Intravascular haemolysis, oxidative stress and acute kidney injury in severe falciparum malaria



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Abstract

Acute kidney injury (AKI) complicating severe falciparum malaria is an independent predictor of death in children and adults, however the magnitude of the problem in both populations is unclear due to divergent AKI definitions. Late recognition of this serious complication results in a mortality of up to 75% without renal replacement therapy, which is frequently unavailable. The aim of this thesis was to characterise malaria-associated AKI along the spectrum of this syndrome, evaluate the pathophysiology and assess the role of paracetamol as a renoprotective adjunctive therapy for the management of severe and moderately severe falciparum malaria.

The main findings of the studies described here include: (1) The incidence of malaria-associated AKI is underrecognised as mild AKI is underreported. AKI defined by the Kidney Disease: Improving Global Outcomes (KDIGO) consensus definition identifies mild AKI and is strongly associated with mortality. (2) Sequestered parasite burden, immune activation, cell-free haemoglobin and lipid peroxidation contribute to the pathophysiology of AKI in severe malaria. The proposed mechanism of kidney injury mediated by cell-free haemoglobin is oxidative damage by ferryl (Fe^{4+})-haemoglobin, which can be reduced by paracetamol. (3) Paracetamol tablets administered at the maximum recommended adult dose (4 g/day) results in sub-therapeutic steady state concentrations for antipyresis in severe and moderately severe malaria in adults, and simulations suggest a loading dose might be indicated. (4) In a randomised controlled trial, it was shown that paracetamol reduces kidney dysfunction and the odds of developing AKI in severe and moderately severe malaria in adults. The protective effect was particularly prominent in patients with high plasma concentrations of cell-free haemoglobin resulting from pronounced intravascular haemolysis.

In conclusion, this thesis shows that AKI defined by the harmonized KDIGO classification system is associated with mortality in falciparum malaria. Multiple mechanisms contribute to the pathophysiology of AKI in malaria and targeted preventative treatment with paracetamol reduces kidney dysfunction associated with high cell-free haemoglobin. The definition of AKI in the WHO malaria guidelines should be reconsidered. A larger trial of the use of paracetamol to reduce kidney dysfunction in severe malaria is warranted.

To my husband, David, and family, Don, Betty, Cam, Geoff and Sue, for their unconditional love and support

যা আমাদের তা আমাদের কাছেই আসবে যদি তা গ্রহণ করার ক্ষমতা আমরা তৈরী করতে পারি।

Everything comes to us that belongs to us if we create the capacity
to receive it.

- Rabindranath Tagore
Nobel Laureate in Literature 1913

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List of abbreviations

ACT	Artemisinin-based combination therapy
AKI	Acute kidney injury
AKIN	Acute kidney injury network
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
ATI	Acute tubular injury (formerly acute tubular necrosis, or ATN)
AUC	Area under the curve
BUN	Blood urea nitrogen
BWF	Blackwater fever
C_{MAX}	Maximum plasma concentration
CFH	Cell-free haemoglobin
CI	Confidence intervals
CKD	Chronic kidney disease
CMCH	Chittagong Medical College Hospital
COX	Cyclooxygenase
CrCl	Creatinine clearance
CRF	Case report form
CTSG	Clinical trials support group
CVP	Central venous pressure
CVVHF	Continuous veno-venous haemofiltration
F2-IsoPs	F2-isoprostanes
FeNa	Fractional excretion of sodium
FST	Fluorescent spot test
G6PD	Glucose-6-phosphate dehydrogenase
GCP	Good clinical practice

GCS	Glasgow coma score
GFR	Glomerular filtration rate
HCT	Haematocrit
HIV	Human immunodeficiency virus
ICH GCP	International conference on harmonization good clinical practice
ICU	Intensive care unit
IHD	Intermittent haemodialysis
IM	Intramuscular drug administration
INR	International normalisation ratio
IQR	Interquartile range
IsoFs	Isofurans
IV	Intravenous drug administration
KDIGO	Kidney disease: Improving global outcomes
LDH	Lactate dehydrogenase
MDRD	Modification of diet in renal disease
MODS	Multi-organ dysfunction syndrome
MORU	Mahidol Oxford Research Unit
NG	Nasogastric
NGAL	Neutrophil gelatinase-associated lipocalin
NO	Nitric oxide
OR	Odds ratio
Pa	Pascals
PCR	Polymerase chain reaction
PD	Peritoneal dialysis
***Pf*HRP2**	*Plasmodium falciparum* histidine rich protein 2
PO	*Per os* drug administration

POX	Peroxidase
PR	Per rectum drug administration
PRBC	Parasitised red blood cells
RBC	Red blood cell
RCD	Red cell deformability
RCT	Randomised controlled trial
RDT	Rapid diagnostic test
RIFLE	Risk, injury, failure, loss and end stage renal disease
ROC	Receiver operating characteristic
RRT	Renal replacement therapy
RTA	Renal tubular acidosis
SAE	Serious adverse event
SBP	Systolic blood pressure
SM	Severe malaria
SS	Shear stress
suPAR	Soluble urokinase-type plasminogen-activator receptor
$t_{1/2}$	Elimination half-life
T_{MAX}	Time to reach the maximum concentration
TMI	Trophozoite maturation inhibition
UM	Uncomplicated malaria
uNGAL	Urine neutrophil gelatinase-associated lipocalin
uNGAL/Ucr	Urine neutrophil gelatinase-associated lipocalin corrected for urine creatinine
Vd	Volume of distribution
WHO	World Health Organization

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Author's contributions

The author's contributions to the original research contained in this thesis includes performing: (1) patient recruitment from 2007 to 2008, and 2010 to 2014, (2) characterisation and analysis of malaria-associated AKI, (3) conception and design, sample collection, performing qualitative G6PD assessment, and interpretation of data for the G6PD characterisation, (4) sample collection, analysis and interpretation of data for the malaria-associated AKI pathophysiology studies, (5) conception, sample acquisition, and interpretation of data for the oral paracetamol pharmacokinetic assessments, (6) conception, design, sample collection and interpretation of data for the *in vitro* paracetamol-artesunate interaction study, and (7) protocol and case report form development, ethics submissions, field site coordinator and team leader, patient enrolment and management, sample collection and processing, database construction and data analyses for the randomised controlled trial of oral paracetamol in severe and moderately severe falciparum malaria. Katherine A. Plewes was not involved in performing: (1) patient recruitment prior to 2007, (2) molecular analysis for G6PD genotyping, (3) quantification assays for *Pf*HRP2, suPAR, NGAL, cell-free haemoglobin, F2-isoprostanes, isofurans or paracetamol concentration determination, (4) design or execution of the 2001 paracetamol crossover randomised controlled trial, (5) non-compartmental, population pharmacokinetic and pharmacodynamics analyses, (6) *in vitro* drug susceptibility assays.

1.0 Introduction

1.1 Aim

The primary aim of this thesis was to study the pathophysiology of acute kidney injury (AKI) in *Plasmodium falciparum* malaria and assess the role of paracetamol as a renoprotective adjunctive therapy in the management of severe falciparum malaria.

The specific objectives were to study:

- Clinical aspects of haemolysis and AKI in adults with severe falciparum malaria (chapter 3)
- The contributions of microvascular obstruction, via sequestration and reduced red cell deformability, and immune activation to the pathophysiology of AKI in Bangladeshi adults with severe falciparum malaria (chapter 4)
- The contribution of cell-free haemoglobin and oxidative stress to the pathophysiology of AKI in Bangladeshi adults with severe falciparum malaria (chapter 4)
- The pharmacokinetics and pharmacodynamics of intramuscular and oral paracetamol in uncomplicated falciparum malaria (chapter 5)
- The pharmacokinetics of oral paracetamol in severe and moderately severe falciparum malaria (chapter 5)
- The *in vitro* drug interaction between artesunate and paracetamol (chapter 5)
- The potential renoprotective effect of paracetamol in severe and moderately severe falciparum malaria using a randomised controlled trial (chapter 6)

1.2 Malaria

1.2.1 Distribution and Epidemiology

The World Health Organization (WHO) classifies malaria into asymptomatic parasitaemia, uncomplicated or potentially fatal severe disease based on clinical and epidemiological criteria [1]. While much of the global burden of severe malaria (SM) is caused by *Plasmodium falciparum,* infection with both *P. vivax* and *P. knowlesi*, although less common, can result in severe and fatal disease [2-5].

There are problems with accurately determining the incidence and distribution of disease for a variety of reasons, related to difficulties in standardised diagnosis and variability in access to health care facilities in malaria endemic countries. Considering these reporting limitations, the global annual incidence of SM is estimated to be 2 million cases with approximately 584,000 deaths (Figure 1.2.1) [6]. While progress has been made in reducing malaria mortality, there is concern that the progress may be undone by the emergence of artemisinin and partner drug resistance in the greater Mekong region.

