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Association between plasma free haem and incidence of vaso-occlusive episodes and acute chest syndrome in children with sickle cell disease

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Summary

We tested the hypothesis that extracellular haem is linked to the incidence of acute complications of sickle cell disease (SCD). Using multivariable regression analysis, higher plasma free haem, but not total plasma haem, was associated with increased odds of vaso-occlusive crisis (VOC) [$P = 0.028$, odds ratio (OR); 2.05, 95% Confidence Interval (CI) = 1.08 – 3.89] and acute chest syndrome (ACS) [$P = 0.016$, OR; 2.56, CI = 1.19, 5.47], after adjusting for age and gender in children with SCD. These findings suggest that haem and factors that influence its concentration in plasma may be informative of the risk of VOC and ACS in SCD patients.

Keywords

extracellular haem; sickle cell disease; vaso-occlusive pain crisis; acute chest syndrome

Sickle cell disease (SCD) is characterized by chronic haemolysis, which intensifies intermittently (Muller-Eberhard *et al.*, 1968). However, the specific pathological role of haemolysis in the clinical complications of this disease remains controversial (Bunn *et al.*, 2010; Gladwin *et al.*, 2004). It is commonly accepted that chronic haemolysis depletes the haemoglobin (Hb) scavenger, haptoglobin, from the plasma of SCD patients (Muller-Eberhard *et al.*, 1968; Reiter *et al.*, 2002). Excess extracellular Hb in the plasma is probably oxidized to methaemoglobin, which is known to readily release haem from its prosthetic pocket (Bunn & Jandl, 1968; Jeney *et al.*, 2002). Extracellular haem is scavenged by plasma proteins including haemopexin and then delivered to the liver for degradation by haem oxygenases into iron, carbon monoxide and biliverdin (which is subsequently reduced to bilirubin) (Bunn & Jandl, 1968; Muller-Eberhard *et al.*, 1968).

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Authorship Contributions

OAA designed the study, collected clinical data and wrote the manuscript; YH performed data analysis and wrote the manuscript, SG performed laboratory analysis; DA and IO collected clinical data; SFOA designed the study, supervised the project and wrote the manuscript.

The plasma concentration of haemopexin is low in SCD patients (Muller-Eberhard *et al.*, 1968). Therefore, haem may accumulate in the plasma and contribute to the clinical complications of SCD. Hitherto, a direct association between plasma haem and clinical outcomes of SCD has not been reported. In this study, we validated a colourimetric assay widely used to directly measure plasma haem (Reiter *et al.*, 2002; Seixas *et al.*, 2009), and then applied it to quantify haem in unfractionated plasma (i.e. total plasma haem; TPH) and in protein-depleted plasma (i.e. plasma free haem, PFH) in children with SCD. Given the compelling evidence that free haem is involved in inflammation we tested the hypothesis that PFH would be associated with the incidence of clinical outcomes in children with SCD.

Study Design and Methods

With Institutional Review Board approval and following informed consent (and assent where appropriate), SCD subjects (3–18 years) were enrolled at steady state i.e. >4 weeks from blood transfusions and acute sickle-related complications. Patients on chronic blood transfusion therapy were excluded. Past medical records were reviewed. Acute chest syndrome (ACS), defined as a radiology report of a new pulmonary infiltrate that was not atelectasis on chest x-ray, was documented. Vaso-occlusive crisis (VOC), defined as a painful sickling event that required treatment with opioids and led to a hospital admission during the two years preceding study enrolment, was documented. The most recent transcranial doppler ultrasound (TCD) reported as being either normal or conditional (according to the Stroke Prevention Trial in Sickle Cell Anaemia [STOP] criteria) and co-existing medical diagnoses of priapism and avascular necrosis were abstracted. Freshly collected EDTA anti-coagulated blood samples were centrifuged at 1500g, 4°C for 10 min to collect plasma.

