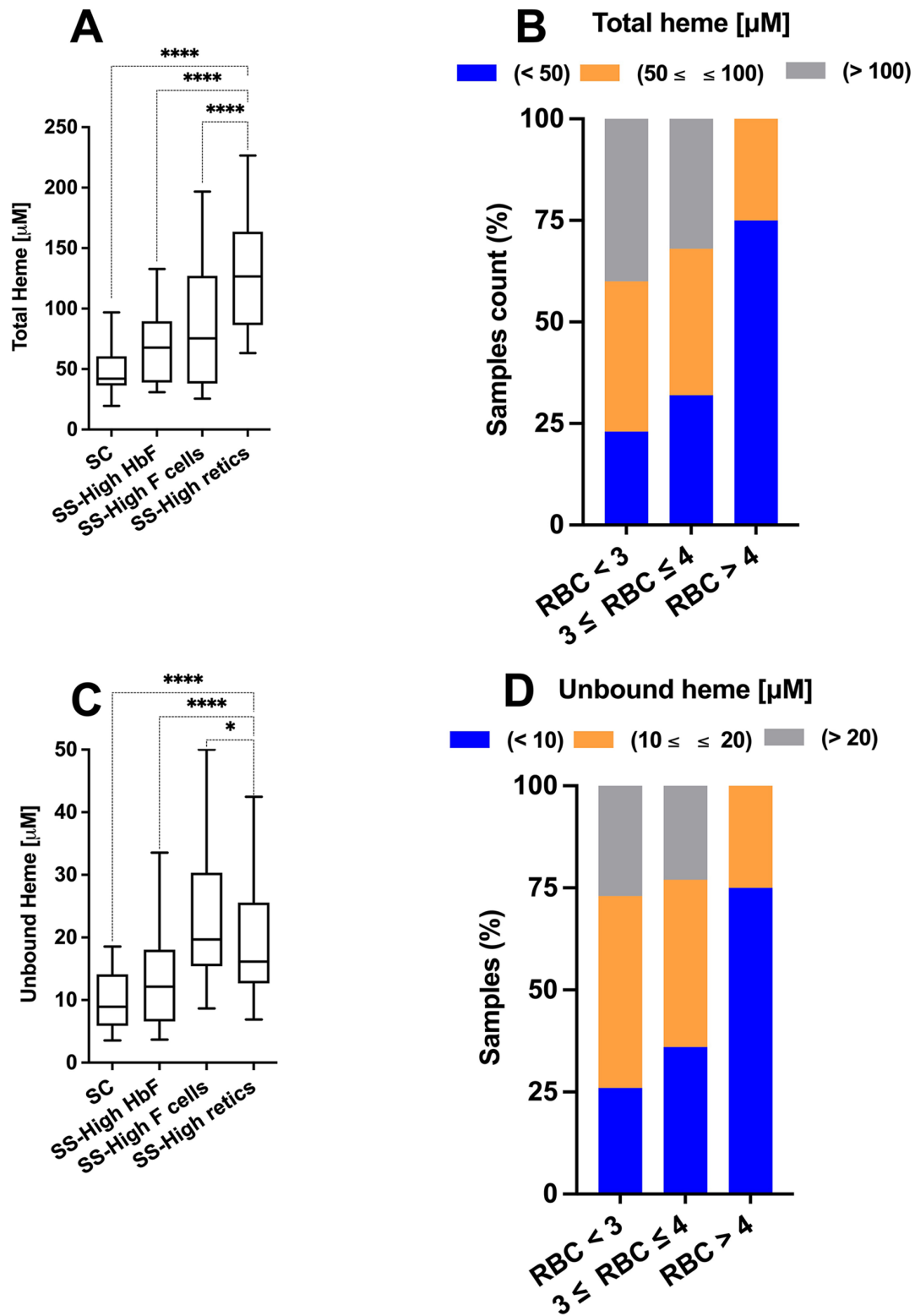
**Figure 5.** Release of heme in plasma of patients with sickle cell disease. Heme detected by extraction of plasma under neutral conditions of a cohort of SCD individuals (HbSC and HbSS) and control (HbAA) individuals is presented as a function of the hemoglobin type of the samples (HbSC,  $n=20$ ; HbSS,  $n=76$ ; HbAA,  $n=24$ ). Mean/median concentration ( $\mu\text{M}$ ) values were HbSC 52/42, HbSS 104/87, HbAA 8.2/8.2. \*\*\*\* indicates  $P$  values of  $<0.0001$ .