It is estimated that 97% of deaths due to severe falciparum malaria occur in children under 5 years old living in sub-Saharan Africa [7]. The transmission of malaria in this region is intense and stable, which results in partial immunity developing in older children and adults, whereas younger children develop severe and often fatal disease (Figure 1.2.1). In regions with low, seasonal, and unstable transmission, SM affects both adults and children

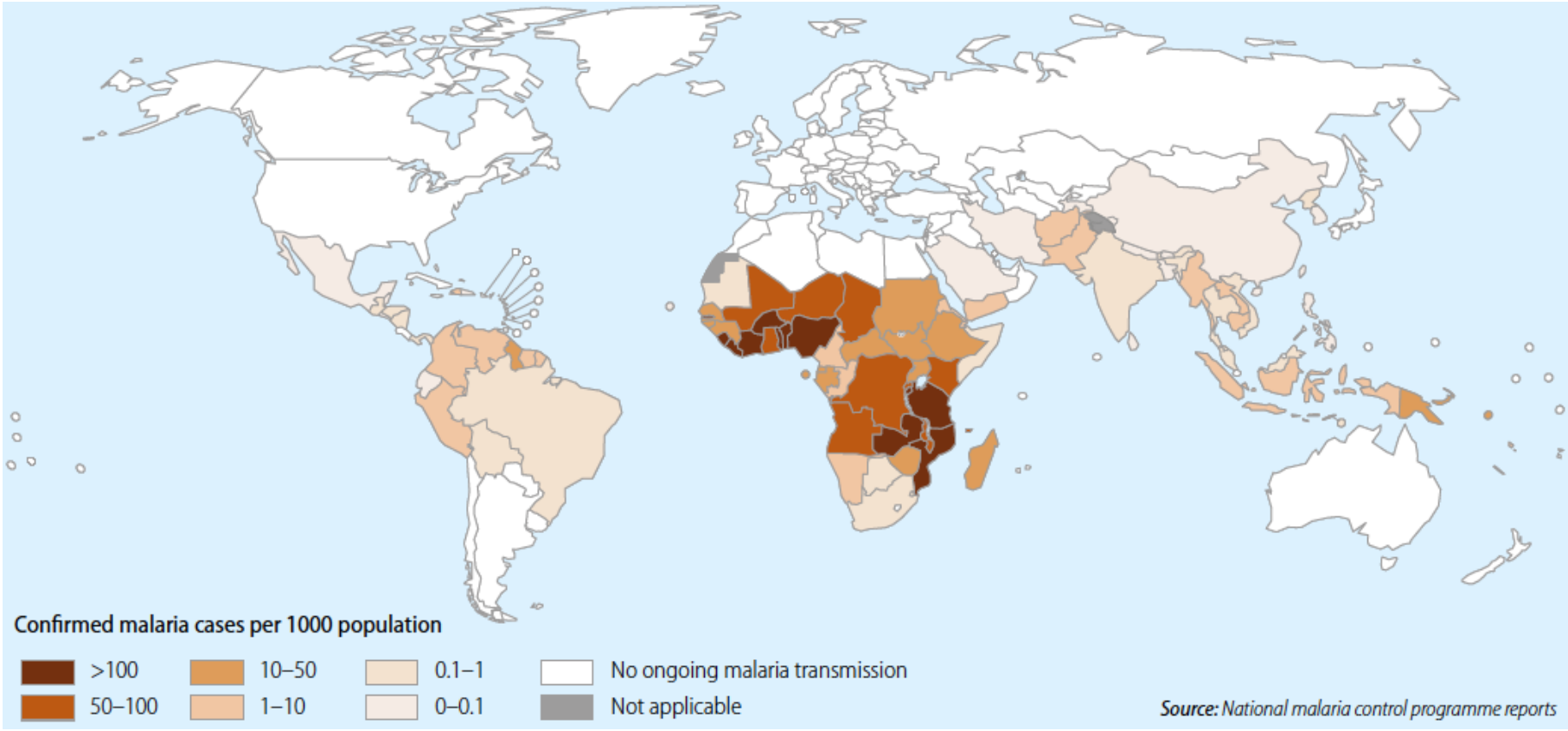


Figure 1.2.1. Global malaria transmission 2013. © World Malaria Report, World Health Organization (page 21) [6].

due to lack of partial immunity. Bangladesh is a low, unstable and seasonal malaria transmission area. The majority of the malaria occurs in the southern Chittagong division [8].

Differences in transmission intensity manifest as distinct clinical epidemiology patterns. In holoendemic areas with stable transmission, severe anaemia, cerebral malaria and metabolic acidosis (acidotic breathing) are the most common presentations. In hypoendemic areas with unstable transmission, cerebral malaria, metabolic acidosis and acute kidney injury (AKI) are the common clinical features [9]. The classical description of AKI being less common in children is discussed in section 1.4.

1.2.2 *Plasmodium* Lifecycle

Malaria is caused by a protozoan parasite of the Apicomplexa phylum and genus *Plasmodium.* There are 5 species that infect humans and cause disease. *P. falciparum* causes the majority of severe and fatal malaria. *P. vivax* and *P. knowlesi* may cause severe and fatal disease, and have been reported to cause AKI. *P. ovale* and *P. malariae* usually cause less severe clinical disease, although recurrent or chronic infections with *P. malariae* have historically been linked to glomerulonephritic kidney disease [10].

Transmission of human malaria begins when a female anopheline mosquito feeds on the host and introduces *Plasmodium* sporozoites into the bloodstream (Figure 1.2.2). Within 1 hour the sporozoites enter the hepatocytes and begin asexual multiplication to produce thousands of merozoites. The duration of the pre-erythrocytic, liver phase is species

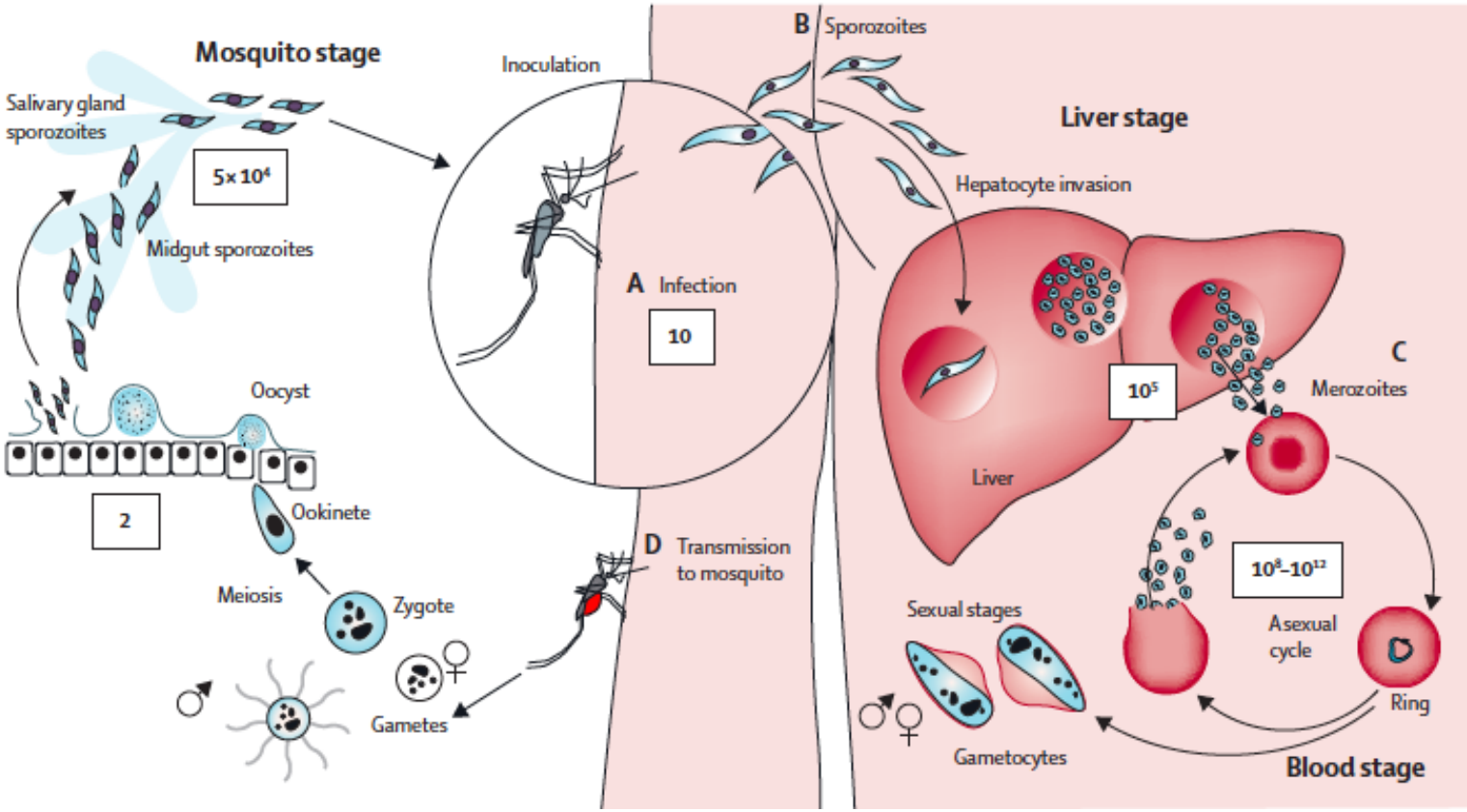


Figure 1.2.2 Malaria life cycle [9].

specific; the median time for *P. falciparum* is 6 days after which the hepatic schizont ruptures and releases merozoites into the bloodstream. The asexual blood stage development involves rapid merozoite invasion of circulating red blood cells (RBC). The parasite matures within the erythrocyte, consuming host haemoglobin and detoxifying toxic haem into haemozoin. After approximately 13-16 hours, during the transition between late-ring to trophozoite stage, the parasitized erythrocytes develop knob-like projections on their surface and begin to adhere to the vascular endothelium of capillaries and venules through specific, molecular-mediated adhesion. This process is known as sequestration and results in microvascular obstruction, which is a unique pathophysiological feature of falciparum malaria. After 36 hours, repeated nuclear division gives rise to multisegmented schizonts, which subsequently rupture to release multiple new merozoites. A new asexual cycle begins when these merozoites rapidly re-invade new erythrocytes. The toxic metabolites, haemozoin, and haemoglobin released upon rupture of infected RBCs contribute to the clinical symptoms of malaria. The role of this cell-free haemoglobin (CFH) in the pathophysiology of AKI and SM is assessed in this thesis.