The unfractionated plasma was subjected to a second spin through a Microcon YM-3 column (Millipore Corporation, Billerica, MA, USA) at 20000g, 4°C for 2 h to prepare a protein-depleted plasma fraction devoid of molecules with a molecular weight > 3000 Daltons. Both plasma samples were aliquoted, and stored at –20°C for batch analysis. The concentration of haem in plasma (whole and protein-depleted) was quantified using a Quantichrome haem assay kit (Bioassay Systems, Hayward, CA) as previously described (Pamplona *et al.*, 2007). Plasma haemopexin concentration was determined using a specific two-site enzyme-linked immunosorbent assay (ELISA) kit (Kamiya Biomedical, Seattle, WA) following the manufacturer's instructions. Laboratory markers were log-transformed to better approximate normal distributions, with which the statistical power can be improved. Logistic regression models were fitted to assess the association between laboratory markers and clinical outcomes, adjusting for confounders such as age, gender, haematocrit (Hct), and white blood cell (WBC) count. Wald tests were performed to assess the significance of associations and obtain p-values, which were considered statistically significant when < 0.05.

Results and Discussion

The principal reported clinical correlates of haemolysis risk in SCD are chronic (e.g. pulmonary hypertension), and they have been defined primarily in adults. To improve the odds of finding a haemolysis risk signal in children, we focused on subjects with diametrically extreme disease phenotypes, as defined by ACS incidence. Eighty-one subjects were enrolled, 75 (92.6%) with HbSS and 6 (7.4%) with HbSβ⁰ thalassaemia genotypes. Forty-six subjects (56.8%) had a history of multiple ACS, while the remainder (n=35) had no history of ACS. Among all the subjects enrolled, 34 (42%) had presented with VOC within the 2 years preceding study enrolment. Four subjects (4.9%) had avascular necrosis (AVN), and 7 (11.67%) conditional TCDs. Priapism had been diagnosed in six

male subjects (18.2%). Mean age was 9.81 ± 4 years and the male to female ratio was 1:1.08. Sixty-five percent of the subjects were on hydroxycarbamide (HC) therapy. The mean values for WBC, Hct and Hb were $10.29 \pm 4 \times 10^9/l$, $26.24 \pm 3.3\%$ and 88.7 ± 10 g/l respectively. These values are similar to those reported in other paediatric studies following 12 months of HC therapy at the maximum tolerated dose (Kinney *et al.*, 1999).

All clinical outcomes were dichotomized into binary codes, and then used in univariate and multivariate analyses. An increase in age was associated with the incidence of AVN ($P = 0.029$), and VOC ($P = 0.008$). Baseline PFH level in patients ranged between 0.74 to 4.78 μM . Next, we investigated whether a unit change in PFH, TPH and plasma haemopexin was associated with the incidence of ACS, VOC, AVN, priapism and conditional TCD. Multivariable linear regression models indicated that PFH was associated with increased odds of ACS and VOC (Table I). Changes in TPH had no significant impact on these dichotomized clinical outcomes. The lack of association with TPH suggests that haemolysis *per se* may not be a major discriminator of ACS, VOC, AVN, priapism and conditional TCD in a paediatric SCD population with widespread use of HC. We did not assess TPH in adults, who are at much higher risk of pulmonary hypertension and leg ulcers, the principal reported clinical correlates of haemolysis.

Patients with low baseline PFH probably have an enhanced innate capacity to neutralize haem and consequently, they are more likely to lessen its inflammatory effect, which has been implicated in the pathogenesis of ACS (Bean *et al.*, 2012; Ghosh & Ofori-Acquah, 2010). The baseline rate of haemolysis within individual SCD patients is stable over time but heterogeneous to each patient (Nouraie *et al.*, 2013). Among the existing conventional markers of haemolysis, higher total serum bilirubin was associated with ACS ($p = 0.013$), while AVN was associated with lower total bilirubin ($p = 0.040$) and lower reticulocyte count ($p = 0.040$) (Table I). It is noteworthy that the associations of clinical outcomes with bilirubin and PFH were not identical (Table I). This lack of concordance suggests that these two laboratory markers did not highly confound each other in this study. In support of this interpretation, there was no correlation between the values of bilirubin and PFH among our subjects ($r = 0.034$, $p = 0.768$).