concentration. This finding established that as heme accumulated in plasma, the unbound fraction also increased, which indicates a failure of plasma proteins in SCD blood to absorb heme as it accumulates (Figure 8(A)). Analysis of the cohort also showed a broad variability in the heme absorption and removal capacity. Differences in total heme concentration did not correlate with the levels of unbound heme. Furthermore, distinct populations of samples could be identified when unbound values were plotted relative to the total heme concentrations (Figure 8(B)). Whereas some patients

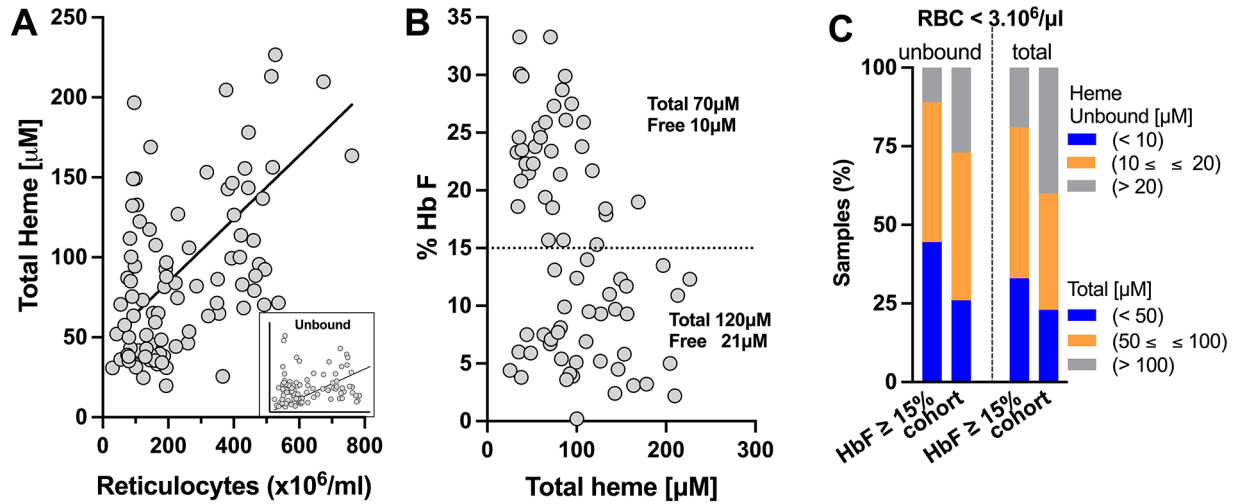
had high levels of heme in plasma without free heme (high total/low unbound), others did not absorb heme even at much lower levels (low total/high unbound). Unexpectedly, an inverse relationship was observed between concentration of heme (total) and the size of the unbound fraction (% unbound) (Figure 8(C)). As also evident in the samples from patients with SCD presented in Figure 4, these findings further emphasized the major contributing role of cell-free Hb to the presence of heme and of the marked differences in the capacity of some patients' plasma to remove heme from the circulation (high total/low %unbound) or inability to absorb or remove heme after release (low total/high %unbound).