1.2.3 Clinical manifestations

The clinical features of malaria range on a continuum from asymptomatic parasitaemia, to ‘mild’ uncomplicated malaria, to severe malaria and potentially death. The presentation of disease depends on host immunity and therefore differs between stable and unstable transmission areas. In unstable transmission areas, all age groups can progress to SM. In high transmission settings, children under 5 are predominantly at risk for

developing SM, whereas older children, adolescents, and adults typically have asymptomatic parasitaemia or uncomplicated malaria (UM). However, severe and fatal infections do occur in older patients, which may represent a challenge with a parasite to which the host is immunologically naïve [11].

The WHO defines SM by clinical and laboratory criteria [1]. Many features of SM represent end organ dysfunction and patients with multiple severity criteria have a higher mortality. AKI is included as a diagnostic criterium of severe disease and is reported more frequently in adults compared to children. Indeed reports of AKI in African children are rare and may contribute to a clinical assumption that this not a complication of paediatric SM. However, metabolic acidosis is one of the main syndromes defining SM in children and kidney dysfunction is an independent predictor for mortality in this population [12]. This thesis explored the diagnosis, pathophysiology and treatment of AKI in adults with SM. This may inform our understanding of the pathophysiology of kidney dysfunction in children and assist with future studies in this area, which are greatly needed.

1.2.4 Pathophysiology

Central to the pathophysiology of severe falciparum malaria is the cytoadherence of infected RBCs to capillary endothelium and the resulting sequestration in the deep microvasculature of different tissues. Sequestered and unsequestered infected RBCs may also adhere to each other, a process known as auto-agglutination [13], to platelets and host leukocytes, and also to uninfected RBCs, a process termed rosetting [14]. These processes result in vascular micro-occlusion with subsequent ischaemia and metabolic

acidosis. The reduced deformability of infected and uninfected RBCs may also contribute to impaired blood flow through already obstructed microvasculature [15]. The degree of sequestration is heterogeneous between organs, whereby some tissues such as brain, gut and subcutaneous fat, exhibit more sequestration than other organs [16].

Host immune responses to infected RBC contents upon schizont rupture also contribute to the pathophysiology of malaria. Activation of macrophages with subsequent pro-inflammatory cytokine release is associated with the febrile episodes experienced during infection [17]. Gaining a better understanding of malaria pathophysiology would facilitate the development of targeted adjunctive therapies. This thesis investigated the contribution of these processes to AKI complicating SM.

1.2.5 Diagnosis

The reference standard for diagnosing malaria is examination of thick and thin peripheral venous blood smears using field Giemsa, Field or Leishman stain and basic light microscopy. In areas without staining or microscopy equipment and trained personnel, rapid diagnostic tests (immunochromatographic strip tests) that identify parasite antigen or enzymes are used to diagnose malaria. Their sensitivity and specificity has been extensively examined and they are now a mainstay of both clinical diagnosis and research-based studies [18].

1.2.6 Management

There are three main pillars to the management of SM following rapid and accurate diagnosis. First, an effective antimalarial must be administered

promptly in the correct dose. Two landmark trials in patients with severe malaria definitively showed that intravenous artesunate reduced mortality by 35% and 23% in adults and children, respectively, compared to quinine [19, 20]. Intravenous artesunate is now the first line treatment for SM as recommended by the WHO [1]. After a minimum of 24 hours of intravenous therapy, an oral artemisinin-based combination therapy (ACT) can be initiated to complete treatment. Despite the best available artemisinin therapy for malaria, mortality remains unacceptably high. In paediatric cerebral malaria, defined by impaired consciousness or convulsions, without multi-organ involvement, the treated mortality is 6% but increases to 43% when kidney dysfunction and metabolic acidosis develop [12]. The goals of the second and third pillars of treatment are directed at reducing this mortality through supportive and adjunctive therapies.

The second critical element of SM management is the provision of adequate supportive treatments to manage complications of the disease [21]. Vigilant patient monitoring for deterioration is crucial particularly in the first 48 hours of admission. Referral to intensive care facilities is often required if respiratory insufficiency and/or haemodynamic instability develop. Renal replacement therapy (RRT) is life saving for patients with established malaria-associated AKI [22]. In the latter case, recognition of AKI is often challenging and frequently delayed due to lack of diagnostic facilities. Strategies to improve diagnosis and early management of AKI are assessed in this thesis.

The third pillar of SM management includes adjunctive therapies targeted to modify the disease process. Many adjunctive therapies, ranging from iron supplementation to more research-based therapies, such as anti-TNF antibodies, have been studied, but unfortunately none have proven to reduce morbidity and mortality from SM [23-25]. This thesis explored the use of paracetamol as adjunctive therapy for severe and moderately severe malaria with the hypothesis that it will reduce renal dysfunction.

1.3 Haemolysis in malaria

1.3.1 Causes of haemolysis in malaria

Acute and prolonged haemolysis accompanies *Plasmodium spp* infections . The mechanisms involved are multifactorial and challenging to differentiate. Three typical patterns of malaria-associated haemolysis following treatment have recently been described (Figure 1.3.1). Acutely, haemolysis results from obligatory destruction of parasitised RBCs at schizogony, which is exacerbated by drug treatment. In SM, there is also rapid haemolysis within the first 3 to 4 days of infection where destruction of unparasitised RBCs is the major contributor to the haemoglobin drop [26-28]. The mechanism of unparasitised RBC haemolysis is unknown. One hypothesis is that the reduced red cell deformability (RCD) observed in both infected and uninfected RBCs makes RBCs more susceptible to splenic clearance and/or haemolysis [29]. An intrinsic RBC abnormality that contributes to haemolysis in malaria is glucose-6-phosphate dehydrogenase (G6PD) deficiency. G6PD deficient cells are more susceptible to haemolysis when exposed to oxidative stress induced by medications, such as primaquine [30], or infections [31-34]. Causes of ongoing haemolysis after parasite clearance

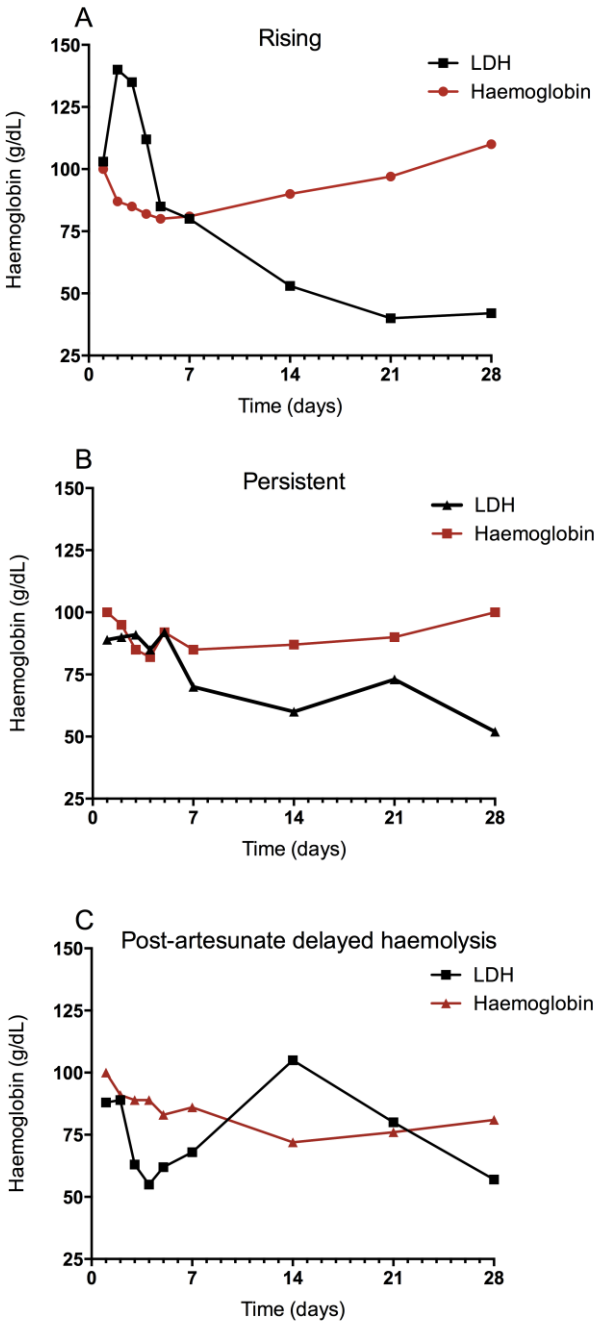


Figure 1.3.1 Patterns of anemia following malaria treated with artesunate in non-immune travellers. (A) Rising pattern: characterised by marked haemolysis until day 4 into illness that progressively increases until day 28 after treatment. (B) Persistent pattern: characterised by moderate haemolysis until day 4 followed by mean haemoglobin rise of 5% per week until day 28 after treatment. (C) Post-artesunate delayed haemolysis pattern: characterised by bimodal haemolysis pattern with moderate haemolysis until day 4, then stabilization followed by a second drop in haemoglobin with evidence of haemolysis between day 7 and day 14. *Abbreviations*: LDH, lactate dehydrogenase /10U per L. Adapted from Jaureguiberry *et. al*. [35].

include autoimmune haemolytic anaemia secondary to parasite antigens and aryl-amino-alcohol containing antimalarials [36, 37], and non-immune haemolytic anaemia of non-parasitised RBCs [38]. Recently, post-artesunate delayed haemolysis has been described that typically occurs in non-immune, hyperparasitaemic patients and manifests 7 to 14 days following the first dose of artesunate (Figure 1.3.1).

