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# Longitudinal changes in iron homeostasis in human experimental and clinical malaria

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## Summary

**Background** The interaction between iron status and malaria is incompletely understood. We evaluated longitudinal changes in iron homeostasis in volunteers enrolled in malaria volunteer infection studies (VIS) and in Malaysian patients with falciparum and vivax malaria.

**Methods** We retrieved data and samples from 55 participants (19 female) enrolled in malaria VIS, and 171 patients (45 female) with malaria and 30 healthy controls (13 female) enrolled in clinical studies in Malaysia. Ferritin, hepcidin, erythropoietin, and soluble transferrin receptor (sTfR) were measured by ELISA.

**Findings** In the VIS, participants' parasitaemia was correlated with baseline mean corpuscular volume (MCV), but not iron status (ferritin, hepcidin or sTfR). Ferritin, hepcidin and sTfR all increased during the VIS. Ferritin and hepcidin normalised by day 28, while sTfR remained elevated. In VIS participants, baseline ferritin was associated with post-treatment increases in liver transaminase levels. In Malaysian patients with malaria, hepcidin and ferritin were elevated on admission compared to healthy controls, while sTfR increased following admission. By day 28, hepcidin had normalised; however, ferritin and sTfR both remained elevated.

**Interpretation** Our findings demonstrate that parasitaemia is associated with an individual's MCV rather than iron status. The persistent elevation in sTfR 4 weeks post-infection in both malaria VIS and clinical malaria may reflect a causal link between malaria and iron deficiency.

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**Keywords:** Malaria; Iron; Iron deficiency; Hepcidin; Controlled human malaria infection; Volunteer infection studies

## Introduction

Malaria and iron deficiency are major public health problems worldwide, with both frequently co-existing in vulnerable populations. Despite the significant global

prevalence of both conditions, the interaction between iron status and malaria is incompletely understood. Iron deficiency has been shown to protect against clinical malaria in African children<sup>1</sup> and pregnant women,<sup>2</sup> and

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### Research in context

#### Evidence before this study

Previous studies have demonstrated that iron deficiency has a protective effect against malaria, and that iron supplementation increases the risk of malaria infection and mortality. Other studies have demonstrated that growth of *P. falciparum* is reduced in red blood cells from iron-deficient donors. However, whether an individual's iron status has an impact on parasite growth *in vivo* has not been determined. In addition, the impact of malaria infection on an individual's iron status is also poorly understood. A causal link between malaria and iron deficiency has been suggested by one study that demonstrated that sickle cell trait (which protects against malaria) was associated with a reduced prevalence of iron deficiency in African children. Whether this causal link occurs in other populations, and the underlying mechanisms, has not been determined.

#### Added value of this study

Our study provides a detailed description of the longitudinal changes in markers of iron status in volunteers experimentally infected with malaria and in Malaysian patients (mostly adults) with clinical malaria. In experimentally-infected volunteers, we found that the main determinant of parasitaemia was the mean corpuscular volume of the red

blood cell, rather than a participant's iron status. In both experimental and clinical malaria, levels of soluble transferrin receptor, a key marker of iron status, remained elevated 1 month following treatment. Finally, in experimentally-infected volunteers we describe an association between participants' iron status and an increase in liver enzymes following treatment, possibly reflecting greater liver inflammation as a result of a more robust immune response.

#### Implications of all the available evidence

Our findings suggest that the previously observed protective effect of iron deficiency on malaria may be mediated through the effect of iron deficiency on the structure of red blood cells. Our findings of a prolonged elevation of soluble transferrin receptor in experimentally-infected volunteers and in Malaysian adults support a previous study in African children suggesting a possible causal link between malaria infection and iron deficiency. Finally, our findings of an association between iron status and post-treatment elevations in liver enzymes in experimentally infected volunteers provide insights into the mechanisms of this finding, and will aid in interpretation of this adverse event in studies evaluating new antimalarial agents.

in African children protects against severe malaria and death.<sup>3</sup> Furthermore, iron supplementation has been associated with subsequent malaria infection and mortality.<sup>4</sup> These clinical studies are supported by *in vitro* data demonstrating that *P. falciparum* infects iron-deficient erythrocytes less efficiently, and that this effect is reversed by iron supplementation.<sup>5</sup> However, the interaction between an individual's iron status and parasite growth *in vivo* has not been defined.

The impact of malaria infection on iron deficiency has also not been fully characterised. Recently, a causal link between malaria and iron deficiency was suggested by a Mendelian randomisation analysis that demonstrated that the sickle cell trait (protective against malaria) was associated with a reduced prevalence of iron deficiency in African children.<sup>6</sup> Whether this causal link occurs in other populations, and the underlying mechanisms, has not been determined.

Characterising the complex interaction between malaria and iron deficiency is further hindered by the difficulties in interpreting the various markers of iron status. Iron metabolism is controlled by the hepatocyte produced hormone hepcidin, which binds to and degrades the iron transmembrane exporter ferroportin.<sup>7-9</sup> Hepcidin therefore reduces iron availability by preventing iron export from the reticuloendothelial system, enterocytes and hepatocytes, subsequently inducing a hypoferraemia.<sup>7-9</sup> Hepcidin is upregulated by interleukin-6 (IL-6) via the JAK/STAT3 pathway,<sup>10</sup> and

has been shown to be increased in African children with falciparum malaria,<sup>11,12</sup> and in adults with vivax malaria.<sup>13</sup> Ferritin, the key intracellular iron storage protein but also an acute phase protein, is also elevated in malaria.<sup>14,15</sup> Soluble transferrin receptor (sTfR) has been proposed as a more reliable indicator of iron status as it is not affected by inflammation.<sup>8,16</sup> However, synthesis of sTfR is also increased by erythropoiesis and haemolysis. Thus, while elevated sTfR levels have been observed in children with malaria,<sup>14,17,18</sup> whether this reflects a functional iron deficiency, erythropoiesis or haemolysis, remains uncertain.

To better understand the complex interaction between *Plasmodium* infection and iron homeostasis, we evaluated the longitudinal changes in hepcidin, ferritin and sTfR in volunteers experimentally infected with blood-stage *P. falciparum*, and in Malaysian patients (mostly adults) with falciparum and vivax malaria. We sought to determine 1) whether there is an association between an individual's iron status and parasite growth, and 2) the impact of experimental and clinical malaria infection on iron homeostasis in adults. Finally, given previous observations of abnormal liver function tests following antimalarial treatment in malaria volunteer infection studies (VIS),<sup>19,20</sup> and the importance of this finding for antimalarial drug development, we investigated whether this adverse finding in malaria VIS was associated with participants' baseline iron status.