#### Plasma heme levels during vaso-occlusive episodes

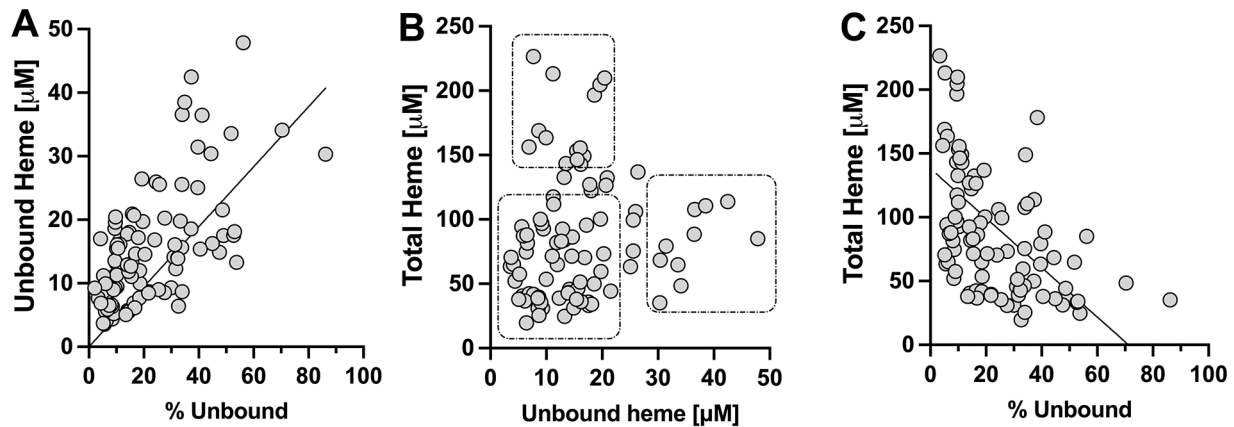
Analysis of plasma heme in a cohort of SCD patients during an acute vaso-occlusive event (VOE) was also performed. Blood samples were obtained at baseline and on day 1 and day 3 of hospital admission for a VOE. In this cohort, patient ages ranged from 6 to 38 years and genotypes included 12 subjects with HbSS, 6 with HbSC, and 2 with HbS $\beta^+$  thalassemia. Although a broad variety of changes were observed (Supplemental Figure S1), small differences in plasma heme concentration were detected in the acute phase compared to the levels measured at baseline (Figure 9). Although the difference in mean values were not significant, an increased number of patients with higher unbound heme was detected during the acute phase (Figure 9(B)), and a 20% increase in unbound heme was measured on the first day of the VOE (means of 18.5–22.5  $\mu\text{M}$ ;  $P=0.07$ ) (Figure 9(D)). To gain further insight into these changes, the levels of the heme scavenger protein hemopexin were measured. As expected, we observed a significant depletion of hemopexin in patients with SCD compared with expected serum concentration of 600–1200  $\mu\text{g}/\text{mL}$ .<sup>31,34</sup> Furthermore, individuals with the HbSS genotype had a 2.5-fold lower concentration of



**Figure 6.** Analysis of plasma heme content in relation to hematology parameters I. Concentrations of total (panels A and B) and unbound heme (panels C and D) were determined in a cohort of plasma samples from SCD patients. The cohort was genotyped and analyzed for various hematology parameters (RBC count, % reticulocytes, % anti-HbF antibody positive Retic (F cells), % of HbF [HPLC]). The cohort was represented by HbSC ( $n=20$ ), HbSS with HbF level above 20% ( $n=34$ ), HbSS with %Fcells above 45% ( $n=11$ ), and HbSS with reticulocytes (retics) above 15% ( $n=31$ ). Note that few samples met several of the criteria but are only represented once in the analysis with priority given to the high retic category, followed by high HbF. Mean/median heme total concentration ( $\mu\text{M}$ ) values were HbSC 52/42, HbSS-high HbF 72/68, HbSS-high Fcells 86/73, HbSS-high retic 126/145. Mean/median heme unbound concentration ( $\mu\text{M}$ ) values were HbSC 9.8/8.9, HbSS-high HbF 14.4/12.1, HbSS-high Fcells 23.1/19.7, HbSS-high retic 20.4/16.1. In panels B and D, the counts of samples with the indicated concentrations of heme (total, panel B; unbound, panel D) were analyzed relative to the RBC counts of the blood samples: less than  $3 \cdot 10^6$  cells/ $\mu\text{L}$ ; from 3 to  $4 \cdot 10^6$  cells/ $\mu\text{L}$ ; more than  $4 \cdot 10^6$  cells/ $\mu\text{L}$ . \* and \*\*\*\* indicate  $P$  values of 0.02 and of  $< 0.0001$ , respectively.



**Figure 7.** Analysis of plasma heme content in relation to hematology parameters II. The concentrations of total heme (panels A and B) presented in Figure 6 were analyzed as a function of the reticulocyte count (panel A) and the percent of HbF (panel B). The inset in panel A shows the plot of unbound heme concentrations relative to reticulocyte counts. The mean values of total and unbound heme ( $\mu\text{M}$ ) of the samples with less or more than 15% HbF, as indicated by the horizontal dashed line, are indicated in panel B. In panel C, the counts of samples with less than  $3.10^6 \text{ RBC}/\mu\text{L}$  and the indicated concentrations of total and unbound heme were analyzed in the samples with an HbF level of at least 15% and compared to the entire cohort.