Higher Hct and WBC count are independently associated with increased incidence of VOC (Platt *et al.*, 1991) and ACS (Castro *et al.*, 1994). Multivariate analysis suggested an independent contribution of PFH to both clinical outcomes, with an Area Under the Receiver Operated Characteristic Curve of 0.69 for VOC and 0.71 for ACS, when Hct or WBC were included in the model, and 0.72 ($P = 0.03$) and 0.74 ($P = 0.028$), respectively, when PFH was also included (Table II). Several antecedent clinical events are associated with the development of ACS yet the mechanism involved in this syndrome remains poorly defined. Recently, experimental data in mice and genetic association studies in a large cohort of patients have suggested a novel role for haem in the pathogenesis of ACS. The experimental data, obtained from studies of the two widely used clinically relevant murine models of SCD (i.e. Townes and Berkeley) by our group, directly links PFH to a severe form of acute lung injury reminiscent of ACS. In support of these experimental findings, a collaborative genetic study of 945 children with SCD, enrolled in the silent infarct transfusion trial, found that a mutation in the *HMOX1* promoter that enhances degradation of haem, is associated with a lower rate of hospitalization for ACS. The present observational study offers critically important laboratory phenotypic data in patients, which advances this emerging new paradigm of ACS pathogenesis. Collectively, these studies provide a strong rationale for a larger study, in the acute setting, to confirm the role of extracellular haem, and the factors that influence its bioavailability, in the pathogenesis of ACS.

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Table I
Association between laboratory markers of haemolysis and clinical outcomes in children with sickle cell disease

Biomarker	ACS		VOC		AVN		Priapism		TCD	
	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P
Haemopexin	0.73 (0.44, 1.19)	0.2	0.62 (0.37, 1.04)	0.07	5.0 (0.72, 34.78)	0.1	0.46 (0.16, 1.34)	0.16	1.33 (0.61, 2.9)	0.48
TPH	1.5 (0.9, 2.48)	0.12	1.34 (0.81, 2.19)	0.25	0.27 (0.06, 1.18)	0.08	1.73 (0.63, 4.73)	0.28	1.34 (0.57, 3.15)	0.51
PFH	2.56 (1.19, 5.47)	0.016*	2.05 (1.08, 3.89)	0.028*	0.63 (0.16, 2.48)	0.5	1.8 (0.68, 4.74)	0.24	0.34 (0.08, 1.51)	0.16
Bilirubin	2.2 (1.18, 4.08)	0.013*	1.02 (0.61, 1.72)	0.94	0.14 (0.02, 0.92)	0.04*	2.95 (0.87, 10.01)	0.08	2.53 (0.82, 7.75)	0.1
Retic count	1.33 (0.8, 2.21)	0.27	0.87 (0.54, 1.42)	0.58	0.02 (0.0, 0.83)	0.04*	0.9 (0.33, 2.44)	0.83	0.87 (0.37, 2.03)	0.75
LDH	1.63 (0.81, 3.31)	0.17	1.51 (0.8, 2.87)	0.21	2.29 (0.15, 56.72)	0.48	1.27 (0.18, 8.86)	0.81	1.02 (0.39, 2.72)	0.96
AST	1.24 (0.75, 2.04)	0.4	1.19 (0.73, 1.94)	0.49	1.24 (0.41, 3.78)	0.71	1.92 (0.65, 5.69)	0.24	0.75 (0.34, 1.64)	0.47

The multivariate logistic regression model adjusted for age and gender was used to obtain the odds ratio (OR), 95% confidence interval and the p-value (P). Values for haemopexin, total plasma haem (TPH), plasma free haem (PFH) and bilirubin were log-transformed to normalize skewed distributions of the raw data. Significant P values are noted (*). ACS, acute chest syndrome; VOC, vaso-occlusive disease; AVN, avascular necrosis; TCD, transcranial doppler ultrasound; LDH, lactate dehydrogenase; AST, aspartate transaminase

Table II

Test for association between PFH and fitted models of VOC and ACS

Phenotype	Model	P-value	AUC
VOC	Basic VOC	-	0.686
	PFH VOC vs Basic VOC	0.031	0.721
ACS	Basic ACS	-	0.719
	PFH ACS vs. Basic ACS	0.028	0.739
	Full vs basic. VOC+WBC	0.025	0.795

Models (Basic VOC= age, gender, Hct; Basic ACS = age, gender, WBC; Full: age, gender, Hct and WBC).

ACS, acute chest syndrome; VOC, vaso-occlusive disease;