## Methods

### Malaria volunteer infection studies (VIS)

Data were retrieved from the records of 55 participants enrolled in 7 induced blood stage malaria (IBSM) VIS conducted in Brisbane, Australia, between 2015 and 2021.<sup>21–25</sup> Study participants were malaria-naïve adults aged 18–55 years with no significant medical co-morbidities or concurrent illness. Participants were inoculated with erythrocytes parasitised with either the *P. falciparum* 3D7 strain (n = 45), or the artemisinin-resistant *P. falciparum* K13 strain (n = 10). Parasitaemia was closely monitored at specific time-points using quantitative PCR (qPCR) targeting the species specific 18S ribosomal ribonucleic (rRNA) gene. The studies evaluated six different antimalarial drugs (including registered antimalarials and new chemical entities in development), with the day of administration being day 8 for the 3D7 strain and day 9 for the K13 *P. falciparum* strain. For many of the studies, sub-curative doses of the antimalarial drugs were intentionally administered, resulting in parasite recrudescence to allow for the calculation of drug minimum inhibitory concentrations. All participants dosed with a new chemical entity were treated with a registered antimalarial drug such as artemether-lumefantrine or atovaquone-proguanil, at the time of recrudescence or at the end of study.

### Parasite data and laboratory measurements

Parasitaemia parameters evaluated in the VIS participants included the log<sub>10</sub> transformed peak pre-treatment parasitaemia (parasites/mL), the total parasite burden (area under the curve of the non-transformed 18S qPCR data from day 4 until time of treatment), and the parasite multiplication rate per 48 h (PMR<sub>48</sub>). The PMR was calculated using a sine-wave mixed effects model, as previously reported.<sup>26</sup> Participants who participated in the VIS had blood collected for standard haematology (including reticulocyte count) and biochemistry prior to inoculation (day 0), prior to antimalarial treatment (day 8 or 9), on day 15 (±2), and at the end of study (EOS, day 30 ± 3). The reticulocyte difference was defined as the difference between the reticulocyte count at EOS and the reticulocyte count prior to inoculation.

Stored frozen aliquots of serum from the studies were tested using commercial ELISA assays for erythropoietin (EPO; BioLegend, Catalogue Number 442907), hepcidin (Intrinsic Lifesciences, Catalogue Number ICE-007) and sTfR (BioVendor, Catalogue Number RD194011100), with all ELISAs performed as per manufacturer's instructions. If CRP and ferritin levels had not been measured at the appropriate timepoints in the clinical trial, they were tested by immunoturbidimetric (CRP) assay and a 2-step chemiluminescent microparticle (ferritin) immunoassay via an external NATA-accredited clinical laboratory using stored frozen serum. As ferritin is an acute phase protein, its level was

adjusted for CRP. The adjustment was based on the Biomarkers Reflecting Inflammation and Nutritional Determinants of Anaemia (BRINDA) project,<sup>27,28</sup> with ferritin levels remaining unchanged if CRP was ≤5 mg/mL, or multiplied by 0.67 if CRP was >5 mg/mL. Participants were defined as iron deficient if they had an adjusted ferritin below 15 ng/mL.

### Malaysian study sites and study procedures

Patients hospitalised with malaria were enrolled as part of observational studies and/or clinical trials conducted at 4 study sites in Sabah, Malaysia, including a tertiary hospital (Queen Elizabeth Hospital; QEH), and 3 district hospitals (Kudat, Kota Marudu and Pitas District Hospitals) between 2010 and 2016.<sup>29–33</sup> For the current analysis only patients with PCR-confirmed falciparum or vivax malaria were included. Patients admitted to the QEH were aged >12 years, were within 18 h of commencing antimalarial treatment, not pregnant and had no major medical co-morbidities. Inclusion criteria at the district hospitals were similar, except patients were all enrolled pre-treatment, and all ages were included. Treatments given were based on local hospital guidelines at the time, including artemisinin-combination (ACT) for uncomplicated *P. falciparum*, ACT or chloroquine plus primaquine for uncomplicated vivax malaria, and intravenous artesunate for severe malaria. Patients enrolled in the clinical trials were randomised to receive either ACT or chloroquine, according to the study protocols.<sup>31–33</sup> Healthy controls were visitors or relatives of the study participants admitted to QEH, with no history of fever within 48 h and blood film negative for malaria.

Venous blood was collected in lithium heparin blood tubes and centrifuged, with plasma stored at –70 °C within 30 min. For patients admitted to the district hospitals, haematology, biochemistry, and parasite parameters were obtained on enrolment (day 0), and 7 and 28 days following admission. Those admitted to QEH only had samples taken at day 0 and day 28. Stored aliquots of plasma, collected at the above timepoints, were tested as described above for hepcidin, ferritin and sTfR levels. The ferritin was not adjusted for CRP as CRP was not available for these patients.

### Ethics statement

All studies were conducted in accordance with the Declaration of Helsinki and the International Committee of Harmonisation of Good Clinical Practice guidelines. All participants provided informed written consent. For the malaria volunteer infection studies, ethics approval was obtained from the Human Research Ethics Committee of the QIMR Berghofer Medical Research Institute (P3540). For the clinical studies in Malaysia, ethics approval was obtained from the Malaysian Ministry of Health (NMRR10\_754\_6684) and the Menzies School of Health Research, Darwin,

Australia (HREC 2010\_1431). Informed written consent was provided by all participating adults and parents/guardians of those under 18 years of age.

### Role of funders

The funders had no role in the study design, data collection, data analysis, interpretation or writing of this report.

### Statistical analysis

Data were analysed using Stata V17.0 and GraphPad Prism V9.1.0. Distribution of continuous variables was assessed by the Shapiro–Wilk test and quantile–quantile (QQ) plots. Normally distributed variables were summarised using the mean and standard deviation (SD), and differences between groups were compared using the Welch’s *t*-test. Non-normally distributed continuous variables were summarised using the median and interquartile range (IQR), and differences between groups compared using the Wilcoxon rank-sum test. Spearman’s correlation was used to investigate the correlations between variables. For the key outcomes of interest in the VIS including total parasite burden (as a measure of parasite growth), peak hepcidin, EOS sTfR and day 15 alanine transferase (ALT), separate multivariable linear regression models were used to assess potential predictors. The outcomes of interest were  $\log_{10}$  transformed, and variables were included in the multivariable models if they had a *P* value of <0.20 in the univariate analyses. Final multivariable models were estimated using backwards elimination of non-significant variables until all remaining variables were below the cut-off of *P* < 0.20. Homogeneity of variance of residuals was checked visually by plotting the residuals against the fitted values and tested using Breusch–Pagan test. The normality assumption of residuals were checked visually using QQ plots and tested using the Shapiro–Wilk test, and linearity assumption was checked by plotting the standardized residuals against each of the predictor variables.

