Journal of Travel Medicine

International Society of Travel Medicine
Established 1991
Promoting healthy travel worldwide

Journal of Travel Medicine, 2023, 1–9
https://doi.org/10.1093/jtm/taad001
Original Article

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Post-treatment haemolysis is common following oral artemisinin combination therapy of uncomplicated malaria in travellers

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Submitted 11 October 2022; Revised 7 December 2022; Editorial Decision 1 January 2023; Accepted 1 January 2023

Abstract

Background: Artemisinin-based combination therapy (ACT) for the treatment of malaria is highly effective, well tolerated and safe. Episodes of delayed haemolysis occur in up to 57.9% of patients with severe malaria treated with intravenous artesunate, mainly caused by 'pitting' of infected red blood cells in the spleen and the delayed loss of these once-infected RBCs (oiRBCs). Several reports indicate that post-treatment haemolysis (PTH) also occurs in uncomplicated malaria treated with oral ACT, calling for systematic investigation.

Methods: A prospective observational study to identify the incidence of PTH after oral ACT, defined as increased lactate dehydrogenase activity and low haptoglobin level on Day 14 after treatment. Patients were enrolled at two study centres in Germany and Italy. Study visits took place on Days 1, 3, 7, 14 and 28. Laboratory investigations included extended clinical routine laboratory tests, quantitative *Pf*HRP2, anti-RBC antibodies and oiRBCs. The state of semi-immunity to malaria was assessed from childhood and ongoing exposure to *Plasmodium* spp. as per patient history.

Results: A total of 134 patients with uncomplicated malaria and 3-day ACT treatment were recruited. Thirty-seven (37.4%) of 99 evaluable patients with *Pf* and none of 9 patients with non-*Pf* malaria exhibited PTH on d14. Patients with PTH had higher initial parasitaemia, higher oiRBC counts on d3 and a 10-fold decrease in oiRBCs between d7 and d14 compared with patients without PTH. In patients with PTH, loss of haemoglobin was 4-fold greater in non-Africans than in Africans (−1.3 vs −0.3 g/dl). Semi-immune African patients with PTH showed markedly increased erythropoiesis on d14 compared with not semi-immune African and non-African patients with PTH.

Conclusions: PTH is common in patients with uncomplicated malaria and oral ACT. While the observed loss of haemoglobin will often not be clinically relevant, it could aggravate pre-existing anaemia, warranting follow-up examinations in populations at risk.

Key words: Post-treatment haemolysis, malaria, Artemisinin combination therapy, once-infected red blood cells, uncomplicated malaria

Introduction

While malaria cases have seen a steady decline since the early 2000s, recent years have shown stagnating case numbers, and malaria still poses a significant health risk to inhabitants of and travellers to endemic regions.[1–3] Artemisinin-based combination therapy (ACT) is the recommended first-line treatment for uncomplicated malaria in all malaria-endemic regions, and an estimated 3 billion ACT treatments have been procured over the past decade.[4] ACTs rapidly eliminate *Plasmodium* spp. while usually being well tolerated and safe.[5]

Episodes of delayed haemolysis were first described in patients with severe malaria at 2–6 weeks after treatment with intravenous artesunate.[6] Post-treatment haemolysis (PTH) or, more specifically, post-artemisinin delayed haemolysis (PADH) describes an increase of haemolytic activity beginning in the second week after treatment initiation with artemisinin drugs. It is estimated to occur in 15–43% of patients with imported severe malaria in non-endemic areas.[7–10] Depending on the setting and definition, PTH following severe malaria may be even more frequent, particularly in patients without malaria semi-immunity, where it can be observed in up to 57.9% of cases.[10] PTH is generally self-limiting, nevertheless close clinical follow-up is advised, and re-hospitalization and blood transfusions have been reported.[6,8,11] PTH has also been described in patients with severe malaria in malaria-endemic areas, yet seems to be far less frequent.[12,13]

The pathophysiology of PADH in the early post-treatment period has been linked to a process called 'pitting', where by parasites are cleared from infected red blood cells (iRBCs) in the spleen resulting in once-infected RBCs (oiRBC) in the circulation.[14] Patients with higher concentrations of oiRBCs have a higher risk of developing PTH.[14] However, this mechanism cannot explain prolonged haemolysis in the late post-treatment period over several weeks, as observed in some patients with PTH.[6] Drug-dependent immune-haemolysis of the immune-complex type has been suggested as a possible additional mechanism, but apart from single cases, no consistent evidence for immune-mediated haemolysis has been demonstrated in cases with prolonged PTH.[14,15]

There are few reports on delayed haemolysis following oral ACT, and these are mostly in patients with high parasitaemia or severe malaria with oral treatment.[16–18] However, uncomplicated malaria and oral ACT treatment are by far more frequent than severe malaria and intravenous treatment on a global scale.[19,20] Therefore, even a loss of smaller amounts of RBCs due to haemolysis after oral ACT could contribute to the high burden of chronic anaemia in malaria endemic regions. Of note, fatal outcome of an episode of PTH after oral ACT has been reported recently.[21]

We hypothesize that PTH also occurs in a substantial number of patients with uncomplicated malaria treated with oral ACTs. This study included patients with imported uncomplicated malaria treated with oral ACTs to assess features of haemolysis and anaemia. An interim analysis of the first 20 patients of this study has been published before.[22] This report includes the entire study population after reaching the predefined sample size of 130 patients.