Understanding the mechanisms of haemolysis in malaria is important since they may affect antimalarial treatment, as exemplified by G6PD deficiency limiting the use of primaquine. The genotypes of G6PD deficiency, and the associated phenotype have not been well described in Bangladeshi patients with malaria. One study of males from Dhaka showed that 4% (N=150) had G6PD deficiency based on enzymatic activity levels [39]. Among 202 individuals of Marma and Khyang ethnic groups living in the Chittagong Hill Tracts, 59% had normal G6PD activity, 35% had mild deficiency (>10-60% residual activity) and 6.5% had severe deficiency (>1-10% residual activity). Further characterisation of G6PD deficiency in Bangladesh is a crucial step towards implementing primaquine treatment in falciparum malaria, as gametocytocidal treatment to reduce transmission, and in vivax malaria, as a radical treatment to kill hypnozoites. The inaccessibility of a point of care G6PD test, together with the limited knowledge of the phenotype and genotype is currently a barrier to implementing these malaria treatment and control strategies.

1.3.2 Effects of cell-free haemoglobin

The inevitable result of haemolysis is the release of intracellular haemoglobin from RBCs into the systemic circulation. When haemoglobin and haem scavengers, haptoglobin and haemopexin, are saturated the extracellular haemoglobin and released haem is filtered through the glomerulus. It is then reabsorbed and catabolized in the proximal tubule and iron deposited as haemosiderin. Haemoglobinuria results when tubular reabsorption capacity is reached [40-43].

Extracellular, or CFH, is involved in disease pathophysiology of several acquired and inherited haemolytic disorders. Early research in this area arose from the blood transfusion field where massive transfusion (>10 units in 24 hours) was associated with increased risk of multiorgan dysfunction syndrome (MODS), respiratory insufficiency, vascular injury, AKI and death [44-48]. The toxicity of CFH is suggested to be dependent upon: (1) extracellular CFH concentration, (2) location of accumulation, and (3) scavenger defense [49]. Molecular mechanisms conferring injury include oxidative and nitric oxide (NO) reactions, and haem release.

Adult severe falciparum and knowlesi malaria are accompanied by intravascular haemolysis where CFH increases with disease severity [50], Barber, B; personal communication). Plasma haptoglobin and haemopexin are depleted in severe adult and paediatric malaria, thus excess CFH can exert its deleterious effects [50-53]. CFH has been shown to quench endothelial NO release in adults with severe falciparum malaria, and was

associated with impairment in NO-dependent endothelial function and proinflammatory cytokinaemia [50]. Similarly, children with SM showed increased haemolysis with higher CFH that was associated with increased NO consumption, pulmonary vascular pressures and myocardial wall stress [54]. Thus, the NO pathway of CFH-mediated toxicity appears to contribute to the pathophysiology but may not explain the complete mechanism of CFH-mediated injury in SM.

CFH is pathogenic as a pro-oxidant and inflammatory agent. In the extracellular environment, ferrous (Fe^{2+}) haemoglobin is oxidized to its ferric state (Fe^{3+}), which confers peroxidase activity to haemoglobin (Figure 1.3.2). Consequently, haemoglobin can reduce hydroperoxides, such as hydrogen peroxide (H_2O_2) and lipid hydroperoxides, resulting in oxidation of haemoglobin to its ferryl state (Fe^{4+}=O) and formation of a globin protein radical [55-58].

$$\text{Haem } (Fe^{3+}) \text{ protein} + H_2O_2 \rightarrow \text{haem } (Fe^{4+}) \text{ protein}^{\bullet\bullet} + H^+ + H_2O$$

Ferryl haemoglobin and a protein radical can then oxidise free and phospholipid-esterified unsaturated fatty acids [59-61]. Arachidonic side chains of membrane phospholipids are particularly vulnerable to this free radical-mediated damage, leading to the generation of F2-isoprostanes (F2-IsoPs) and isofurans (IsoFs) F2-IsoPs and IsoFs, have been shown to be the most reliable measures of oxidative stress *in vivo* [61, 62]. IsoFs are formed preferentially at higher oxygen tension compared to F2-IsoPs, thus

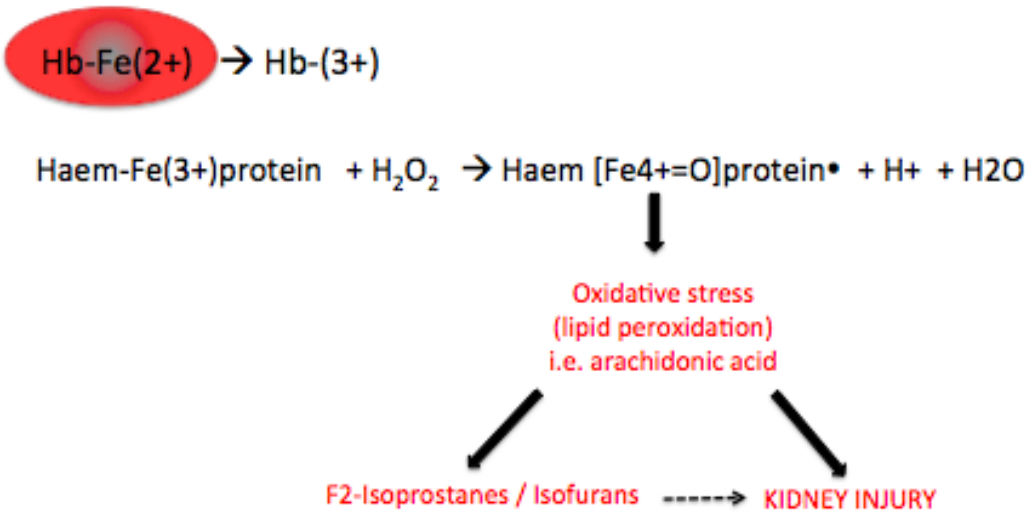


Figure 1.3.2. Cell-free haemoglobin mediated oxidative stress. Extracellular haemoglobin is oxidized to its ferric state (Fe^{3+}) conferring a peroxidase activity to haemoglobin. Ferric- haemoglobin can reduce hydroperoxides (i.e. H_2O_2). This process oxidises haemoglobin to its ferryl state (Fe^{4+}) and a protein radical, which can initiate oxidation of free and phospholipid-esterified unsaturated fatty acids. This generates F2-isoprostanes and isofurans, which are well-substantiated *in vivo* measures of oxidative stress. F2-isoprostanes are potent vasoconstrictors associated with decreased renal blood flow and glomerular filtration [63].

measuring both lipid peroxidation products provides a more reliable measure of oxidative stress [62].

In vitro ferryl haemoglobin was shown to cause endothelial injury via apoptotic cell death [64]. In animal models, CFH is potentially toxic to several organ systems including vascular, myocardial, renal and central nervous system tissues [64]. Systemic and intrarenal F2-IsoPs were shown to be potent vasoconstrictors associated with decreased renal blood flow and glomerular filtration rate (GFR). Evidence suggests that F2-IsoPs and IsoFs generated by haemoprotein-catalyzed oxidation of lipids contribute to oxidative damage and vasoconstriction associated with AKI in the setting of haemolytic disorders, rhabdomyolysis, sepsis and post-cardiopulmonary bypass [65-69]. Urinary F2-IsoPs were shown to be elevated in SM compared to uncomplicated falciparum and vivax malaria [70].

The evidence supporting CFH-mediated pathology emphasizes that inhibiting this pathway may be an effective therapeutic target. In an animal model of CFH-mediated injury, transfusion of older RBCs resulted in haemoglobinuria, vascular injury, hypertension and AKI [44]. The role of CFH mediating this toxicity was supported by treatment with haptoglobin infusion as a scavenger of CFH that attenuated these adverse outcomes. Smaller case series' and case studies in humans have shown a similar beneficial effect of haptoglobin infusion in the prevention of haemoglobinuria and AKI [71-81]. The potential for an intervention to inhibit CFH-mediated

damage is promising as a therapy to prevent AKI in patients with haemolytic conditions.

The role of high CFH and oxidative stress in the pathophysiology of AKI complicating SM is unknown. This thesis assessed the pathophysiologic role of CFH-mediated oxidative stress in adults with SM.

1.3.4 Blackwater fever

Fulminant haemolysis (blackwater fever) is a manifestation of SM with passage of dark or red urine (haemoglobinuria). Despite extensive research, the exact mechanism of blackwater fever (BWF) is unclear. Historically, this complication was seen in non-immune expatriates in Africa who may or may not have been taking quinine [82]. The reported incidence of BWF decreased upon the introduction of chloroquine from 1950 to 1980, but re-emerged in the literature since the reintroduction of quinine. It is proposed to occur in four circumstances: (1) non-immune people exposed to malaria taking quinine, (2) G6PD deficient patients with or without malaria who take oxidant drugs, such as primaquine, (3) G6PD deficient patients with malaria who receive quinine, and (4) G6PD normal patients with SM irrespective of antimalarial treatment [16, 33]. A detailed study of Vietnamese adults with the clinical syndrome of BWF found a particularly strong association with G6PD deficiency, in addition to presence of *plasmodium* parasites and history of quinine ingestion [33]. However, in African children with falciparum malaria, BWF was not associated with G6PD deficiency [83, 84]. This is likely due to differences in the G6PD variants with the corresponding level enzymatic deficiency between these two populations. Interestingly, there is a

similar prevalence of BWF among Asian adults and African children with SM treated with quinine or artesunate, in the range of 4-7% [19, 20].