Longitudinal measurements in the malaria VIS were analysed using a repeated measures ANOVA, with non-normally distributed variables  $\log_{10}$  transformed, and sphericity tested using Mauchly’s Test of Sphericity and corrected using the Greenhouse–Geisser correction. For the longitudinal measurements in the Malaysian studies, a linear mixed effects model was used to account for missing observations at day 7 (*n* = 77 for *P. falciparum* and *n* = 6 for *P. vivax*), therefore incorporating all available observations at day 0, day 7 and day 28 timepoints. The linear mixed effects model included a main effect for time as a categorical variable and a random intercept for study participant, assumed to follow a normal distribution. Non-normally distributed variables were  $\log_{10}$  transformed, and normality and homoscedasticity of residuals were assessed with QQ plots of conditional residuals and plotting the conditional residuals against predicted values. *P* values for

comparisons between timepoints were obtained from the repeated measures ANOVA for the malaria VIS or the mixed effects model for the Malaysian studies; for these models, displayed *P* values have been adjusted using the Bonferroni correction to account for the number of pairwise comparisons performed.

## Results

### Malaria volunteer infection studies

#### Demographics and parasite parameters

A total of 55 participants were enrolled in the malaria VIS. The median age of participants was 26 years (IQR 22–36), and 19 (35%) were female (Table 1). Compared to males, at baseline females had lower ferritin (median 46 [IQR 22–75] vs 101 [IQR 56–171] ng/mL, *P* = 0.0013), lower baseline sTfR (mean 1.50  $\mu$  g/mL (SD 0.55) vs 2.01  $\mu$  g/mL [SD 0.63], *P* = 0.0014), and higher mean corpuscular volume (MCV; median 89 [IQR 85–92] vs 86 [IQR 84–88] fL, *P* = 0.010). Only one male participant, and no female, was iron deficient at baseline, defined as a ferritin <15 ng/mL. Participants had a median pre-treatment peak parasitaemia of 16,973 parasites/mL [IQR 4753–57,602], and a median total pre-treatment parasite burden of 70,920 parasites/mL [IQR 26,017–255,370], with no differences between males and females. There was no evidence of correlation between either baseline ferritin or sTfR and peak pre-treatment parasitaemia, total pre-treatment parasite burden, or parasite multiplication rate (Table 2, Supplementary Table S1). However, peak pre-treatment parasitaemia and total pre-treatment parasite burden were both associated with baseline MCV (*r* = 0.35, *P* = 0.0089; and *r* = 0.39, *P* = 0.0039, respectively). The association between baseline MCV and total pre-treatment parasite burden remained significant on multivariable analysis (*P* = 0.0053, Table 2).

#### Longitudinal changes in iron markers

Hepcidin rose following inoculation, peaking at day 8, before falling to baseline levels by day 15 (Fig. 1). Ferritin levels remained unchanged between baseline (median 73 ng/mL [IQR 66–82]) and day of treatment, but then increased following treatment to a median 101 ng/mL (IQR 91–114) on day 15 (*P* = 0.0021 for day 8 vs day 15), before returning to baseline levels by day 30. Median sTfR also remained unchanged from inoculation to the day of treatment but increased following treatment from 1.70  $\mu$  g/mL (IQR 1.63–1.78) to 1.97  $\mu$  g/mL (IQR 1.88–2.05) at day 15, increasing further to 2.27  $\mu$  g/mL (IQR 2.17–2.36) by day 30 (*P* < 0.0001 for day 8 vs day 30). Like sTfR, median EPO remained stable between baseline and day 8 but increased significantly between day 8 and day 15, and then increased further between day 15 and day 30 (*P* = 0.019 for day 8 vs day 15). Median CRP increased slightly from 1.5 mg/L (IQR 0.3–2.7 mg/L) at baseline to 3.6 mg/L

	All (n = 55)	Male (n = 36)	Female (n = 19)	P value
Age, years	26 (22–36)	25 (22–32)	31 (22–40)	0.17
PP <sub>Pre</sub> , parasites/mL	16,973 (4753–57,602)	11,674 (3646–31,620)	32,898 (9062–74,773)	0.054
TPB <sub>Pre</sub> , parasites	70,920 (26,017–255,370)	51,978 (24,997–247,779)	138,073 (29,987–255,370)	0.29
Baseline haemoglobin, g/L	145 (136–152)	149 (145–154)	133 (127–138)	<0.0001
End of study haemoglobin, g/dL	136 (127–143)	141 (135–145)	121 (116–131)	<0.0001
Baseline ferritin <sup>a</sup> , ng/mL	73 (66–82)	101 (56–171)	46 (22–75)	0.0013
End of study ferritin <sup>a</sup> , ng/mL	78 (61–76)	94 (63–179)	42 (14–63)	<0.0001
Baseline sTfR, $\mu$ g/mL, (mean, SD)	1.87 (0.66)	2.01 (0.63)	1.50 (0.55)	0.0014
End of study sTfR, $\mu$ g/mL,	2.27 (2.17–2.36)	2.40 (2.10–2.86)	1.87 (1.63–2.52)	0.010
Baseline EPO, mIU/mL	5.71 (5.12–6.36)	5.75 (3.91–8.94)	4.44 (3.17–11.16)	0.60
End of study EPO, mIU/mL	7.69 (6.90–8.57)	6.69 (4.34–12.61)	6.86 (5.32–9.62)	0.99
Baseline hepcidin, ng/mL	25 (22–29)	28 (15–44)	23 (12–42)	0.50
Day 8 hepcidin, ng/mL	30 (26–34)	33 (19–53)	32 (10–67)	0.98
Baseline MCV, fL	86 (85–89)	86 (84–88)	89 (85–92)	0.010
End of study MCV, fL (mean, SD)	87 (4.2)	86 (3.7)	88 (4.6)	0.023

Variables are median (interquartile range) unless otherwise stated. Pf, *P. falciparum*; PP<sub>Pre</sub>, peak parasitaemia pre-treatment; SD, standard deviation; TPB<sub>Pre</sub>, total parasite burden pre-treatment; sTfR, soluble transferrin receptor; EPO, erythropoietin; MCV, mean corpuscular volume. <sup>a</sup>The ferritin was adjusted for inflammation as per the BRINDA project.<sup>27,28</sup>

**Table 1: Clinical and laboratory characteristics of volunteers experimentally infected with *P. falciparum*.**

(IQR 2.4–4.8 mg/L; P = 0.22) on day 8, increasing further to 9.3 mg/L (IQR 8.1–10.5 mg/L) on day 15 (P < 0.0001 for day 8 vs day 15), before returning to baseline by EOS. By day 30, 6 participants (11%) met the criteria for iron deficiency, including the single participant who had iron deficiency at baseline.