Methods

A prospective observational study was conducted at Charité—Universitätsmedizin Berlin (Berlin, Germany), Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) and Ospedale Sacro Cuore—Don Calabria di Negrar (Negrar, Italy). The study protocol was approved by the Ethics Committee of Charité—Universitätsmedizin Berlin (EA1/283/13) and the Comitato Etico per le Province di Verona e Rovigo. The study is registered at the WHO International Clinical Trials Registry Platform (DRKS00007104).[22]

Patient recruitment

All patients with microscopically confirmed, uncomplicated *Plasmodium* spp. infection, seeking medical treatment at one of the study centres, who were willing to attend follow-up visits were included in the study after written informed consent was obtained. Patients with severe malaria (except for isolated uncomplicated hyperparasitaemia) according to WHO criteria were excluded.[23] Patients were either treated with artemether/lumefantrine (ARM/LUF, Riamet, Novartis, Basel, Switzerland) or dihydroartemisinin/piperaquine (DHA/PPQ, Eurartesim, Sigma-Tau, Pomezia, Italy) according to the current guidelines. Patients were excluded if they had received antimalarial treatment (excluding prophylaxis) in the past 12 weeks, were currently taking medication potentially causing haemolysis or had pre-existing conditions that potentially cause haemolysis (e.g. glucose-6-phosphate dehydrogenase deficiency [G6PDD], sickle cell disease [SCD], mechanic heart valve). During hospitalization, study visits were conducted on the day of admission (d0) and on Day 3 of treatment (d3). Follow-up visits after discharge from hospital were performed on d7 (range = d5–9) and d14 (range = d11–17). In case of laboratory evidence of haemolysis on d14, further follow-up visits were conducted on d28 (range = d24–32), and thereafter, as determined by the treating physician. Based on their ancestry, patients were grouped in patients of African descent and patients of non-African descent. Patients were categorized as being 'semi-immune' to malaria if they (i) were born and passed childhood in a malaria

endemic region according to WHO[19] and (ii) had ongoing regular contact with *Plasmodium* spp., defined as at least two episodes of malaria within the last 10 years. Others were considered as not 'semi-immune'.

Laboratory analyses

Standard haematological (differential blood count), biochemical (haptoglobin, lactate dehydrogenase [LDH], C-reactive protein [CRP], potassium and sodium levels, renal and liver function tests and screening for G6PDD) and parasitological (thick and thin blood smears) examinations were performed by accredited laboratories at Charité—Universitätsmedizin Berlin and IRCCS. Absolute parasitemia per μl was calculated as (parasitemia [%] * erythrocytes [per nl) * 1.000.000]/100); reticulocyte production index (RPI) was calculated as (reticulocytes [%] * haematocrit [l/l])/(0.45 * haemoglobin [Hb]-dependent correction factor) as described elsewhere.[24] Anaemia was defined as Hb <13 g/dl in males and Hb <12 g/dl in females according to the definitions of anaemia of WHO.[25] The direct antiglobulin test (DAT) was performed on fresh EDTA samples using commercial anti-IgG (Bio-Rad, Dreieich, Germany), anti-IgA (Dako/Agilent, Hamburg, Germany), anti-IgM (Bio-Rad, Dreieich, Germany) and anti-C3d (Dako, Hamburg, Germany). In all patients with positive DAT, an eluate from the patients' RBCs was prepared using the acid technique (BAG, Lich, Germany). RBC antibodies were investigated with standard techniques using gel cards and commercially available test RBCs (Bio-Rad, Cressier sur Morat, Switzerland). Antibody screening test was routinely performed in the indirect antiglobulin test (IAT) with untreated RBCs as well as with papain-treated RBCs using neutral cards in all patients.

Quantitative measurement of *Plasmodium falciparum* histidine-rich protein 2 (*Pf*HRP2) was performed on serum samples from d0 by double sandwich ELISA as described before.[26–28] In short, anti-*Pf*HRP2 IgM (MBS563506) at 1 μg/ml and HRP-conjugated anti-*Pf*HRP2 IgG (MBS563505, both MyBioSource Inc, San Diego, CA, USA) at 0.2 μg/ml were used as primary and secondary antibodies, respectively. Samples were pre-diluted in PBST depending on parasitaemia and measured in three dilution series (1:2) along with a *Pf*HRP2 standard dilution series starting at 10 ng/ml (kindly provided by DJ Sullivan, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD). Extinction measurement was performed at 450 nm with FilterMax F5 (Molecular Devices, LLC, San Jose, CA, USA).