While BWF is reported to be increasing, the prevalence varies in adults and children due to differences in definition, methods of detection (i.e. urine dipstick, microscopy, spectrophotometry, or gross visual colour inspection alone), and the population denominator. Retrospective studies report haemoglobinuria in up to 25% of adults [85] and 19-23% of children [83, 86] with SM. In comparison, studies with a denominator population of patients admitted with AKI report haemoglobinuria associated with falciparum malaria in up to 43% of adults [87] and 43-62% of children [84, 88]. However, the definitions of BWF and/or the method of haemoglobinuria are poorly defined in these studies. A rigorous study of Nigerian children with SM reported the incidence of haemoglobinuria to be 19.1% based on urine dipstick, microscopy and spectrophotometry, which was associated with age (above 5 years), jaundice, pallor and dark urine [83].

Historically BWF is associated with low or absent parasitaemia, however studies do not report this association consistently [83, 89]. The association of BWF with total parasite biomass, estimated from plasma *P. falciparum* histidine rich protein 2 (*Pf*HRP2), is not known. Since peripheral parasitaemia reflects schizogony during the 48-hour lifecycle, if the infection is unsynchronized there is potential to miss observing a high parasitaemia in haemoglobinuric patients. Furthermore, there may be a time lag between high parasitaemia and appearance of ‘blackwater’. Alternatively, since

haemoglobinuria is considered a manifestation of severe disease that often has a high mortality, it is possible that these patients present late in disease after the majority of their parasite biomass has sequestered, thus the peripheral parasitaemia would falsely represent a low parasite burden. In either scenario, it is highly unlikely that haemolysis of only parasitized RBCs can account for the large drop in haemoglobin observed in BWF patients. On admission, these patients already have a ~25% loss of RBC mass, which can continue until day 7 to reach a ~35% loss of RBCs [33]. To explain this degree of RBC loss when peak parasitaemia is usually less than 10-15%, the haemolysis occurring during an episode of BWF may also be due to lysis of uninfected RBCs.

Patients with SM exhibit reduced RCD and haemolysis of both infected and uninfected RBCs [90, 91]. Potentially, elevated CFH may contribute to reduced RCD via lipid peroxidation of RBC arachidonic acids, which renders them more susceptible to haemolysis and/or splenic clearance [92]. This may present a unifying explanation for different circumstances in which BWF arises, where different populations (i.e. G6PD deficient, malaria infected or both) have a similar decreased RBC deformability and increased fragility.

In general terms, the development of haemoglobinuria is dependant upon (1) plasma CFH concentration, (2) scavenging capacity of plasma CFH, (3) glomerular filtration of CFH dimers, and (4) proximal tubular reabsorption [41-43, 92]. Higher CFH result concentrations have been shown to result in higher urine haemoglobin concentrations in the absence of renal injury [41-

43]. However, in experimental models of BWF with 70% parasitaemia, not all animals exhibit haemoglobinuria [93]. Similarly, not all SM patients with massive haemolysis and elevated CFH exhibit BWF [50]. Thus, elevated plasma CFH may be a prerequisite for developing haemoglobinuria but it is not sufficient. Healthy control subjects administered haemoglobin infusions have different thresholds of plasma protein-bound CFH before haemoglobinuria is observed [41]. This may be due to haptoglobin genotype 2-2 conferring less efficient clearance of CFH [94, 95]. Alternatively, the lack of haemoglobinuria in all patients with high CFH may be due to decreased dimerisation of the haemoglobin tetramer resulting in less glomerular filtration and thus, absence of BWF. Haemoglobin dimerisation is enhanced by dilution [42], which may explain why patients frequently develop BWF after admission and fluid resuscitation (personal communication). In healthy controls infused with haemoglobin, inulin clearance was decreased compared to normal, which was attributed to renal vasoconstriction induced by CFH [41]. In SM patients, a situation arises akin to a continuous infusion of CFH and resultant renal vasoconstriction could induce a reduction in glomerular filtration. Further, SM patients may have a degree of pre-renal AKI with an already decreased GFR, which could be compounded by CFH-mediated vasoconstriction and result in lack of haemoglobinuria.

Partial immunity, and autoimmune reactions to drugs have been proposed to be involved in the development BWF. While these factors may contribute, they are not sufficient to cause haemoglobinuria since animal and human studies have shown the development of BWF in the absence of these

features [33, 93]. Further prospective studies with standardized definitions of haemoglobinuria are necessary to verify the reported increasing trends in paediatric BWF and unravel the mechanisms involved.

1.4 Acute kidney injury in severe malaria

In adults with severe falciparum malaria, AKI develops in up to 40% of patients, whereas in children the reported incidence of 10% is likely an underestimate [9, 12, 19, 20]. Kidney dysfunction in malaria is an independent predictor of mortality in all ages [12, 19]. Apart from antimalarial treatment with artesunate, RRT is the only treatment modality shown to reduce mortality in SM: from 75% to 26% in patients with AKI [22]. Prevention of AKI is an important step in reducing mortality, particularly since RRT is unavailable in endemic areas that need it most. Gaining a better understanding of the pathophysiology of AKI in malaria will help towards the development of targeted therapies.

1.4.1 Definition

The WHO classifies malaria into uncomplicated or severe disease based on clinical and epidemiological criteria [1]. The criteria for SM caused by any human *Plasmodium* species includes AKI, defined as a plasma or serum creatinine > 265μM (3 mg/dl) or a blood urea > 20 mM [1]. This definition is specific to AKI, which is differentiated from the chronic nephrotic syndrome that has historically been associated with *P. malariae* [10]. There is currently lack of an appropriate WHO AKI definition in paediatric malaria, potentially related to reports that AKI is rare in children, and is not included among the clinical syndromes defining severe paediatric malaria.

Differing terminology in the literature describing AKI associated with SM obscures the clinical presentation. References to falciparum malaria-associated AKI include: malaria nephropathy, malaria nephritis, and malarial acute renal failure [22, 96]. In contemporary nephrology literature and clinical practice, the term AKI has been adopted to replace acute renal failure (ARF) to encompass the broad clinical spectrum of this syndrome including clinically relevant, smaller increments of creatinine that are associated with increased morbidity and mortality but do not necessarily result in complete organ failure [97]. Thus the term includes both kidney injury and/or kidney functional impairment. While the AKI definition has evolved in the nephrology field, the 2014 WHO malaria guidelines adopted this updated *terminology* to reflect kidney dysfunction in malaria. However, this was not accompanied by a harmonisation of the WHO *definition* of malaria-associated AKI with the criteria proposed by the Kidney Diseases Improving Global Outcomes (KDIGO) to better integrate global AKI epidemiology and pathophysiology research [97].

The WHO definition of malaria-associated AKI has been modified over the years (Table 1.4.1). In 2010, the criteria for urine output less than 400 mg/24h in adults and <12 mg/kg/24h in children was excluded since AKI in malaria is non-oliguric in approximately 30-40% of adult SM patients [9]. This proportion may not apply to children, as among the few studies specifically describing AKI complicating paediatric SM, one study reports that 94% are

Table 1.4.1 WHO definitions of malaria-associated AKI

Year	Adults	Children
1990	Urine output <400ml/24h despite rehydration AND Serum creatinine > 265 µmol/l	Urine output <12mg/kg/24h despite rehydration AND Serum creatinine >265 µmol/l
2000	Urine output <400ml/24h despite rehydration AND Serum creatinine > 265 µmol/l	Urine output <12mg/kg/24h despite rehydration OR Serum creatinine above age related normal range
2006	No laboratory cut off defined	No laboratory cuff off defined
2010	Serum creatinine > 265 µmol/l	Serum creatinine > 265 µmol/l
2012	Serum creatinine > 265 µmol/l OR blood urea >10mM (>60mg/dl)	Urine output <1ml/kg/h AND Urea +/- creatinine >95th centile for age
2014	Clinical: Anuria Research: plasma/serum creatinine >265 µM (3mg/dl) OR plasma/serum blood urea >20mM (>120mg/dl)	plasma/serum creatinine >265 µM (3mg/dl) OR plasma/serum blood urea >20mM (>120mg/dl)
2015	Clinical: Anuria Research: plasma/serum creatinine >265 µM (3mg/dl) OR plasma/serum blood urea >20mM (>120mg/dl)	plasma/serum creatinine >265 µM (3mg/dl) OR plasma/serum blood urea >20mM (>120mg/dl)

References: [1, 21, 98-102]

oliguric (<12 ml/kg/24 h) [86]. Urine output is often difficult to accurately assess in malaria endemic countries, particularly in children, and may delay diagnosis for 24 hours. Therefore prior to 2010, malaria-associated AKI may have been under-recognised, under-diagnosed and under-reported, given the need to fulfill both creatinine and urine output criteria. It is critical to emphasize that the absolute creatinine of > 256 μmol/L and blood urea of > 20 mM thresholds are proposed definitions for malaria research and epidemiology, and the clinical diagnosis of AKI must be considered using all available patient data, since up to 16% of those with a kinetic GFR of < 35 ml/min have a creatinine < 3 mg/dL (unpublished). Further this threshold is not applicable to children given their smaller muscle mass and lower age-adjusted creatinine ranges. This highlights that the current WHO AKI definition based on a single creatinine or urea cut-off is imperfect, and should consider that the consensus diagnostic criteria for AKI is based on changes in serum creatinine and/or urine output [103].