Peak hepcidin (day 8) correlated with baseline hepcidin ( $r = 0.76$ , P < 0.0001) and baseline adjusted ferritin

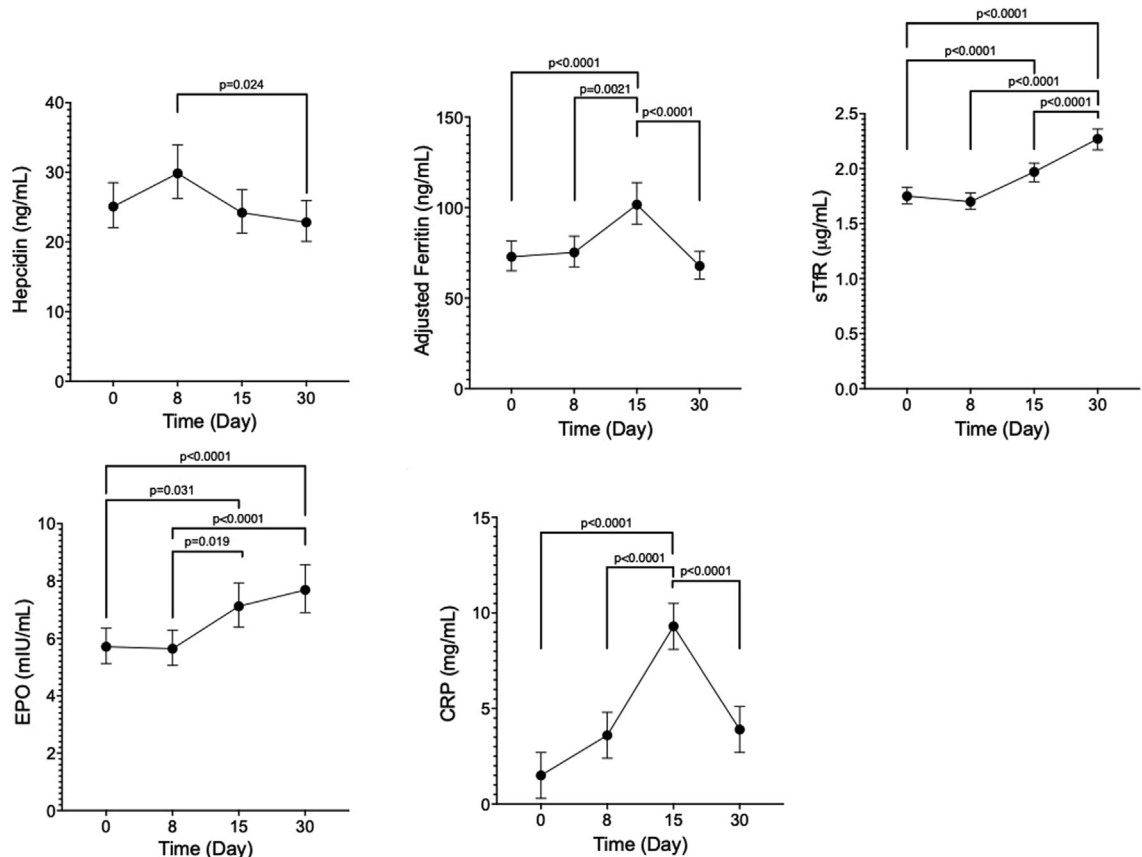
( $r = 0.28$ , P = 0.039), although not pre-treatment total parasite burden (Table 2). The correlation between baseline and peak hepcidin remained significant on the multivariable model (P < 0.0001).

Regarding predictors of iron parameters at the end of study (day 30), day 30 sTfR was strongly associated with the baseline sTfR ( $r = 0.86$ , P < 0.0001), and inversely associated with baseline MCV ( $r = -0.42$ , P = 0.0024)

Associations	Spearman correlation		Regression model	
	R-value (95% confidence interval)	P value	Co-efficient (95% confidence interval)	P value
<b>Total pre-treatment parasite burden</b>			<b>(R<sup>2</sup> = 0.13)</b>	
Baseline MCV	0.39 (0.27–0.50)	0.0043	0.08 (0.02–0.13)	0.0053
Baseline adjusted ferritin	0.19 (0.06–0.32)	0.17		
Baseline sTfR	-0.24 (-0.37 to -0.12)	0.071		
Baseline hepcidin	-0.10 (-0.25 to 0.06)	0.49		
<b>Peak hepcidin</b>			<b>(R<sup>2</sup> = 0.61)</b>	
Baseline hepcidin	0.76 (0.66–0.85)	<0.0001	0.79 (0.62–0.96)	<0.0001
Baseline adjusted ferritin	0.28 (0.15–0.41)	0.039		
Day 8 CRP	0.16 (0.02–0.30)	0.25		
Total pre-treatment parasite burden	-0.10 (-0.24 to 0.05)	0.49		
<b>EOS sTfR<sup>a</sup></b>			<b>(Adjusted R<sup>2</sup> = 0.75)</b>	
Baseline sTfR	0.86 (0.79–0.90)	<0.0001	0.58 (0.47–0.69)	<0.0001
Baseline MCV	-0.42 (-0.54 to -0.29)	0.0024	0.00 (-0.01 to 0.01)	0.17
Peak hepcidin	-0.30 (-0.42 to -0.18)	0.025		
Total pre-treatment parasite burden	-0.06 (-0.19 to 0.06)	0.63		
EOS EPO	0.12 (0.00–0.25)	0.38		
EOS reticulocytes	-0.01 (-0.18 to 0.16)	0.94		

Multivariable linear regression analysis of log<sub>10</sub> transformed biological plausible factors using backward elimination. R<sup>2</sup>: coefficient of determination of the model; EOS, end of study; EPO, erythropoietin; MCV, mean corpuscular volume; sTfR, soluble transferrin receptor. <sup>a</sup>n = 39.