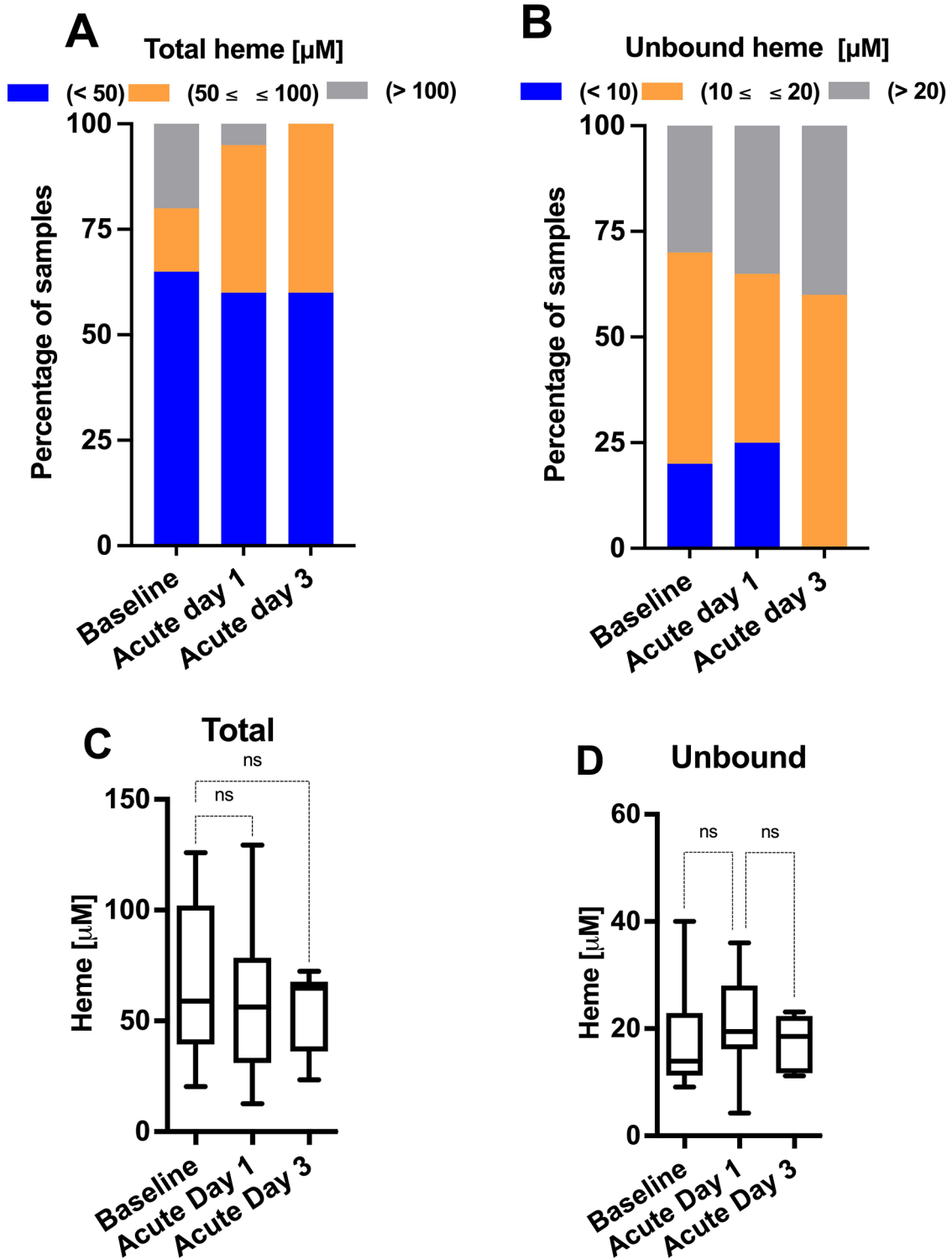
**Figure 8.** Analysis of unbound heme fraction in plasma of sickle patients. Panel A: Concentration of unbound heme of the sickle cohort was plotted relative to the unbound fraction, which was calculated as the percent of unbound heme relative to the total heme concentration. Panels B and C: Concentration of total heme was plotted relative to the concentration of unbound heme and to the unbound fraction. In panel B, samples are grouped (boxes) based on relative high or low total and unbound heme.

hemopexin compared to individuals with the HbSC genotype, though any changes observed between the steady state and acute phase were not statistically significant (Figure 10(A)). However, when hemopexin levels were analyzed in relation to the concentration of unbound heme, a twofold to threefold depletion of hemopexin was measured in samples with unbound heme values above the mean values of the cohort (Figure 10(B)). These findings are consistent with the interpretation that samples with above-average heme concentrations represent a combination of events leading to higher hemolysis, higher heme released, and deficiency in absorption and in removal (Figure 1).