Immunofluorescence microscopy of oiRBCs

Presence of oiRBCs in peripheral blood was measured by fluorescence microscopy on Days 3, 7, 14 and 28 as previously described.[14,29] In short, thin blood smears were air-dried, fixed with methanol, frozen and stored at −20°C for batch analysis. After thawing, cells were incubated with anti-RESA IgG (clone 28/2, Walter and Elisabeth Hall Institute Antibody Facility, Melbourne, Australia) at 5 μg/ml and were stained with Alexa Fluor 488 Goat Anti-Mouse IgG antibody (AB_2534088) and Hoechst 33342 (both Thermo Fisher Scientific, Waltham, MA, USA) at 0.5 μg/ml. Fluorescence microscopy was performed on Keyence BZ-X700 fluorescence microscope. Frequency of oiRBC (oiRBC %) was calculated as the mean of RESA-positive RBCs/total number of erythrocytes per 100 high-power fields; absolute oiRBC count (oiRBC/nl) was calculated as oiRBC (%) * (erythrocytes/nl) * 100. The pitting rate was calculated as (oiRBC d3 [%]/parasitaemia d0 [%]) * 100.

Study outcomes and statistical analysis

The primary objective of this study was to identify the proportion of patients with PTH after oral ACT. The primary endpoint PTH was defined as LDH levels above the age-dependent upper normal and low haptoglobin levels (<0.3 g/l) on d14 after the first dose of treatment. Secondary objectives were to assess possible risk factors for PTH (age, descent, sex, previous exposure to *Plasmodium* spp., initial parasitaemia and oiRBC after treatment) and to analyse the course of anaemia and compensation for loss of oiRBC through erythropoiesis. Data were pseudonymised, entered into password encrypted electronic case-report forms, transferred into a purpose-built data base and checked by two investigators for plausibility before analysis.

Student's *t*-test for normally distributed data and Mann–Whitney U test for non-normally distributed data as determined by Shapiro–Wilk test were used for between-group comparison of continuous data and Fisher's exact test for binary data at a two-sided significance level of $\alpha = 0.05$. Adjustment for parasitaemia was performed using a logistic regression model, as indicated. Data are presented as mean and 95% confidence interval (CI) unless otherwise specified. Statistical analysis was performed using JMP Pro version 14 (SAS Institute Inc, Cary, NC, USA), and graphs were plotted using Prism 8 (GraphPad, San Diego, CA, USA). The sample size was calculated to detect a 20% incidence of PTH with a 95% CI, ±7.5% precision and 15% lost to follow-up, resulting in a sample size of 130 patients.

Results

From May 2014 to December 2018, a total of 134 patients with uncomplicated malaria and 3-day ACT treatment were recruited at the two study centres, 8 in Negrar and 126 in Berlin. These included 123 cases of *Plasmodium falciparum* malaria, 5 cases each of *Plasmodium ovale* and *Plasmodium vivax* and 1 case of *Plasmodium malariae*. Baseline characteristics of patients are shown in Table 1. There was no difference in the baseline characteristics between the two study centres (data not shown). All patients showed rapid clinical improvement and complete parasite clearance within 72 hours after initiation of treatment. There were no cases of treatment failure. Five patients were excluded from the study: four due to G6PDD diagnosed during the study and one due to concomitant SCD, which had not been indicated by the patient at inclusion. Further 14 patients were lost to follow-up before reaching the primary endpoint at d14. Of the remaining 115 patients, complete datasets for Hb, LDH and haptoglobin on d14 were evaluable for 99 cases with *P. falciparum*, 1 with *P. malariae*, 5 with *P. ovale* and 3 with *P. vivax*.

The criteria of PTH on d14 were met by 37 of 99 (37.4%) patients with *P. falciparum* malaria. None of the nine patients

Table 1. Baseline characteristics of patients with uncomplicated malaria and ACT treatment

Characteristic	All ACT, *n* = 134	*Pf* malaria, *n* = 123	Non-*Pf* malaria, *n* = 11
Age in y	38 [36–40]	38 [36–40]	40 [31–50]
Children, *n*/*N* (%)	4/134 (3.0%)	4/123 (3.3%)	0/11 (0.0%)
African descent, *n*/*N* (%)	90/134 (67.2%)	83/123 (67.5%)	7/11 (63.6%)
Male sex, *n*/*N* (%)	95/134 (70.9%)	88/123 (71.5%)	7/11 (63.6%)
Treatment with ARM/LUM, *n*/*N* (%)	31/134 (23.1%)	26/123 (21.1%)	5/11 (45.5%)
Treatment with DHA/PPQ, *n*/*N* (%)	103/134 (76.9%)	97/123 (78.9%)	6/11 (54.5%)
Parasitaemia d0 in %, median [IQR]	0.3 [0.1–1.0]	0.5 [0.1–1.0]	0.1 [0.09–0.4]
Parasitaemia d0 per μl, median [IQR]	16 400 [4875–53 100]	19 500 [5100–54 000]	5000 [3330–16 800]
Hb d0 in g/dl	13.5 [13.2–13.8]	13.5 [13.1–13.8]	13.4 [12.0–14.7]
LDH d0 in U/l	345 [316–375], *n* = 118	346 [314–378], *n* = 108	338 [280–396], *n* = 10
Thrombocytes d0 per nl	112 [102–123]	114 [103–125]	96 [69–123]
CRP d0 in mg/dl	100.9 [83.1–118.7]	74 [41–131]	144.3 [0.7–288.0]

Data are presented as mean and 95% CI unless otherwise specified.