**Table 2: Associations between markers of iron metabolism and parasite parameters in volunteers experimentally infected with *P. falciparum*.**



**Fig. 1: Longitudinal changes in the markers of iron metabolism in 55 volunteers experimentally infected with *P. falciparum*.** Data points represent the means, and error bars the 95% confidence intervals. A repeated measures ANOVA was performed on  $\log_{10}$  transformed data which were back-transformed to original scale for presentation. Displayed P values have been adjusted using the Bonferroni correction to account for the 6 pairwise comparisons performed. Ferritin was adjusted as per the BRINDA project.<sup>26,27</sup>

and peak hepcidin ( $r = -0.30$ ,  $P = 0.025$ ). There was no correlation between day 30 sTfR and either day 30 EPO or day 30 reticulocyte count. In the multivariable analysis, the only predictor of day 30 sTfR was baseline sTfR ( $P < 0.0001$ ). An increase in reticulocytes was observed from baseline to end of study (median  $14 \times 10^9/L$ , [IQR 3–20];  $n = 39$ ,  $P < 0.0001$ ), with this increase associated with baseline ferritin ( $r = 0.34$ ,  $P = 0.033$ ).

#### Longitudinal changes in liver transaminases

Median ALT remained unchanged between day 0 (20 U/L [IQR 18–22]) and day 8 (19 U/L [IQR 17–21]) but was transiently increased on day 15 (41 U/L [IQR 37–46];  $P < 0.0001$ ), returning to baseline at EOS (Fig. 2). Median AST was also transiently increased on day 15 compared to baseline and day 8 levels. The bilirubin did not vary across the duration of the study, with no significant changes.

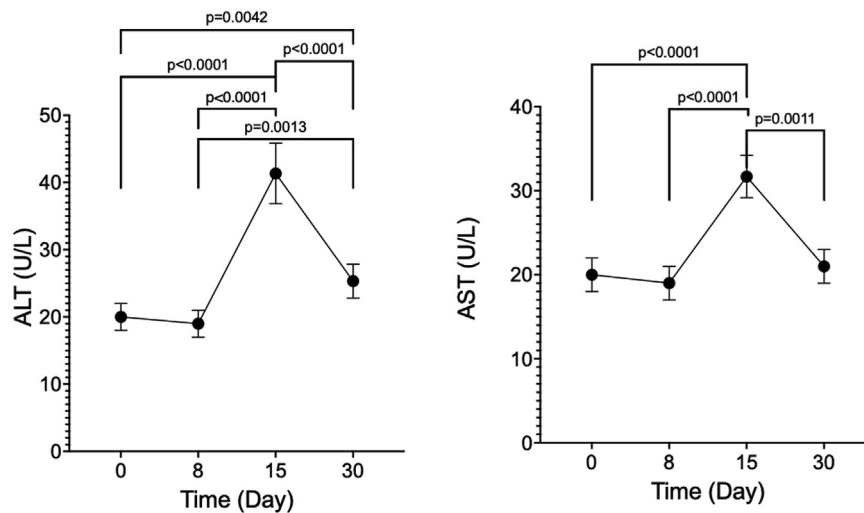
Day 15 ALT and AST were both associated with baseline adjusted ferritin (ALT:  $r = 0.51$ ,  $P < 0.0001$ ; AST:  $r = 0.43$ ,  $P = 0.0001$ ) as well as the day 8 adjusted

ferritin (ALT:  $r = 0.48$ ,  $P < 0.0001$ ; AST:  $r = 0.42$ ,  $P = 0.0021$ ) and day 15 adjusted ferritin (ALT:  $r = 0.52$ ,  $P = 0.0012$ ; AST:  $r = 0.52$ ,  $P = 0.0014$ ) (Table 3). Hepcidin and sTfR were not significantly associated with ALT and AST, at any timepoints. Day 15 ALT and AST were also associated with day 15 CRP levels (ALT:  $r = 0.26$ ,  $P = 0.042$ ; AST:  $r = 0.28$ ,  $P = 0.041$ ), as well as with total parasite burden pre-treatment (ALT:  $r = 0.35$ ,  $P = 0.0092$ ; AST:  $r = 0.33$ ,  $P = 0.013$ ), although not with peak parasitaemia. In multivariable analysis, total parasite burden pre-treatment ( $P = 0.041$ ) and baseline ferritin ( $P = 0.0012$ ) remained significantly correlated with day 15 ALT.

#### Malaysian patients hospitalised with malaria

##### Demographics and admission parasitaemia

A total of 201 patients were enrolled and had iron parameters evaluated, including 109 with falciparum and 62 with vivax malaria, as well as 30 healthy controls. Appropriate samples were collected on days 0, 7 and 28 from 33 patients with falciparum and 56 with vivax



**Fig. 2: Longitudinal changes in the markers of liver transaminases in 55 volunteers experimentally infected with *P. falciparum*.** Data points represent the means, and error bars the 95% confidence intervals. A repeated measures ANOVA was performed on  $\log_{10}$  transformed data which were back-transformed to original scale for presentation. Displayed P values have been adjusted using the Bonferroni correction to account for the 6 pairwise comparisons performed.

malaria, with the others having samples collected on days 0 and 28. The median age was 28 years (IQR 19–43, range 7–72) for patients with falciparum malaria, and 18 years (IQR 10–35, range 5–66) for those with vivax malaria (Table 4). Most patients and controls were male (73% [79/109] of patients with falciparum malaria, 76% [47/62] of patients with vivax malaria, and 57% [17/30] of controls). The median parasitaemia on presentation was higher in patients with *P. falciparum* (6783 parasites/ $\mu$ L [IQR 1836–20,020]) compared to those with *P. vivax* (3629 parasites/ $\mu$ L [IQR 1226–8832]). There was no correlation between parasitaemia and MCV on admission in either falciparum or vivax malaria.

#### Markers of iron metabolism in patients with falciparum and vivax malaria

Hepcidin on admission was significantly higher in patients with both falciparum and vivax malaria compared to the healthy controls (median 136 ng/mL [IQR 80–221], 130 ng/mL [IQR 54–205] and 22 ng/mL [IQR 16–31], respectively;  $P < 0.0001$  for each *Plasmodium* species

compared to controls; Table 4), and in patients with falciparum malaria was associated with parasitaemia ( $r = 0.36$ ,  $P = 0.0013$ ; Supplementary Table S2). By day 28, hepcidin in patients with falciparum and vivax malaria had returned to similar levels as the control participants.