Nephrologists are focused on establishing a universal and standardized definition of AKI in order to facilitate research in this area since lack of a consensus definition is a limitation. In 2004, the staged RIFLE (Risk, Injury, Failure, Loss and End stage kidney disease) definition was created and subsequently adapted to establish the paediatric RIFLE (pRIFLE) criteria for use in children [104, 105]. In 2007, this definition was modified, the AKI Network (AKIN) criteria [106], to include a creatinine rise of ≥ 0.3 mg/dl within a 48 hour period since minor elevations in creatinine were associated with increased mortality [107, 108]. In 2012, the KDIGO definition and

Table 1.4.2 KDIGO definition and staging criteria of acute kidney injury

Definition of AKI		
Criteria	Any one of the following: Increase in SCr by ≥ 26.5 µmol/L (0.3 mg/dl) within 48 hours Increase in SCr to ≥ 1.5 times baseline, presumed to occur within prior 7 days Urine volume <0.5 ml/kg/h for 6 hours	
Staging of AKI		
Stage	**Serum creatinine**	**Urine output**
1[#]	SCr ≥ 26.5 µmol/L (≥ 0.3mg/dL) increase *or* 1.5 - 1.9 times baseline	< 0.5 ml/kg/h for 6-12 hours
2	2.0 - 2.9 times baseline	< 0.5 ml/kg/h for ≥ 12 hours
3*	3.0 times baseline *or* Increase SCr > 353.6 µmol/L (>4 mg/dl) *or* Initiation of RRT *or* if < 18years, decreased in eGFR to <35 ml/min per 1.73m2	< 0.3 ml/kg/h for ≥ 24 hours *or* anuria for ≥ 12 hours

[#] Increase in creatinine occurring within 48-hour time period

* Stage 3 also includes patients requiring RRT independent of the stage (defined by SCr and/or UO) they are at the moment they initiate RRT

staging classification of AKI was proposed, which harmonized the above definitions to create a single definition for standardized clinical practice and research (Table 1.4.2). Global adoption of the KDIGO definition is important, as this will facilitate achieving the goals set forth by the International Society of Nephrology's '0-by-25' initiative for AKI aiming for zero preventable deaths from AKI by 2025 [109].

Assuming that the majority of patients with SM have normal baseline renal function, an admission creatinine of > 3 mg/dL would give a KDIGO stage of 2 or 3 in adults and all children stage 3 AKI. In-hospital mortality associated with AKIN stage 2 and 3 are 12 - 67% and 33 - 58%, respectively [110]. Few studies have considered AKIN, RIFLE or KDIGO criteria in malaria-associated AKI. A recent study of Nigerian children admitted with AKI classified by the paediatric RIFLE score showed that 11.4% of cases were due to malaria, of whom 87.5% presented with 'Failure' [111]. There is a potential danger in managing patients, particularly children, relying strictly on the WHO creatinine definition, as early referral for monitoring and potential dialysis may be delayed should an admission creatinine/urea not meeting the definition falsely reassure the treating clinician. The RIFLE criteria studied in adult malaria, showed that requirement for RRT and in-hospital mortality increased with RIFLE class [112]. Limitations in this study were the retrospective design and RIFLE classification based on estimated GFR and not creatinine or urine output [112]. A prospective study of malaria-associated-AKI described by KDIGO criteria has not been reported. However, adopting the KDIGO criteria into the WHO malaria guidelines may

result in improved recognition and outcomes of malaria-associated AKI and facilitate harmonization of this population on a global level.

However, assuming a normal baseline in patients with malaria may be problematic given the increasing global burden of hypertension and diabetes associated nephropathy [113]. In Bangladesh, the prevalence of hypertension and type 2 diabetes mellitus has increased since 1995, rising to 14% and 7%, respectively [114]. Comprehensive national registries of renal diseases are not available in Bangladesh, however the most common reported causes of end stage renal disease are glomerulonephritis, (primarily mesangial proliferative in adults and post-streptococcal in children), diabetic nephropathy and hypertensive nephropathy [115]. One study in Bangladeshi adults reports that medical causes of AKI (creatinine > 5.66 mg/dl) are associated with a 40% mortality and the common aetiologies include gastroenteritis, rapidly progressive glomerulonephritis of unknown etiology and sepsis, however comorbid conditions were not reported [116].

Unfortunately, repeated creatinine measurements may not be possible in resource-limited settings and estimated baseline creatinine using the Modification of Diet in Renal Disease (MDRD) formula has not been validated in many populations suffering from malaria, which potentially limits application of KDIGO AKI diagnosis. Developing solutions to these issues is critical. As an alternative to the MDRD calculation to estimate baseline creatinine, establishing age and country specific population means of creatinine may facilitate KDIGO AKI diagnosis on admission. The

development of a practical clinical malaria-associated AKI severity score and a robust, simple test that does not rely on costly and potentially unavailable laboratory tests is badly needed. Urine microscopy is tool that could be used where slide microscopy is performed. Thus validating a urine microscopy score that is predictive for KDIGO stages, RRT and/or outcomes that may assist in appropriately monitoring and triaging patients may be a practical solution for resource-limited settings. In a study of hospitalized patients referred for nephrology consultation, the urine sediment score (based on granular casts and renal tubular epithelial cells) was associated with severity of AKI at the time of consultation and was a better predictor of worsening AKI than the change in creatinine or oliguria [117]. A systematic review of the limited studies of urine microscopy in patients with AKI, found that scoring granular casts and renal tubular epithelial cells may be useful to diagnose ATI, and predict need for RRT and non-recovery of AKI [118]. A study evaluating urine microscopy as a predictor of ATI and AKI outcomes in Bangladeshi adults with falciparum malaria is currently ongoing. Urine microscopy has its limitations, thus the combination with a biomarker available in RDT form could provide the best way forward [117, 119]. Overall, there is pressing need to standardize AKI diagnostic criteria for adults and children with malaria, and conduct prospective studies in both populations looking at accurate prediction of AKI, RRT, and outcomes.

1.4.2 Epidemiology

The prevalence of AKI complicating malaria in adults and children varies by country and denominator patient population. In adults, the prevalence of

malaria-associated AKI is inadvertently cited as ranging widely from 0.4-60% [120]. In children, there is a paucity of research into the prevalence and description of malaria-associated AKI, and cited estimates range from 1-51% [121, 122]. These broad ranges are problematic, as they do not accurately reflect the magnitude of this potentially fatal complication. It is critical to describe the true incidence of malaria-associated AKI in adults and children in order to implement adequate management strategies, and determine need for increased research in this area.

The epidemiology of malaria-associated AKI is difficult to ascertain, particularly in African children. This is related to the lack of laboratory facilities to measure kidney function, under-appreciation of AKI as a complication in paediatric SM, and divergent definitions. (Supplementary Table 1.4.1A,B, 1.4.2, 1.4.3; Figures 1.4.1 and 1.4.2). Most data come from single-centre studies conducted in urban centres, and variation in AKI estimates from different regions may represent publication bias from countries with well-established renal centres [123]. In African children with SM, AKI might not be appreciated as an important problem and may be easily missed, especially in cases of non-oliguric AKI, which is reported in at least 15% of paediatric AKI cases [86]. Using adult creatinine cut-off values to define AKI in children is another obvious cause for underreporting of AKI in African children. The 2010 WHO AKI definition of creatinine >265 μmol/L is clearly not appropriate for children. Using this definition, the average prevalence of malaria-associated AKI among adults versus children with SM is 23% versus 3% in Africa [124-132], 34% versus 5% in Southeast Asia

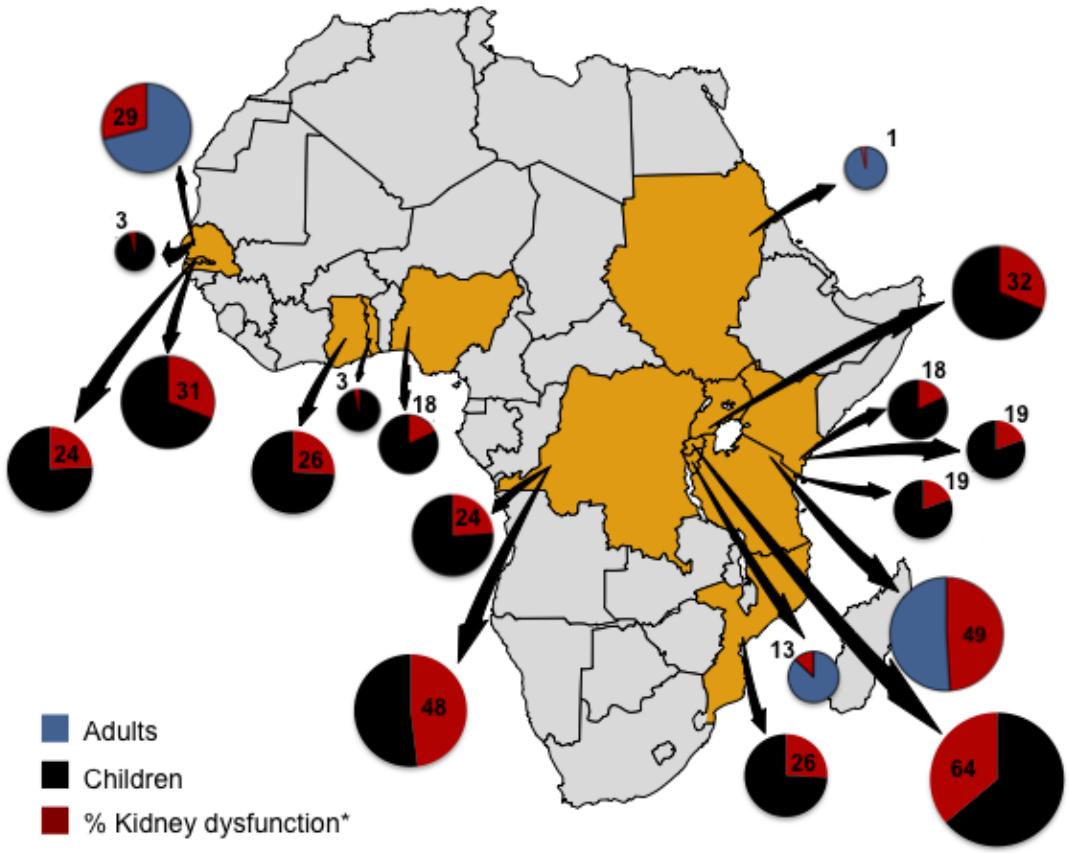


Figure 1.4.1 Prevalence of malaria-associated AKI by country in adults and children with falciparum malaria in Africa. *See Supplementary tables 1.4.1A, 1.4.1B and 1.4.2 for AKI definitions used in each study.