Table 2. Baseline characteristics in *P. falciparum* Malaria patients with and without PTH on d14

Characteristic	No PTH, *n* = 62	PTH, *n* = 37	*P* value	OR (95% CI)
Age in y	37 [33–41]	39 [36–43]	0.33	
Children	4/62 (6.5%)	0/37 (0%)	0.29	
African descent, *n*/*N* (%)	38/62 (61.3%)	23/37 (62.2%)	1.0	OR 1.0 (0.4–2.4)
Male sex, *n*/*N* (%)	38/62 (61.3%)	31/37 (83.8%)	0.01	OR 3.3 (1.2–9.0)
			0.06	aOR 2.7 (1.0–7.7)
Treatment with ARM/LUM, *n*/*N* (%)	13/62 (21.0%)	7/37 (18.9%)	1.0	OR 0.9 (0.3 to 2.5)
Treatment with DHA/PPQ, *n*/*N* (%)	49/62 (79.0%)	30/37 (81.1%)	n/a	n/a
Parasitaemia d0 in %, median [IQR]	0.3 [0.1–0.8]	1.0 [0.3–2.0]	<0.001	
Parasitaemia d0 per μl, median [IQR]	12 450 [4680–40 200]	46 000 [11 100–102 000]	<0.001	
*Pf*HRP2 d0 in ng/ml, median [IQR], n = 44	10 [1–38], *n* = 25	121 [11–204], *n* = 19	0.008	
Hb d0 in g/dl	13.5 [13.1–13.9]	14.0 [13.4–14.5]	0.18	
Anaemia d0, n/N (%)	18 (29.0%)	7 (18.9%)	0.34	
LDH d0 in U/l	307 [277–337]	372 [334–409]	0.009	
Thrombocytes d0 per nl	132 [117–147]	85 [65–104]	<0.001	

Data are presented as mean and 95% CI unless otherwise specified. Anaemia was defined as Hb <13 g/dl in males and Hb <12 g/dl in females according to the definitions of anaemia of WHO. OR, odds ratio; aOR, adjusted odds ratio after correcting for initial parasitaemia (logistic regression model).

with non-*P. falciparum* malaria showed signs of PTH. All further analyses are therefore restricted to patients with *P. falciparum* malaria.

Risk factors for PTH

All cases of malaria were acquired in Africa (Supplementary Figure 1). Patients with PTH had higher levels of parasitaemia, *Pf*HRP2, LDH and CRP and lower thrombocytes at d0. Male sex was associated with occurrence of PTH in univariate analysis, however, this association was not statistically significant when corrected for parasitaemia ($P = 0.06$, logistic regression). Treatment with either ARM/LUM or DHA/PPQ did not affect the occurrence of PTH (Table 2).

Haematologic parameters during follow-up

Patients with PTH showed a median decrease in Hb levels of −0.7 g/dl (95% CI = −1.0 to −0.3) in the post-treatment period (d3–14) compared with −0.2 g/dl (95% CI = −0.5 to 0.02) in patients without PTH ($P = 0.048$, Figure 1A). Overall (d0–14), the loss of Hb was −1.7 g/dl (95% CI = −2.1 to −1.4) and − 0.9 g/dl (95% CI = −1.1 to −0.6) in patients with and without PTH, respectively ($P < 0.001$, Supplementary Table 1). LDH levels were higher in patients with PTH than in those without PTH throughout the study period d0–14 (Figure 1B, Supplementary Table 1). An increase in reticulocyte production after treatment could be seen in both groups, yet RPI on d14 was significantly higher in patients with PTH than in patients without PTH (2.2, 95% CI = 2.0–2.4 vs 1.8, 95% CI = 1.6–2.0, respectively; $P = 0.003$). In 11 (30%) out of all 37 patients with PTH, the loss of Hb due to PTH was entirely compensated through erythropoiesis and no decrease in Hb in the post-treatment period was noted. There was no difference in absolute Hb levels on d14 between both groups (median = 12.2 and 12.6 g/dl in patients with and without PTH, respectively, $P = 0.18$, Figure 1B, upper panel, Supplementary Table 1). However, while no differences were seen on d0, d3 or d7, anaemia on d14 was more prevalent in patients with PTH than in patients without PTH (27/37, 73.0% vs 29/62, 46.8%; $P = 0.01$; Table 2, Supplementary Table 1).