The median ferritin on admission was also significantly higher in patients with both falciparum and vivax malaria compared to the controls (301 ng/mL [IQR 202–430], 209 ng/mL [IQR 146–313] and 53 ng/mL [IQR 20–90], respectively;  $P < 0.0001$  for each *Plasmodium* species compared to controls), and was higher in patients with falciparum compared to vivax malaria ( $P = 0.0062$ ). By day 15, ferritin increased in those treated for vivax but not falciparum malaria. By day 28, median ferritin had fallen significantly in patients with falciparum and vivax malaria (Fig. 3), but remained significantly higher than that of controls (127 ng/mL [IQR 52–201], 84 ng/mL [IQR 35–142], and 53 ng/mL [IQR 20–90], respectively;  $P < 0.0001$  and  $P = 0.017$  for *P. falciparum* and *P. vivax*, respectively, compared to controls, Table 4). There were no associations in either

Association with day 15 ALT	Spearman correlation		Regression model (Adjusted $R^2 = 0.30$ )	
	R value (95% confidence interval)	P value	Co-efficient (95% confidence interval)	P value
Baseline adjusted ferritin	0.51 (0.40–0.61)	<0.0001	0.39 (0.16–0.62)	0.0012
Total parasite burden	0.35 (0.22–0.48)	0.0093	0.13 (0.05–0.25)	0.041
Day 15 CRP	0.26 (0.13–0.40)	0.042	0.11 (–0.05 to 0.28)	0.18
Peak hepcidin	–0.03 (–0.16 to 0.11)	0.84		

Multivariable linear regression analysis using backward elimination of log-transformed biological plausible factors. Adjusted  $R^2$ : coefficient of determination of the model. ALT, alanine transferase; CRP, C-reactive protein.

**Table 3: Associations between markers of iron metabolism and liver enzymes in volunteers experimentally infected with *P. falciparum*.**



	Control (n = 30)	P. falciparum				P. vivax				P value (Pf vs Pv)	
		All (n = 109)	Male (n = 79)	Female (n = 30)	P value (male vs female)	All (n = 62)	Male (n = 47)	Female (n = 15)	P value (male vs female)	P value (Pv vs control)	P value (Pf vs Pv)
Age, years	36 (27-43)	28 (19-43)	26 (19-39)	38 (20-52)	0.13	18 (10-35)	24 (11-36)	11 (9-18)	0.028	0.0022	0.0071
Haemoglobin, g/L		131 (113-144)	137 (115-146)	120 (104-130)	0.0014	124 (104-133)	124 (109-139)	108 (93-121)	0.0073		0.016
Mean corpuscular volume, fL		79 (71-86) [n = 65]	79 (71-87) [n = 47]	78 (68-88) [n = 18]	0.71	81 (75-88) [n = 59]	84 (77-88) [n = 45]	78 (71-83) [n = 12]	0.024		0.23
Parasitaemia on presentation		6783 (1836-20,020)	6848 (1732-21,760)	6161 (1917-17,263)	0.71	3629 (1226-8832)	3739 (1226-8832)	3518 (625-9736)	0.69		0.033
Admission hepcidin, ng/mL		136 (80-221)	139 (80-216)	120 (69-235)	0.53	<0.0001	130 (54-205)	87 (30-154)	0.083	<0.0001	0.21
Day 28 hepcidin, ng/mL		29 (13-45)	33 (16-48)	22 (11-39)	0.053	0.23	19 (12-37)	17 (14-27)	0.38	0.89	0.13
Admission ferritin, ng/mL		53 (20-90)	301 (202-430)	330 (203-501)	0.016	0.0010	209 (146-313)	207 (146-313)	0.64	<0.0001	0.0062
Day 28 ferritin, ng/mL		127 (52-201)	152 (71-241)	70 (29-112)	0.0011	<0.0001	84 (35-142)	87 (35-159)	0.32	0.0173	0.023
Admission sTfR, µg/mL		1.24 (1.11-1.57)	1.24 (0.96-1.45)	1.23 (1.01-1.70)	0.10	0.35	1.24 (1.04-1.60)	1.21 (1.00-1.59)	0.46	0.73	0.44
Day 28 sTfR, µg/mL		1.99 (1.63-2.42)	1.91 (1.63-2.27)	2.18 (1.61-2.60)	0.19	<0.0001	2.02 (1.57-2.42)	2.01 (1.44-2.36)	0.26	<0.0001	0.99

Variables are median (interquartile range). Pf, P. falciparum; Pv, P. vivax; sTfR, soluble transferrin receptor.

Table 4: Clinical and laboratory characteristics of Malaysian patients with falciparum and vivax malaria.

species between day 0 or day 28 ferritin and parasitaemia (Supplementary Table S2).

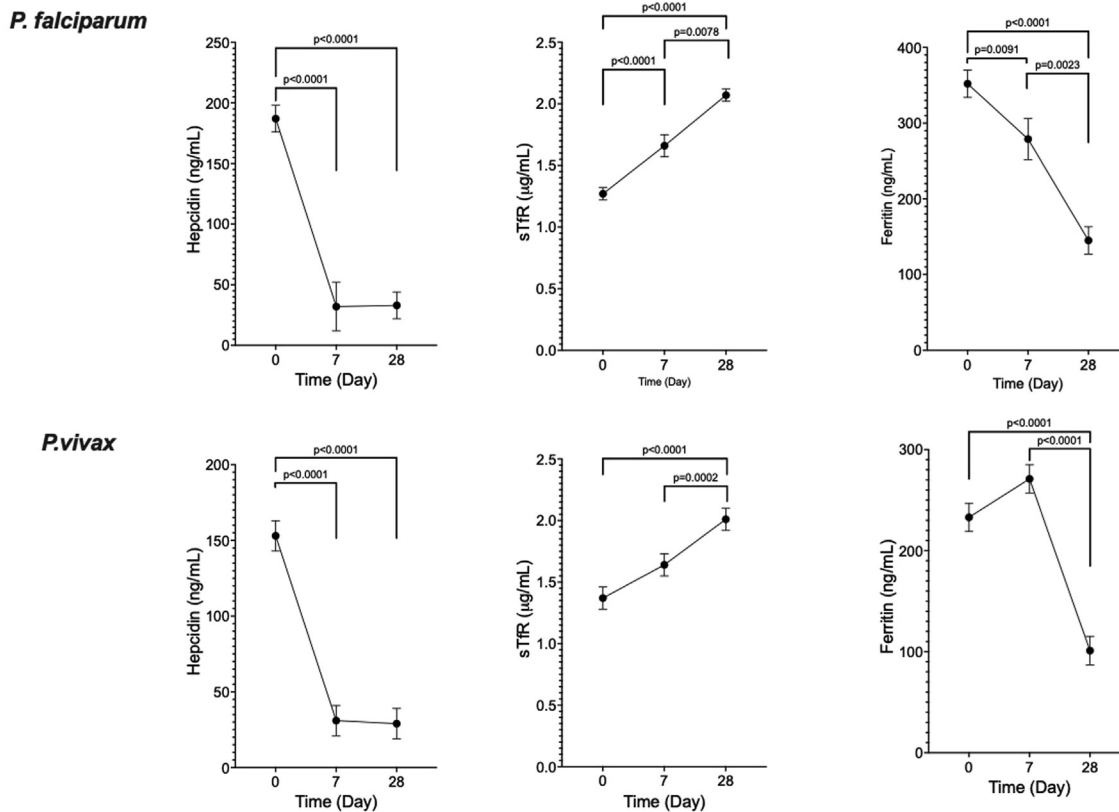
In patients with falciparum and vivax malaria, the sTfR levels on admission were similar to those of the controls, however were significantly higher than the controls by day 30 (median 1.99 µg/mL [IQR 1.63–2.42], 2.02 [IQR 1.57–2.42], and 1.24 [IQR 1.11–1.57], respectively; P < 0.0001 for each *Plasmodium* species compared to controls). Despite the similar levels of sTfR between patients with malaria on admission and controls, sTfR levels were inversely associated with parasitaemia (r = -0.20, P = 0.042) in patients with falciparum malaria, possibly reflecting decreased erythropoiesis in patients with higher parasitaemia.