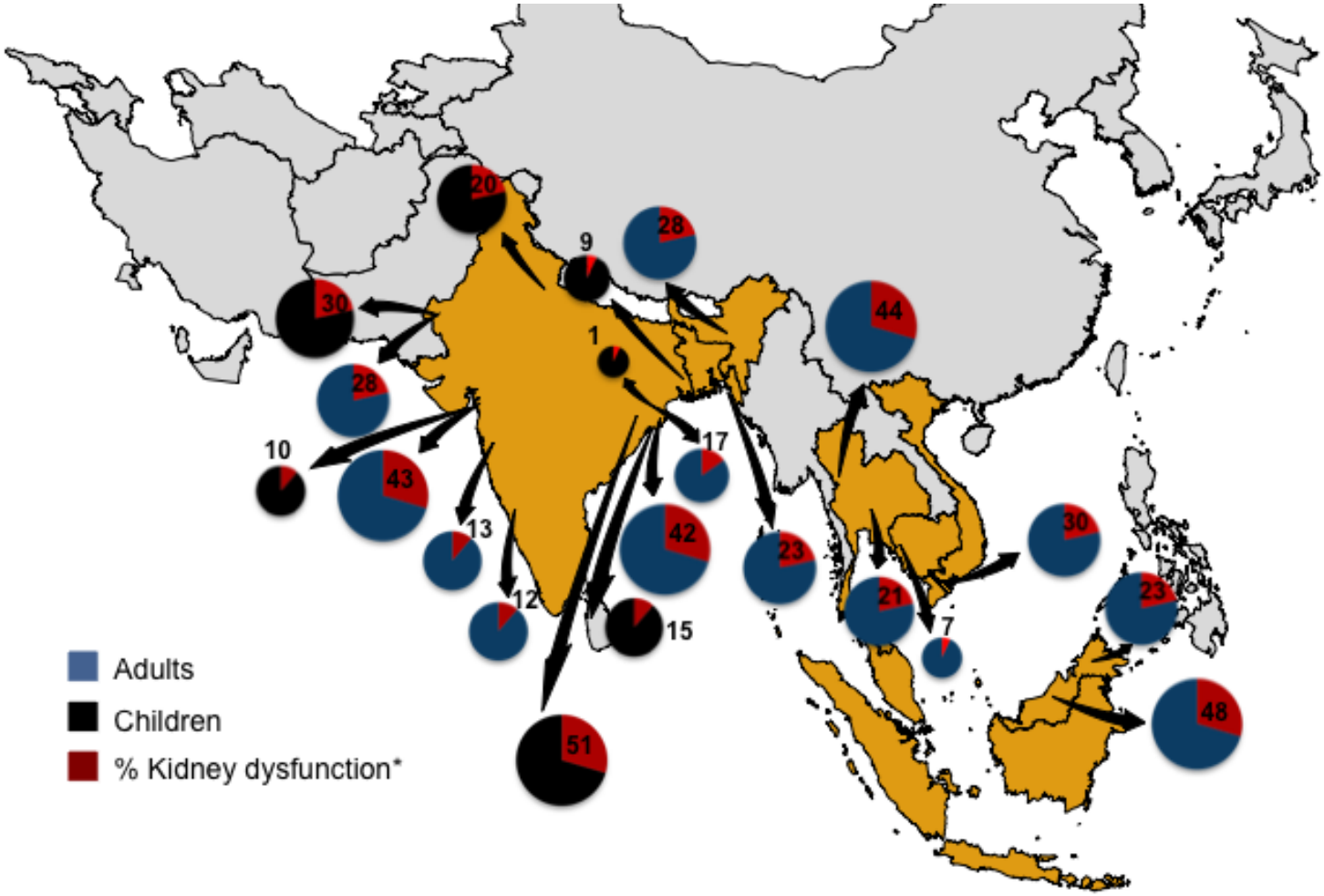


Figure 1.4.2 Prevalence of malaria-associated AKI by country in adults and children with falciparum malaria in South and Southeast Asia. *See Supplementary tables 1.4.1A, 1.4.1B and 1.4.2 for AKI definitions used in each study.

[3, 52, 112, 133-135], and 21% versus 8% in South Asia [121, 136-140](Table 1.4.3; Supplementary Tables 1.4.1, 1.4.2, and 1.4.3). For instance, a study of paediatric SM in Togo found only 3% with AKI as defined by the adult WHO criteria [125]. In comparison, malaria-associated AKI prevalence, defined by varying local standards, is reported to be much higher in African children: 31% [84, 88, 111, 141-145] and South Asian children: 21% [122, 146-149]. A recent study of paediatric SM in Kinshasa found that 24% had AKI when classified by modified AKIN criteria [86]. AKI diagnosis only based on oliguria also likely severely underestimates prevalence: in a Gambian study of paediatric SM only 0.6% presented with anuria [128]. These differences point to the importance of standardizing a definition for malaria-associated AKI. In addition, reported prevalence of malaria-associated AKI, according to local definitions, differs in the denominator: children with AKI, 31% [84, 88, 111, 141-145, 150], or children with SM, 14% [86, 125-128, 132, 151] (Table 1.4.3; Supplementary Table 1.4.2 and 1.4.3).

Although there are increasing reports of malaria-associated AKI in children from several African countries [111], reliable epidemiological information is still largely lacking and highly likely to be underestimated. What is clear is that mortality of paediatric malaria-associated AKI is high, with reported mortality rates between 13-43% [86, 127]. Improved recognition of paediatric malaria-associated AKI in Africa is thus clearly warranted, which is critical for targeted, region specific interventions to be implemented.

Table 1.4.3 Summary of differing malaria-associated AKI prevalence by AKI definition and denominator population

Definition		**WHO**	**OTHER**	**WHO**	**OTHER**	**WHO**	**OTHER**
Region		Africa		Southeast Asia		South Asia	
Denominator	**Population**						
Severe malaria	Adults	23%	--	34%	31%	25%	15%
	Children	3%	18%	5%	--	12%	9%
AKI	Adults	--	--	--	--	14%	9%
	Children	--	31%	--	--	--	1%

A total of 67 studies were assessed for the prevalence of AKI. Mean prevalence presented. WHO= World Health Organization definition of AKI (creatinine >3mg/dl ± urine output < 400 mg/dl); OTHER= local definition of AKI used in publication (see Supplementary Tables 1.4.1, 1.4.2 and 1.4.3 for specific details).

Co-infection with human immunodeficiency virus (HIV) may impact the epidemiology of malaria-associated AKI, particularly in Africa. There is little known about the effect of HIV co-infection on the kidney in malaria patients. A post-mortem study of 43 HIV-1 infected Congolese individuals incidentally revealed that 28% (12/43) had renal histologic evidence of malaria infection including potential sequestration, 85% had severe acute tubular injury (ATI), and all had evidence of renal dysfunction prior to death (mean serum creatinine 10.7 +/-3.1 mg/dL)[152]. A study of African paediatric SM showed that HIV co-infected patients had higher blood urea nitrogen (BUN) compared to SM alone [153]. African HIV-positive adults hospitalized with acute malaria or SM were more likely to have impaired renal function, AKI (defined by creatinine >265 μmol/L) and require dialysis compared to HIV-negative malaria patients [154, 155]. The mechanism of increased renal dysfunction in HIV co-infected malaria patients is unclear and is not thought to be due to concomitant antiretrovirals or underlying HIV nephropathy.

The impact of AKI complicating SM also seriously affects individuals in resource rich settings. There are high rates of AKI among imported malaria patients ranging from 40 - 54%, associated with a high mortality of 15 - 23% [156, 157]. In a study from France reporting on 42 cases of imported malaria, 31% (13/42) had AKI or developed AKI during the course of their illness; of whom 15% (2/13) died, 85% (11/13) required prolonged intensive care unit (ICU) stays, and 90% were non-immune [158]. This may suggest that immune mechanisms are involved in renoprotection either directly or indirectly by reducing the parasite burden. In a large retrospective study of

400 cases of imported SM, 40% had AKI, of whom 51% required dialysis and 23% died [157]. These studies underscore the high mortality of malaria-associated AKI (15-23%) despite optimal supportive therapy, including RRT administered to 50-58% of patients. Therefore, determining preventative or adjunctive therapies that can reduce malaria-associated AKI is critical to reducing malaria mortality.

