Accurate cell-free heme dosage in plasma correlates with anemia, hemolysis and sickle cell nephropathy severity

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INTRODUCTION

Sickle cell disease (SCD), a chronic hemolytic anaemia, is characterized by hemolysis, inflammation and vaso-occlusive crises. Hemolysis results in the release of red blood cell (RBC) content into the circulation, including hemoglobin (Hb), which is then oxidized into cell-free heme. Excess heme is highly toxic due to its ability to promote oxidative stress and inflammation. It is known that heme triggers damage to endothelial cells through TLR4 and promotes complement activation, contributing to vasculopathy (Merle et al., 2019; Belcher et al.,2014; Ghosh et al.,2013). These phenomena lead to chronic organ damage, such as severe kidney complications, known as sickle cell nephropathy (SCN). To date, methods to dose plasma heme are mostly inaccurate, specifically due to the hemoglobin signal leading to overestimation. As both an actor of the pathophysiology and a potent biomarker of hemolysis, accurate plasma heme dosage is of great interest for SCD.

OBJECTIVES

Investigate relationships between plasma heme concentration measured using our new method and other routinely used biomarkers of anemia, hemolysis and SCN severity in SCD patients.

METHODS

FCDREP-1 cohort of SCD patients :

- Inclusion criteria :
  - Adults ≥ 18 years
  - Diagnosis of sickle cell disease with HbSS or HbSβ^0 genotype
  - Written consent must be given by the patient after being informed of the purpose, progress and potential risks
- Exclusion criteria :
  - Pregnant and nursing women
  - Patients transfused within the preceding 3 months
  - Patients who were hospitalized with a VOC within the preceding month
  - Patients taking Voxelotor, Crizanlizumab or L-glutamine at inclusion
  - Patients with non-stabilized infectious or inflammatory pathologies
  - Homeless patients
  - Patients deprived of their liberty by a court or administrative order or under guardianship
  - Patients unable to understand the purpose and conditions of carrying out the study, who are unable to give consent

- **110 SCD patients** treated or not treated with hydroxycarbamide (HU) were included in the FCDREP-1 study (Table 1). Patients' biological data was collected from a computerized database at Henri Mondor Hospital. SCN severity was assessed by urine microalbumin to creatinine ratio (ACR). Patients were separated into three groups according to ACR value: normal (ACR≤3 mg/mmol); microalbuminuria (3<ACR≤30 mg/mmol) and macroalbuminuria (ACR>30 mg/mmol).

- Correlations were calculated using Spearman test. Comparisons between HU-treated, or non-treated patients were done using the Mann-Whitney test. Comparisons between the three ACR groups were done using the Kruskal-Wallis test. Statistical significance was set at $p < 0.05$.

Hemolysis parameters measurement :

Plasma heme and hemopexin were measured by spectrophotometry from purified plasma. The dosage is based on light absorption of samples using mathematical conversion of the signal by use of reference spectra of the different species and chemical modifications of the iron redox and ligation states (Kiger et al.,2019, Figure 1). Albumin-bound heme was calculated by subtracting the hemopexin-bound heme (according to hemopexin concentration) from the total heme concentration

FCDREP-1 (n=110 SCD patients)	
Sex, n (%)	Female : 63 [57%]
	Male : 47 [43%]
Age, mean (±SD)	38 (±11)
Hydroxyurea treated, n (%)	70 (64%)
Hemoglobin (g/dL), median [IQR]	8.8 [8.1 - 10]
Hematocrit (%), median [IQR]	25.5 [22.9 - 28.9]
LDH (U/L), median [IQR]	398.5 [313.8 - 555.5]
Reticulocytes (G/L), median [IQR]	165.5 [118.3 - 238.7]
Reticulocytes (%), median [IQR]	6 [4 - 8]
eGFR (mL/min), median [IQR]	117.5 [100.8 - 128]
Albumin-to-creatinin ratio, median [IQR]	3 .4 [1 - 12.1]
Fetal Hemoglobin (%), median [IQR]	12 [5-19]
Aspartate aminotransferase (U/L), median [IQR]	45 [36 - 53.8]
Total Bilirubin (µM), median [IQR]	28.3 [18.5 - 43.1]
Free Hemoglobin (µM), median [IQR]	65.6 [3.1 - 8.6]
Plasma Heme (µM), median [IQR]	2 [1 - 4.5]
Hemopexin (g/L), median [IQR]	0.2 [0.1 - 0.3]

Table 1. FCDREP-1 cohort characteristics. *SD : standard deviation, IQR : interquartile range, LDH : lactate dehydrogenase, eGFR : estimated glomerular filtration rate.*