**Discussion**

When bound to appropriate hemoproteins, heme plays a significant role in human physiology including delivery

of oxygen to tissues and electron transfer in mitochondrial membranes. Due to its lipophilic nature, when heme is released from proteins like Hb, it distributes between different cellular and extracellular pools, intercalates into cell membranes, and provides a focus point for the production of reactive oxygen species leading to activation of inflammation and cellular damage.<sup>5-13,16-18</sup> Heme scavengers such as hemopexin mitigate this effect<sup>14,20,25,28-32,35</sup> by binding heme with high affinity and removing it from circulation. Hence, in the setting of chronic hemolysis, high levels of heme can lead to the relative depletion of hemopexin, as production does not keep up with removal. This in turn will lower the ability of hemopexin to effectively remove heme from the other pools in plasma and cellular damage will occur. In hemolytic disorders such as SCD, it has been hypothesized that when plasma heme-binding capacity is saturated, the unbound heme plays an important role in pathogenesis.<sup>8,11,14,17,29,34</sup>

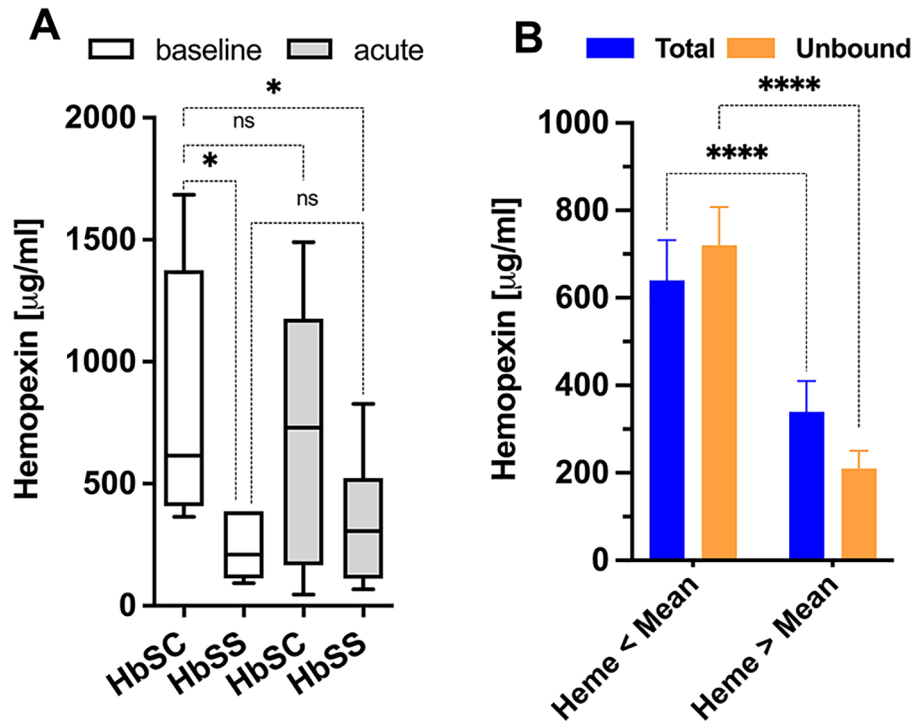


**Figure 9.** Heme plasma content of SCD patients in vaso-occlusive crisis. Concentrations of total and unbound heme in plasma samples of patients in VOE were measured at day 1 and day 3 and compared to baseline levels. Panels A and B: The percentage of samples with the indicated concentrations of heme were analyzed at baseline ( $n=20$ ), day 1 ( $n=20$ ), and day 3 ( $n=10$ ). Panels C and D: Note that the differences in unbound heme did not reach statistical significance but were more pronounced than total heme. Mean/median of total heme: baseline (68/59), day 1 (57/56), day 3 (55/65), and for unbound heme: baseline (18.5/14), day 1 (22.5/19.5), day 3 (17.5/18.5). *ns*, non-significant.