Exemplary courses of laboratory values over time for patients with uncompensated and compensated PTH as well as without PTH are shown in Figure 1C. Ten (27%) of 37 patients with PTH had follow up visits on d28. Nine of them showed persistently

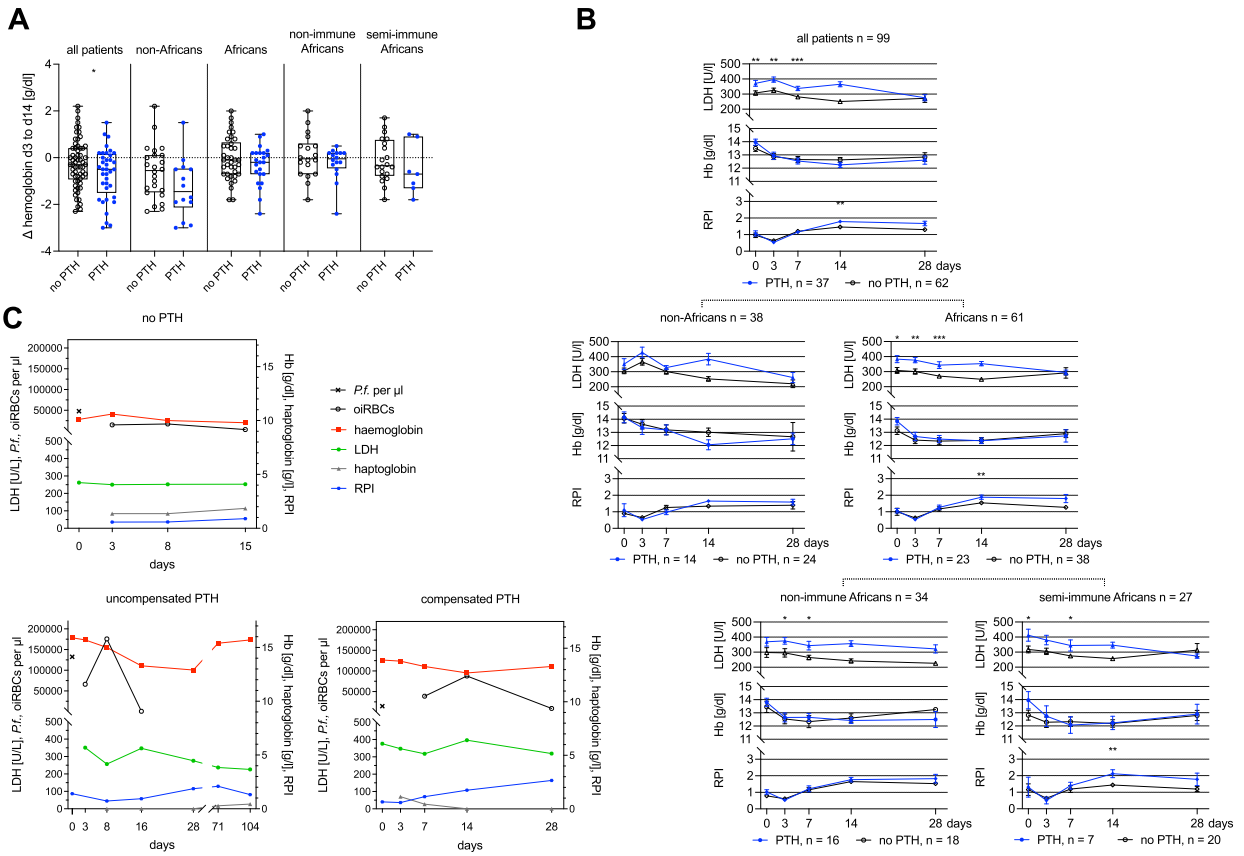

Figure 1. Laboratory data over time for patients with and without PTH; (A) changes in Hb levels during the post-treatment phase (d3–14) for patients with and without PTH and subgroups; boxes indicate median and interquartile range; whiskers indicate min to max values; points are individual patient data; (B) RPI, Hb and LDH were measured on d0, d3 and d7 (±2 days), d14 (±3 days) and d28 (±4 days) in all patients ($n = 99$); haematologic parameters for patient subgroups (non-African and African patients, non-immune and semi-immune African patients) are shown; mean and standard error of the mean are depicted; closed blue symbols, patients with PTH; open black symbols, patients without PTH; (C) exemplary laboratory values over time in three representative patients without, with compensated and with uncompensated PTH; *Pf*, *Plasmodium falciparum*; statistical significance was investigated by *t*-test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

low haptoglobin levels and seven exhibited elevated LDH levels. Further follow-up showed that three patients still had low haptoglobin and elevated LDH on d35 and one continued to have low haptoglobin and elevated LDH levels until d49. By d60, haptoglobin and LDH had returned to normal levels in all three patients. One patient with normalized LDH on d28 had low haptoglobin until d71, which returned to normal on d104 (Figure 1C).

Influence of descent and previous exposure to *Plasmodium* spp.

Based on the results of the first interim analysis,[22] where a compensation of Hb loss in all patients of African descent with PTH but in none of the non-Africans was observed, we specifically explored whether descent or anamnestic criteria of 'semi-immunity' were associated with occurrence of PTH, with decrease of Hb levels or with compensation of Hb loss by increased erythropoiesis. Patients were classified into groups of African descent (61/99, 62%) or non-African descent (38/99, 38%). Patients of African descent were further classified into patients without (34/61, 56%) and with malaria 'semi-immunity' (27/61, 44%) as defined above.