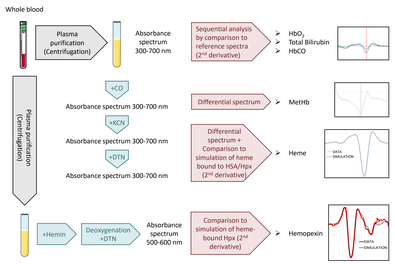

Figure 1. Diagram of hemolysis measurement. *DTN : sodium dithionite, HSA : human serum albumin, Hpx : hemopexin*

RESULTS

Plasma heme was successfully measured in the 110 SCD patients of our cohort with a median of 2 µM (heme measured on healthy control samples is usually <1 µM). A significant difference was observed between HU-treated and non-treated patients (Figure 2A), suggesting that HU could reduce intravascular hemolysis. HU is also able to reduce the heme fraction bound to albumin (Figure 2B), while increasing hemopexin level (Figure 2C, normal values above 0.5 g/L). However, the plasma free Hb level was not significantly different between groups (Figure 2D), similarly to lactate dehydrogenase (LDH, Figure 2E) and hemoglobin (Figure 2G), only reticulocytes were significantly decreased in the HU-treated group (Figure 2F). As expected, plasma heme is inversely correlated to hemopexin level (Figure 3A) and shows a positive linear correlation with LDH level (Figure 3B), but with a relatively high variation of LDH values for the same heme value, as shown on the residual plot (Figure 3C). We can notice that most patients with more than 5 µM of heme show hemopexin levels < 0.1 g/L (Figure 3A). Heme was also positively correlated with other hemolysis parameters including hematocrit (Figure 4A), hemoglobin (Figure 4B) and negatively correlated with total bilirubin (Figure 4C), aspartate transaminase (ASAT, Figure 4D), plasma free hemoglobin (Figure 4E) and reticulocytes (Figure 4F). Heme was also negatively correlated with fetal hemoglobin (HbF, Figure 4G) and positively correlated with lymphocyte count (Figure 4E). These two correlations were statistically significant when the HU-treated group was analyzed individually, but not in the non-treated group.

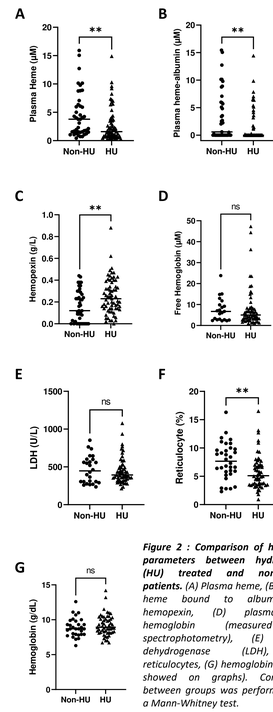

Figure 2 : Comparison of hemolysis parameters between hydroxyurea (HU) treated and non-treated patients. *(A) Plasma heme, (B) plasma heme bound to albumin, (C) hemopexin, (D) plasma free hemoglobin (measured by spectrophotometry), (E) lactate dehydrogenase (LDH), (F) reticulocytes, (G) hemoglobin (median showed on graphs). Comparison between groups was performed with a Mann-Whitney test.*

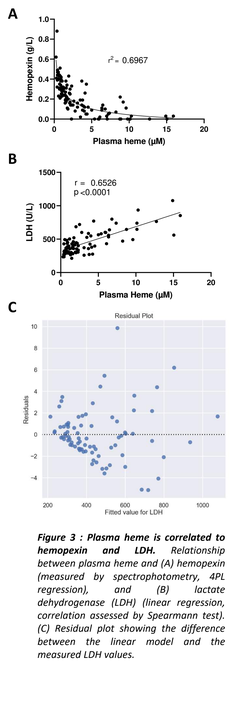

Figure 3 : Plasma heme is correlated to hemopexin and LDH. *Relationship between plasma heme and (A) hemopexin (measured by spectrophotometry, 4PL regression), and (B) lactate dehydrogenase (LDH) (linear regression, correlation assessed by Spearmann test). (C) Residual plot showing the difference between the linear model and the measured LDH values.*

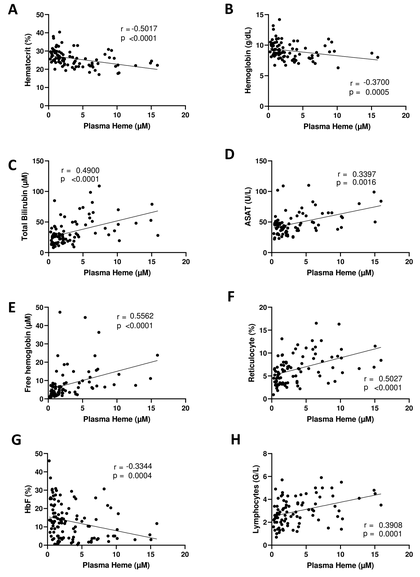

Figure 4 : Plasma heme is correlated to hemolysis parameters, fetal hemoglobin and lymphocyte count. *Linear regression between plasma heme (measured by spectrophotometry) and (A) hematocrit, (B) hemoglobin, (C) total bilirubin, (D) aspartate transaminase (ASAT), (E) plasma free hemoglobin (measured by spectrophotometry), (F) reticulocytes, (G) fetal hemoglobin (HbF, measured by high-performance liquid chromatography) and (H) lymphocytes. Correlations were assessed by Spearmann test.*