There are several heme-binding proteins in plasma, most notably hemopexin and albumin. The levels of these proteins can be measured by commercial assays, but these assays cannot distinguish between protein that is available for heme binding and that which is saturated.

Here, we describe a method for the quantification of the unbound cell-free heme fraction in relation to the total cell-free heme in plasma. This assay may also be used for the assessment of the heme-binding/removal capacity of plasma as unbound heme will only be present when the various





**Figure 10.** Levels of the heme scavenger hemopexin and heme. Panel A: Concentrations of the plasma protein hemopexin were measured in the cohort of sickle patients presented in Figure 5. Panel B: The mean values of hemopexin concentration were calculated in samples with a heme concentration (total and unbound) below or above the mean values of the cohort (mean total, 93 µM; mean unbound 16.5 µM). Error bars represent standard error of the mean. *ns*, non-significant; \* and \*\*\*\* indicate *P* values of <0.05 and <0.0001, respectively.

heme-binding sites have been saturated. We first show that total and unbound cell-free heme may be reliably measured after extraction with AA and NA, respectively. Then we use this method to determine heme measurements in plasma samples from individuals with SCD and correlated our findings with hematologic parameters known to be associated with disease severity. The measurement of total heme was similar to values measured using commercial kit-based assays in other cohorts.<sup>16,28</sup> Our findings were consistent with the hypothesis that in individuals with more severe disease as expressed by hemolysis rate, reticulocytosis, lower RBC count, and lower HbF, heme is released and accumulates in the unbound pool. We also describe the impressive capacity of the blood system, even in chronic hemolytic disorders of such as SCD, to sequester free heme.

Whereas this assay provides reliable measurements requiring a small amount of plasma, limitations on biological relevance should be considered. In patients with chronic hemolysis, measurement of cell-free Hb could be included to assess its contribution to the total heme pool. Similarly, hemolysis from venipuncture and/or blood storage may impact the level of total heme measured in plasma. As shown above, however, the measurement of the unbound fraction of heme is not impaired by the presence of cell-free Hb. Regardless, care should be taken to collect and store blood and plasma to reduce *ex vivo* hemolysis. The method as described measures the presence of bound and unbound heme in plasma. When heme is released into plasma *in vivo*, heme will distribute between the plasma phase and the cellular membranes of the blood and vascular cells (Figure 1). The presence of heme in plasma likely reflects the presence

of heme in these membranes as these pools are in equilibrium with each other. However, these membrane pools cannot be measured from plasma analysis. The endothelial membrane pools cannot be assessed in a blood draw. In addition, the RBCs represent a high membrane surface area to provide a binding pool for heme, but the overwhelming amount of heme derived from the RBC Hb extraction will make it difficult to correctly assess the heme in the RBC membrane. Finally, the method presented requires generation of a standard curve in heme-spiked acetone extracts of plasma for quantification. Though we were able to reproduce our standard curve using a large stock of pooled plasma and consistent techniques, this step in the method might represent some challenges for its implementation in a clinical setting.

In conclusion, we present a novel method to quantify total and unbound heme in biological samples. A direct application would be the assessment of heme in plasma to determine the heme-binding capacity of the plasma and to assess the therapeutic value of treatment with compounds like hemopexin and antisickling agents leading to an increase in RBC lifespan.<sup>11,20,26,29,33</sup> This assay may be applied to understand the pathophysiologic impact of heme and heme-binding proteins in hemolytic disorders such as SCD and acute illness such as sepsis. In addition, these methods may be adapted to investigate the therapeutic potential of novel agents that target hemolysis and cell-free heme.

#### AUTHORS' CONTRIBUTIONS

All authors contributed to the design, conduct, and analysis of the study and generation of the manuscript. MV, ES, and FAK

wrote the manuscript, MV, ES, and SKL conducted the experiments. EPV and FAK assisted with data analysis.

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#### DECLARATION OF CONFLICTING INTERESTS

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#### ORCID IDS

Madhav Vissa  <https://orcid.org/0000-0002-1088-5856>

Frans A Kuypers  <https://orcid.org/0000-0003-0731-1007>

#### SUPPLEMENTAL MATERIAL

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