At presentation, patients of non-African descent had a higher mean Hb than those of African descent (14.2 vs 13.4 g/dl, $P = 0.02$) as well as lower parasitaemia (10 800 vs 34 400 per μl, $P = 0.02$) and lower *Pf*HRP2-levels (4 vs 34 ng/ml, $n = 44$, $P = 0.04$, Supplementary Table 2). Africans without 'semi-immunity' had higher levels of initial parasitaemia than Africans with 'semi-immunity' (53 000 vs 14 700 per μl, $P = 0.03$), with no difference in *Pf*HRP2-levels (Supplementary Table 3).

The frequency of PTH did not differ significantly between patients of African and non-African descent (Supplementary Table 2). Within the group of Africans, frequency of PTH was 25.9% (7/27) in patients with 'semi-immunity' compared with 47.1% (16/34) in patients without 'semi-immunity'; this difference was not statistically significant ($P = 0.11$, Supplementary Table 3).

Among patients with PTH, non-Africans had a significantly greater loss of Hb in the post-treatment phase than Africans (−1.3 vs −0.3 g/dl, $P < 0.001$, Figure 1A) despite non-Africans with PTH presenting with lower initial parasitaemia than Africans with PTH (Supplementary Table 2). Interestingly, while the prevalence of anaemia did not differ in African patients with and without PTH during follow-up, anaemia on d14 in non-Africans was significantly more prevalent in patients with PTH

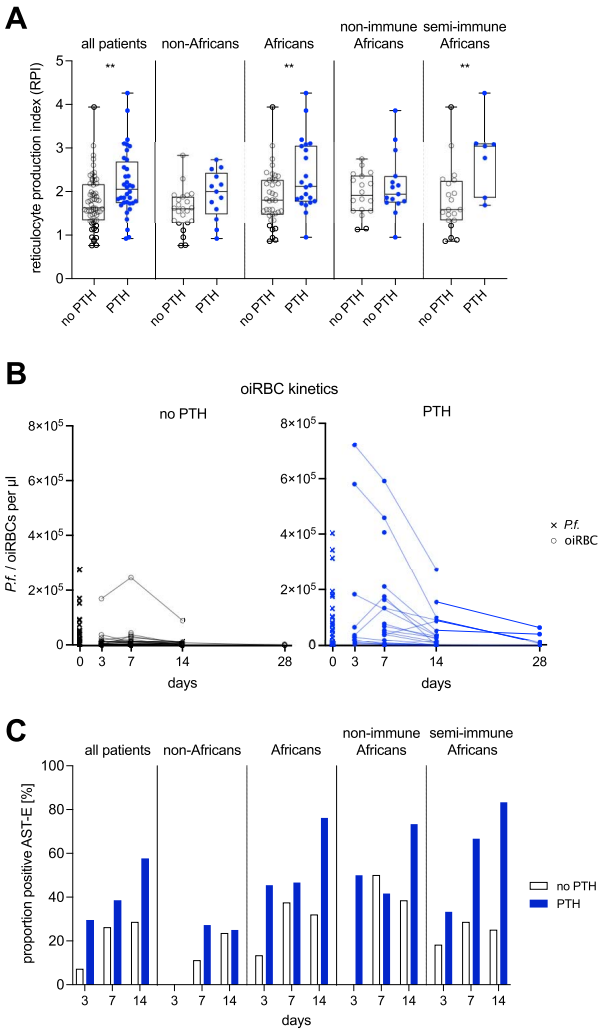

Figure 2. RPI, occurrence of oiRBCs and antigen screening test differ in patients with and without PTH; (A) RPI on Day 14 in patients with and without PTH and in subgroups; (B) initial parasitaemia (×) and oiRBCs (○) during follow-up in patients without and with PTH; parasitaemia was determined by microscopy, oiRBCs were measured per µl of blood by fluorescence microscopy on thin blood smears; (C) occurrence of positive antibody screening test with papain-treated RBCs (AST-E) in patients with and without PTH in different subgroups; in (A) and (C), patients were classified as of African or non-African descent and semi-immune or non-immune according to their ancestry and history of exposure to *Plasmodium* spp., respectively; blue symbols, PTH; black symbols, no PTH; crosses, parasites d0 per µl

than in those without (Supplementary Table 2). In line with these findings, in non-African patients, the RPI at d14 did not differ significantly between patients with and without PTH (1.9 vs 1.6, $P = 0.06$), whereas in African patients, a significantly increased RPI was seen in patients with PTH compared with those without PTH (2.4 vs 1.9, P = 0.01) (Figures 1B and 2A). This increase in RPI in patients with vs without PTH was particularly pronounced in the subgroup of semi-immune Africans (2.8 vs 1.8, $P = 0.006$) but was not observed in the subgroup of non-immune Africans (2.2 vs 2.0, $P = 0.35$) (Figures 1B and 2A). Notably, again absolute Hb levels on d14 did not differ between patients with and without PTH in both subgroups.