### Discussion

In this study we evaluated the longitudinal changes in markers of iron metabolism in volunteers experimentally infected with blood-stage *P. falciparum*, and in Malaysian patients with falciparum and vivax malaria, to better understand the complex interaction between *Plasmodium* infection and iron homeostasis. We demonstrated that in human experimental malaria parasite growth was not associated with participants' baseline iron status but was associated with their MCV. We found that hepcidin levels increased in experimental and clinical infection but fell rapidly following treatment, while sTfR remained elevated following recovery in both experimental and clinical malaria. These findings highlight the prolonged disturbance that occurs in iron biomarkers during malaria and extend previous studies in African children<sup>6</sup> suggesting a possible causal link between malaria infection and functional iron deficiency.

Many pathogens require iron for growth, development, and pathogenicity, and hence it has long been postulated that iron deficiency may be protective against severe manifestations of malaria. Previous studies have demonstrated that iron deficiency is associated with a reduced risk of malaria in African children<sup>1</sup> and pregnant women,<sup>34,35</sup> and that iron supplementation increases risk of malaria infection and mortality.<sup>3</sup> Furthermore, *in vitro* studies of parasite growth in red blood cells (RBCs) from iron replete donors have demonstrated increased parasite growth,<sup>5</sup> and a modelling study has suggested an association between host iron bioavailability and parasite multiplication rate.<sup>36</sup>

In contrast to these studies, in the current study where parasite growth rates were evaluated in 55 experimentally infected volunteers, we did not find any correlation between any of the iron parameters at baseline with either peak parasitaemia, total pre-treatment parasite burden, or parasite multiplication rate. We did however find a positive correlation between RBC mean corpuscular volume (MCV) and parasitaemia, with MCV being associated with total pre-



**Fig. 3: Longitudinal changes in the markers of iron metabolism in Malaysian patients with *P. falciparum* (n = 109) and *P. vivax* (n = 62) malaria.** Data points and error bars represent marginal means and 95% confidence intervals, respectively. Data were analysed using a linear mixed effects model, as day 7 samples were available for only 33/109 patients with falciparum malaria, and 56/62 patients with vivax malaria. Non-normally distributed variables were log<sub>10</sub> transformed, and back-transformed for presentation. Displayed P values have been adjusted using the Bonferroni correction to account for the 6 pairwise comparisons performed. sTfR, soluble transferrin receptor.

treatment parasite burden on multivariable analysis. This finding is consistent with the above-mentioned study by Clark et al. in which parasite growth was increased in RBCs from iron replete donors (all of whom had an MCV >80 fL), compared to RBCs from iron-deficient donors (with an MCV <80 fL).<sup>5</sup> Furthermore, Clark et al. reported that *P. falciparum* growth and invasion rates increased when old RBCs (which have lower MCV) were replaced with young RBCs (with a higher MCV).<sup>5</sup> These findings are also consistent with *ex-vivo* findings in Gambian children with anaemia; multivariable analysis demonstrated that MCV and haemoglobin, but not other markers of iron status, were independently associated with parasite growth.<sup>37</sup> Taken together, these findings suggest that the protective effect of iron deficiency on clinical malaria and parasitaemia observed in clinical studies may be mediated through the effect of iron deficiency on the structure of the RBCs, rather than by depletion of the iron available to the parasite. Although we did not find an association between MCV and parasitaemia in Malaysian patients with clinical malaria, this may relate to greater

heterogeneity in this population and other factors contributing to parasitaemia such as duration of infection and/or host immunity.

Our study in experimentally infected volunteers provided a unique opportunity to evaluate the longitudinal changes in iron biomarkers that occur in early malaria infection. One previous study has evaluated iron biomarkers in experimental human malaria infection; however this study was small, reporting data from only 5 participants with sporozoite-induced malaria.<sup>38</sup> In the current study we demonstrate that hepcidin increases very early in infection, peaking at day 8 following inoculation with blood-stage *P. falciparum*, when parasitaemia levels were only just at the threshold for microscopic detection. This is consistent with a previous study by de Mast et al., who demonstrated that hepcidin concentrations were also elevated in Indonesian children with asymptomatic *P. falciparum* and *P. vivax* parasitaemia, despite low CRP levels in these children.<sup>11</sup> The mechanism of elevated hepcidin at these low parasitaemia levels is uncertain. Hepcidin is known to be produced by the liver in response to IL-6,<sup>10</sup> and it is

possible that the early increase in hepcidin reflects an early inflammatory response. However, we found that CRP was only very mildly elevated by day 8, and like other studies,<sup>39</sup> we did not find an association between CRP and hepcidin. IL-6 independent mechanisms of hepcidin regulation have been reported, including production of hepcidin by peripheral blood mononuclear cells stimulated by parasitised RBCs,<sup>40</sup> and may account for the early increase in hepcidin observed in the malaria VIS.