Since heme is suspected to be involved in the development of kidney damage in SCD, through complement activation (Merle et al.,2018; Ofori-Acquah et al.,2020), we studied the link between plasma heme and microalbumin to creatinine ratio (ACR). In this cohort, a significant positive correlation was found between heme and ACR ($r = 0.32$; $p = 0.0011$). The patients were divided into three groups according to their plasma heme value (heme tertiles, T1≤1.3 µM, 1.3<T2≤3.78 µM, T3>3.78 µM) and ACR was plotted against age (Figure 5A). The number of patients with macroalbuminuria (ACR > 30 mg/mmol) was reduced in the low heme group, specifically for the patients under 45 years old, while in the high heme group we observed less patients with a normal ACR value (≤ 3 mg/mmol). The difference in heme and heme-albumin concentrations between the three ACR groups was statistically significant (Figure 5B, $p = 0.017$ and $p = 0.0008$ respectively) and heme-albumin can significantly discriminate between normal and microalbuminuria groups ($p = 0.0097$). We also observed a significant difference of LDH levels between ACR groups but less discriminatory than heme-albumin ($p = 0.0021$). Since the duration of HU treatment is variable in our cohort and could differentially affect heme and kidney function, we compared heme and ACR in the patients with less than 3 µM of heme.

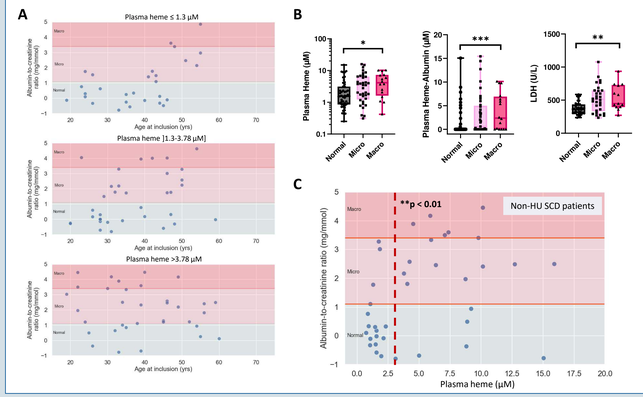

Figure 5 : Plasma heme is correlated to albumin/creatinine ratio. *Sickle cell disease patients were separated into three groups according to microalbumin to creatinine ratio (ACR) value : normal (ACR≤3 mg/mmol); micro (3<ACR≤30 mg/mmol) and macro (ACR>30 mg/mmol), natural log transformation. (A) ACR was compared to age at inclusion (in years) in heme tertiles of FCDREP-1 cohort (T1≤1.3 µM, 1.3<T2≤3.78 µM, T3>3.78 µM). (B) ACR groups compared to plasma heme, heme bound to albumin and lactate dehydrogenase (LDH) using Kruskal-Wallis test. (C) ACR compared to plasma heme in the non-HU treated group. Patients with heme ≤ 3 µM were compared to patients with heme > 3 µM with a Kruskal-Wallis test.*

CONCLUSIONS

We demonstrated the feasibility and the interest of measuring plasma heme and hemopexin to monitor intravascular hemolysis and chronic kidney damage in SCD patients. Our data also suggest that HU treatment can lower intravascular hemolysis, better than LDH and hemoglobin levels in our cohort. We did not observe any significant effect of HU treatment on plasma free hemoglobin concentration, which may be due to unspecific hemolysis arising during the sampling or in the tube and which could limit the use of this measure. We also determined heme-albumin, which represents the heme fraction that is not bound to hemopexin and thus the potentially toxic amount of circulating heme. The determination of heme-albumin (also called methemalbumin) requires the measurement of hemopexin but seems more efficient to discriminate patients with normal, micro and macroalbuminuria compared to total heme and LDH.

An ongoing longitudinal cohort study on a cohort of SCD patients with a 12 years follow-up will help us to correlate more precisely complement activation, heme level and chronic kidney damage and to evaluate the effect of HU treatment on clinical and biological parameters.

Our results indicate the complexity of determining a precise threshold of hemolysis and its impact on organ damage for clinical use in SCD, based on the measurement of the routinely used biomarkers. Thus, as a reliable biomarker and a mediator of both hemolysis and SCN, accurate quantification of plasma heme should be assessed for all patients suffering from hemolytic anemia. Our observations also highlight the importance of therapies aiming at decreasing plasma heme in SCD patients to prevent kidney damage.

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