Once infected RBCs

Data on the presence of oiRBCs were available for 54 patients, including 23 with and 31 without PTH. Patients with PTH had a significantly higher pitting rate (119.0 vs 40.4%, $P = 0.02$, $n = 33$) and significantly higher levels of oiRBCs throughout the post-treatment phase than patients without PTH (Supplementary Table 4, Figure 2B). In patients with PTH, decrease in oiRBCs levels between d7 and d14 was 10-fold that of patients without PTH (−33.29 vs −3.11 per µl, $P = 0.006$, $n = 41$). OiRBC frequencies and kinetics did not differ significantly between patients receiving ARM/LUM or DHA/PPQ (data not shown). There was no difference in the presence of oiRBC between African and non-African patients or those with or without semi-immunity (Supplementary Table 5).

During prolonged follow-up, oiRBCs were found in two patients with persistent haemolysis on Days 35 and 49 as well as in one patient without persistent haemolysis on Day 35. In two other patients without persistent haemolysis, no oiRBCs could be detected during follow-up on Days 49 and 70.

Detection of anti-RBC autoantibodies

Immuno-haematological data were available for 75 patients (Supplementary Table 6). DAT was positive on d14 in 4 (12.1%) of 33 patients with PTH and 3 (7.1%) of 42 patients without PTH ($P = 0.69$). All patients with positive DAT were of African descent. IAT was positive on d14 in two African patients, one with PTH and one without PTH (data not shown).

In the antibody screening test with enzyme-treated erythrocytes (AST-E) on d14, antibodies were significantly more frequent in patients with PTH than in patients without PTH (19/33, 57.6% vs 12/42, 28.6%, respectively; $P = 0.02$). In subgroup analysis, this higher prevalence of positive AST-E in patients with PTH was only detectable in patients of African descent (16/21, 76.2% vs 8/25, 32.0%, $P = 0.004$) but not in patients of non-African descent (3/12, 25.0% vs 4/17, 23.5%, $P = 1.0$). There was no difference with regard to AST-E between Africans with and without semi-immunity. In general, the proportion of patients with positive AST-E increased over time after infection (Figure 2C).

Discussion

Episodes of delayed haemolysis following parenteral artemisinin treatment of severe malaria have been reported over the past 10 years,[6,7,10,12,13] and artemisinin-dependent pitting, i.e. splenic removal of parasites from erythrocytes, has been identified as a relevant underlying pathophysiological mechanism.[14] Occasional reports of delayed haemolysis following oral artemisinin treatment[16–18,21] called for systematic, prospective investigation of the phenomenon among patients treated for uncomplicated malaria with oral ACT.

More than one-third of patients with uncomplicated malaria met the criteria of PTH on Day 14 after the initiation of treatment in our study. Previously described risk factors for PTH in severe malaria,[7,14] such as high parasitaemia and increased *Pf*HRP2, were confirmed in this study population with uncomplicated

malaria. We also found comparatively higher levels of oiRBC after treatment in patients with PTH than in those without, with a particularly pronounced (10-fold) decrease of oiRBC between Days 7 and 14 in patients with PTH. Of note, oiRBCs could be found until d49 in two patients with PTH, and haemolysis could be observed up to 10 weeks after treatment in some patients, meriting further investigations of these extended haemolytic episodes.

A very sensitive definition of PTH was applied in this study, aiming to assess even mild and asymptomatic late haemolytic episodes. Accordingly, the mean decrease in Hb of −0.7 g/dl during the post-treatment period in PTH patients was subtle. As reported before, patients with PTH showed a tendency towards higher initial Hb levels before treatment initiation.[14,22,30,31] Thus, the absolute Hb level at d14 was not significantly different in patients with and without PTH. While no differences were noted on d0, d3 or d7, anaemia on d14 was more frequent in patients with PTH than in those without. However, this effect was caused mainly by the subgroup of non-African patients.

Three patients with PTH, all non-African, showed a loss of Hb between 2.5 and 3.0 g/dl during follow up (d3–14). While clinically insignificant in these otherwise healthy individuals, such a loss in Hb may have a more severe impact on patients with pre-existing anaemia or with chronic conditions like heart failure, among others. In general, our study provides no indication that ACT should not be used in certain patients at risk for anaemia; extended follow-up may, however, be advisable in this patient group.