In contrast to the early increase in hepcidin, in experimental malaria infection ferritin did not rise until after treatment, with the rise coinciding with a rapid fall in hepcidin. While ferritin and hepcidin had both returned to baseline by the end of study in experimental malaria, sTfR concentrations rose markedly after treatment, and were at peak levels by the end of study. This may reflect erythropoiesis, with erythropoietin and the reticulocyte count also both increased by the end of study. However, we did not observe any correlation between day 30 sTfR levels and either erythropoietin or reticulocyte count, possibly suggesting that the increased sTfR may also reflect a hepcidin-mediated reduction in total iron body content. Hepcidin depletes available iron by reducing gastrointestinal absorption as well as preventing release of iron from intracellular sources. A hepcidin-mediated iron deficiency resulting from malaria has been suggested by Muriuki et al.,<sup>29</sup> who evaluated sickle cell trait (a genetic variant protective against malaria) in Mendelian randomisation analyses and demonstrated that sickle cell trait was associated with a reduced prevalence of iron deficiency in African children. Our findings from the malaria VIS suggest that hepcidin-mediated iron deficiency following malaria may occur in early primary infections, even at very low parasitaemia levels.

It was notable in our malaria VIS that sTfR concentrations at end of study were strongly correlated with the sTfR concentrations at baseline. While not unexpected, this highlights the fact that individuals who are iron deficient, although perhaps at lower risk of acquiring malaria, may nonetheless be most vulnerable to further depletion in iron stores. We also noted a correlation between baseline ferritin and magnitude of reticulocyte response on recovery, suggesting that individuals with iron deficiency may be at greater risk of malarial anaemia due to impaired erythropoiesis.

In the current study, we also evaluated longitudinal changes of iron parameters in Malaysian patients (mostly adults) with falciparum and vivax malaria. To our knowledge longitudinal analysis of iron parameters in adults with clinical malaria has not previously been conducted, although elevated hepcidin has been reported in adults with vivax malaria.<sup>13</sup> In the current study the temporal changes in the iron biomarkers in Malaysian patients were generally consistent with those of the VIS, and as previously reported in African

children. As expected, hepcidin and ferritin were both elevated on admission, and were higher than the levels observed in the VIS. As in the VIS, hepcidin levels fell rapidly following antimalarial treatment. The post-treatment increase in ferritin observed in the VIS was observed in clinical vivax but not falciparum malaria, possibly reflecting a greater post-treatment inflammatory response seen with *P. vivax*.<sup>41</sup> In both species ferritin had reduced by day 28, although remained significantly higher than the level of healthy controls. This is consistent with a previous study by Castberg et al., which demonstrated that in African children ferritin remained elevated up to 4 weeks following malaria infection (when compared to the 6 week post-treatment and presumed baseline level).<sup>15</sup> Whilst it seems likely that in the current study ferritin may have declined further beyond 28 days, we cannot exclude the possibility that, compared to controls, the patients with malaria had increased ferritin prior to infection, and may have been more susceptible to malaria infection, consistent with the above-mentioned studies reporting increased susceptibility to malaria infection in more iron-replete individuals.

As with the VIS, sTfR remained significantly elevated at day 28 in Malaysian patients with clinical malaria, and again raises the possibility that a hepcidin-mediated functional iron deficiency following malaria may also occur in adults. This persistent elevation of both ferritin and sTfR up to one month following malaria infection in adults also suggests that, as with children, these parameters may be unhelpful in determining iron status in adults in malaria endemic regions. In contrast, hepcidin returns rapidly to baseline, and may be a more informative marker.

Finally in this study we evaluated a possible link between iron status and post-treatment elevation in the liver transaminases, alanine transferase (ALT) and aspartate transferase (AST). Post-treatment elevation in ALT/AST are common in malaria VIS<sup>19,20</sup> and in clinical malaria in non-immune patients.<sup>42</sup> In malaria VIS these transient elevations in liver transaminases have the potential to be misinterpreted as being attributable to the investigational antimalarial, thereby inappropriately halt drug development. Thus, understanding the factors contributing to these post-treatment changes is important for antimalarial drug development. Post-treatment transaminitis in malaria VIS has previously been attributed to inflammation, with peak ALT associated with peak CRP.<sup>20</sup> However, in this study we also found that baseline ferritin strongly predicted day 15 ALT/AST, even after controlling for other contributing factors such as parasitaemia and CRP. We hypothesise that this post-treatment increase in ALT/AST may reflect a hepatic immune response, with this response more exaggerated in those who are more iron replete and thus have a more robust immune response. This latter hypothesis is consistent with a recent study demonstrating

attenuated hepatic immune response and reduced hepatic pathology in *P. chabaudi*-infected transgenic mice carrying a mutation in the transferrin receptor causing decreased cellular iron uptake.<sup>43</sup>

Our study had several limitations. The sample sizes in both the malaria VIS and the Malaysian studies were relatively small, and it is possible that we were underpowered to detect significant correlations. In addition, the strength of many of the observed correlations, including adjusted correlations, was weak to moderate, suggesting that there are likely other factors contributing to the outcome variables evaluated such as the parasitaemia parameters, as would be expected in clinical studies such as these. We used backwards elimination to select and adjust for potential confounders; however, this approach may not have accounted for all confounders. In both the malaria VIS and the Malaysian studies follow up was limited to approximately 4 weeks, and thus we were unable to determine any changes beyond this time. Finally, CRP was not measured in the Malaysian patients, and so we were unable to adjust ferritin for CRP.

In summary, our studies provide a detailed description of the longitudinal changes that occur in iron homeostasis during a malaria infection. We demonstrate that in experimental infection MCV, rather than iron status, is associated with parasitaemia, and demonstrate the very early increase in hepcidin in blood stage malaria infection. Importantly we provide information on the duration of the disturbance in iron homeostasis following recovery from clinical malaria in adults, demonstrating that while hepcidin declines rapidly to the level of healthy controls, ferritin and sTfR both remain above the levels of healthy controls up to 1 month post recovery. Finally, we demonstrate an association between iron status and risk of post-treatment elevations in liver transaminases in malaria VIS, providing insights into the potential mechanisms of this finding, and providing further reassurance that these changes are a common response to treatment of malaria rather than specific drug effect.

#### Contributors

S.D.W., J.S.M., N.M.A. and B.E.B. conceived and designed the study. B.E.B, M.J.G, T.W. and G.S.R. conducted the clinical studies Malaysia. S.D.W., B.E.B., and J.S.M, conducted the malaria volunteer infection studies. K.P., J.S.E.G., A.S.N., and F.M.A. conducted the assays. S.D.W., L.M. and B.E.B. analysed the data. S.D.W. and B.E.B. wrote the paper, with input from L.M., D.M.F., S.C., J.S.M., and N.M.A. All authors approved the final draft of the manuscript. S.D.W. and B.E.B have accessed and verified the data.

#### Data sharing statement

Data will be made available upon request through contact with the corresponding author, with an appropriate data sharing agreement in place.

#### Declaration of interests

None of the authors have conflicts of interests to declare.

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#### Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.ebiom.2024.105189>.

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