In the interim analysis of this study,[22] we had observed differences in the compensation of PTH through increased erythropoiesis in patients of African vs European descent: loss of Hb had been compensated in all patients with PTH of African but not of European descent. The larger cohort now allowed for a more detailed assessment of this observation. In line with the interim analysis, we observed a four times greater mean decrease of Hb in PTH patients of non-African descent than in those of African descent (−1.3 vs -0.3 g/dl). Interestingly, the average loss in Hb observed in non-African patients with PTH was comparable to that observed in patients with PTH after severe malaria and parenteral artesunate treatment in a larger French study.[30]

The greater Hb decrease in non-African patients in our study could not be attributed to higher parasite burden, as these patients had lower parasite counts and *Pf*HRP2 levels compared with Africans. However, our data show consistently that Africans included in this study had a higher potential of compensating, through increased erythropoiesis, the loss of Hb following PTH than non-African patients, which was particularly true for African patients with persistent semi-immunity to malaria. One possible explanation for this finding could be an accelerated clearance of iRBCs in the spleen in patients with semi-immunity.[32] Intriguingly, semi-immune Africans with PTH in our study exhibited higher median levels of *Pf*HRP2 but lower median initial parasite levels than non-immune Africans with PTH, possibly supporting this hypothesis. This early loss of iRBCs is known to be accompanied by a substantial loss of uninfected RBCs[31] before the initiation of treatment and may thus explain the higher activation of reticulocyte production in the semi-immune. A set of genetic and also acquired factors like a reduced susceptibility to the suppression of erythropoiesis by tumour necrosis factor-alpha or malaria pigment hemozoin[33] may further contribute to these observations and should be investigated in further studies.

Remarkably, none of the patients with non-falciparum malaria showed signs of PTH. Whether this can be explained by the small number of non-falciparum patients in this study or rather by specific characteristics of non-falciparum malaria, such as lower parasitaemia or differences in parasite-RBC interaction, requires further future investigation.

Autoantibodies against RBCs have lately been discussed as possible contributors to PTH,[15] and a recent review reported positive DAT in nearly half (17/39) of patients with malaria and subsequent PTH.[34] Artesunate-induced haemolysis with positive IAT with papain-treated RBCs has been described as late as 12 months following treatment.[35] Following systematic prospective immunohaematological testing in our study, low and comparable frequencies of DAT and IAT in both patients with and without PTH on Day 14 were found. The most prominent difference between patients with and without PTH was seen in AST-E results in the subgroup of African patients with semi-immunity, among whom 83% (5/6) vs 25% (3/12) had positive results on Day 14 with and without PTH, respectively. The role of potential immune-mediated haemolysis in PTH is illustrated, e.g. by Fernandez-Arias *et al*.[36] They found high levels of anti-phosphatidylserine (PS) antibodies in patients with PTH (nadir 2–3 weeks after treatment), but not early haemolysis (i.e. 3–7 days post-treatment), and antibody levels correlating with progression of anaemia.[36] In a malaria mouse model, these anti-PS antibodies were found to bind to uninfected RBCs promoting opsonization and phagocytosis, thus effectively contributing to Hb loss in malaria.[36]

Limitations

For logistical reasons, it was not possible to recruit more patients from the second study centre, resulting in an imbalance of representation of centre contributions in the dataset. However, no significant demographic differences were found between the two centres. Classification of patients as African or non-African descent and particularly as semi-immune relied on anamnestic and not on universally defined criteria, limiting the accuracy of patient classification in the subgroup analyses carried out. Data on *Pf*HRP2, oiRBCs and immunohaematology were not available for all patients, limiting the sample size in subgroup analyses.

Conclusion

This study provides a comprehensive analysis of PTH in patients with uncomplicated malaria in travellers treated with oral ACTs. While PTH was clinically mild in the patients observed in this study, it caused a significant loss of Hb in patients of non-African descent in the post-treatment phase, up to 2.5–3 g/dl in three patients. The observed loss of Hb is usually not clinically significant for the vast majority of patients but may be of concern in patients with chronic disease in whom even mild anaemia poses a clinical risk. Patients of African descent exhibited a comparatively smaller loss of Hb and particularly semi-immune

African patients were able to compensate for loss of Hb caused by PTH through increased erythropoiesis. The role of oiRBC and pitting for the pathophysiology of PTH was confirmed in this group of patients with uncomplicated malaria, but further research is needed to elucidate the mechanisms of PTH not attributable to pitting.

Supplementary Data

Supplementary data are available at *JTM* online.

Acknowledgements

We thank the physicians of the Department of Infectious Diseases and Respiratory Medicine for their help in collecting biosamples, and Cüneyt Acar, Ute Gläser, Claudia Conrad, Doris Frey, Philipp Georg and Sarah Volkers as well as the lab-technicians of the Institute of Transfusion Medicine at Charité for their advice and assistance in sample preparation. We would also like to thank Salo Tober-Lau, Friederike Münn and Marcel Schmude for their support in managing data and preparing this manuscript.

Funding

This research was funded in part by Deutsche Forschungs Gemeinschaft as part of IRTG 2290 Molecular Interactions in Malaria.

Conflict of interest

None declared.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

Authors' contributions

F.K. and T.Z. initiated the study and acquired funding and conceptualized and supervised the study. F.K., P.T.-L., T.L., L.B., J.K., A.A., F.G.G., L.M., M.S.S., K.M.H., F.P., N.M., M.S., A.M., M.W., L.E.S. and T.Z. acquired samples and clinical data. P.T.-L. and B.M. conducted the experiments. F.K. and P.T.-L. analysed the data and wrote the original manuscript draft, and P.T.-L. and L.B. visualized the results. All authors reviewed the manuscript and agreed to publication.

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