1.4.3 Other *Plasmodium species* and AKI

While the majority of malaria-associated AKI complicates *P. falciparum* infection, the other human malaria parasites can cause a spectrum of renal pathologies [1-4]. *P. vivax* and *P. knowlesi* cause severe disease less commonly but can be associated with AKI. The less malignant forms of chronic infection with *P. malariae* were reported to cause “quartan malarial nephropathy” due to recurrent infection, a high burden of soluble parasite antigen and glomerular deposition of immune complexes, leading to membranous and membranoproliferative glomerulonephritis, chronic or relapsing nephrotic syndrome. However, the concept of “quartan malarial nephropathy” has been recently challenged [10]. Infections with *P. ovale* are rarely associated with AKI.

Severe vivax malaria is mainly been reported from India, Indonesia, and Pakistan [159]. In India, the reported prevalence of AKI associated with *P. vivax* ranges from 24 - 32% in adults and 15 - 45% in children, associated with a mortality of 8 - 21% [159-162]. However, a recent meta-analysis assessing the prevalence of severe vivax malaria showed an AKI prevalence

of 5.9% in hospitalized patients, and 0.5% in pooled inpatients and outpatients [159]. In a series of 25 *P. vivax* patients with AKI, factors associated with AKI included volume depletion, intravascular haemolysis, and hyperparasitaemia [162]. Limitations of severe *P. vivax* studies include lack of polymerase chain reaction (PCR) diagnosis to exclude falciparum co-infections, other diagnostics to rule out concomitant sepsis and comorbidities, and reporting bias. Given the different pathophysiology of falciparum malaria, the mechanisms of AKI in vivax patients warrants more detailed study.

Severe knowlesi infection is now recognized as a significant cause of human malaria in Southeast Asia, which causes severe disease in approximately 7% of patients [163]. Recent studies show that 24-55% of severe knowlesi infections are complicated by AKI, associated with hyperparasitaemia and intravascular haemolysis [3, 163, 164]. There are a small number of children with severe knowlesi reported and AKI was not associated with severe disease [165]. Histopathology of AKI-associated fatal *P. knowlesi* infection in one case report showed ATI with intravascular parasitised RBCs [166].

Overall the prevalence of AKI in *P. knowlesi* patients is high, similar to the prevalence among falciparum malaria patients, whereas the prevalence of AKI associated with vivax infection is approximately 4 - 10 times lower. This suggests that there may be a common mechanism of AKI in *P. knowlesi* and *P. falciparum*.

1.4.4 Pathophysiology

The pathogenesis of AKI in falciparum malaria is multifactorial, and likely involves both pre-renal and intrinsic renal insults. Decreased renal function due to pre-renal causes occurs due to (1) intravascular dehydration, and (2) hypotension, which is present in approximately 10% of cases. [167, 168]. Proposed intrinsic renal causes include: (1) renal ischaemia resulting in ATI as a consequence of prerenal factors (2) CFH-mediated oxidative damage, and nitric oxide scavenging, (3) microvascular obstruction due to sequestration of parasitised RBCs, and congestion of infected and uninfected RBCs, that may be compounded by reduced RCD and increased blood viscosity [169, 170], and (4) intrarenal inflammation [171].

It has been proposed that early stages of AKI in malaria are pre-renal due to catecholamine-induced vasoconstriction, which could be reversed with an alpha-blocker [172]. In a pathophysiologic study of 3 patients with SM, those who presented with higher serum creatinine were found to have reduced cortical renal blood flow, as measured by radioactive isotope (^{133}Xenon), with ischaemia proposed to be due to hypovolaemia and blood hyperviscosity [168, 173]. However, one of these patients had AKI with normal renal blood flow with increased perfusion to the juxtamedullary cortex and outer medulla. In paediatric SM, total and intracellular body water were decreased suggestive of dehydration, but these measures did not correlate with serum creatinine, and there was no evidence of intravascular volume depletion [174]. A larger study of paediatric SM found that volume depletion was present at admission, as measured by central venous pressure (CVP),

which improved with volume expansion along with normalisation of creatinine [175]. A randomized controlled trial of dextran versus hydroxyethyl starch in paediatric severe malaria found that both fluids corrected shock with a concomitant improvement in urine output and creatinine [176]. A detailed haemodynamic study of adult SM (n=28) showed that all patients were hypovolaemic on admission; however, renal function was unrelated to volume status on admission and did not improve after fluid resuscitation [177]. Administering dopamine to malaria patients with AKI showed that increased renal blood flow was accompanied by a net decrease in oxygen extraction but no net change in oxygen consumption [178]. This suggests that increased renal blood flow does not improve perfusion of vessels obstructed by sequestered parasitised RBCs, albeit “renal dose’ dopamine in bacterial sepsis has also shown not to improve renal function despite an increase in renal blood flow. Taken together, these findings suggest that impairment of renal microvascular blood flow might be involved in the pathogenesis of malaria-associated AKI.

Hyperparasitaemia associated with AKI complicating falciparum and knowlesi malaria is inconsistently reported [5, 122, 146-149, 163, 171, 179-181]. While high peripheral parasitaemia is recognised to be an important feature of SM, in falciparum malaria microvascular obstruction due to sequestration of parasitised RBCs is a key pathophysiologic mechanism [182]. Renal histopathology from adult falciparum patients shows sequestration of parasitised RBCs in glomerular and tubulointerstitial vessels, which correlates with pre-mortem AKI [171]. African children with

SM and renal dysfunction were found to have increased kidney volume on ultrasound, which may reflect capillary and/ or post-venule sequestration resulting in increased hydrostatic pressure and renal fluid retention [183]. These studies suggest that sequestration within the renal microvasculature may contribute to falciparum malaria-associated AKI via vessel obstruction and ischaemia. Since the peripheral parasitaemia does not represent the sequestered parasite biomass, this may be an explanation of why parasitaemia has inconsistently correlated with malaria-associated AKI. Reduced RCD of infected and uninfected RBCs is predictive of severe disease and outcomes by contributing to the microvascular congestion [169, 184]. Recently, reduced RCD was associated with renal dysfunction in diabetic and non-diabetic control patients [185]. One hypothesis is that reduced RCD may contribute to AKI in malaria by limiting microvascular blood flow through renal vessels containing parasitised RBCs.

Since Hippocrates first noted an association with BWF and anuria in 430 BC, there has been a potential role for CFH in AKI complicated malaria [186]. However, it is still unclear why some individuals with BWF develop AKI while others do not. When the degree of intravascular haemolysis exceeds the scavenging capacity of plasma haptoglobin and haemopexin, CFH dimers are filtered by the glomeruli and reabsorbed by the proximal tubule [43]. Once the absorptive capacity is exceeded, haemoglobin appears in the urine. The renal threshold for haemoglobinuria of 0.3-0.6 g/L of CFH has been proposed [41]. Early pathology studies showed that malaria patients with renal insufficiency showed evidence of haemoglobinuric nephrosis,

where deeply pigmented casts in distal convoluting and collecting tubules were associated with necrosis of tubular epithelium [187]. Many of the early pathological studies describe ‘haemoglobinuric nephrosis’ in terms of luminal obstruction as being pathological, similar to descriptions of myoglobin in rhabdomyolysis [188]. However, the ability of CFH to mediate lipid peroxidation and renal vasoconstriction are likely more prominent mechanisms of renal injury. Recent research suggests CFH can initiate lipid peroxidation of arachidonic acid via redox cycling that generates F2-isoprostanes and isofurans. F2-isoprostanes have been shown to be potent vasoconstrictors that can cause a reduction in renal blood flow and GFR [61, 66, 189]. CFH is also likely to cause vasoconstriction through its quenching of endothelial nitric oxide (NO) in severe falciparum malaria, where it has been associated with impairment in NO-dependent endothelial dysfunction [50]. Arachidonic acids in membrane phospholipids are particularly vulnerable to this free radical-mediated lipid peroxidation, which can disrupt cell membranes by altering membrane organisation and integrity. In malaria, the high arachidonic acid content of infected RBC membranes reduces with parasite maturation, suggesting membrane peroxidation during parasite development [190]. Thus, reduction of RCD may contribute to AKI both by reducing blood flow in the renal microvasculature, and by the resulting F2-isoprostanes and isofurans that mediate renal vasoconstriction.

CFH-mediated oxidative stress is associated with AKI in diseases and medical conditions characterised by high CFH [65, 66, 69, 191]. While it is known that malaria is accompanied by intravascular haemolysis that

increases with disease severity [50], the role of CFH in AKI complicating SM is unknown. Furthermore, since both knowlesi and falciparum SM have similarly high rates of AKI and high CFH (unpublished), a common mechanism of CFH-mediated oxidative stress may contribute to AKI in these two infections. Of interest, while SM patients have been shown to have high concentrations of CFH and malondialdehyde, a marker of lipid peroxidation, not all severe patients develop AKI [50, 192]. One hypothesis is that the CFH-mediated damage may exhibit a quantitative effect, i.e. higher CFH concentration leads to incremental renal injury. Higher doses of infused haemoglobin results in quantitatively more haemoglobinuria, however this study did not look at renal function or injury [43]. An experimental model showed that haemoglobin-mediated AKI was associated with prior dehydration [193]. In studies on rhabdomyolysis, myoglobin at acid pH induced more profound vasoconstriction with associated GFR reduction [194]. Thus, a 'multiple-hit' hypothesis may accentuate malaria-associated AKI where patients who are hypovolaemic and/or more acidotic are particularly susceptible to CFH-mediated oxidative kidney damage.

Haemozoin is the byproduct of parasite host haemoglobin digestion that can remain in ghosted erythrocyte membranes after schizont rupture, and can be phagocytosed by host leukocytes causing immune activation and release of cytokines [195]. Deposition of pigment in renal vessel walls and glomeruli is commonly seen in biopsy and post-mortem specimens [171]. Histopathology also shows accumulation of mononuclear cells in the peritubular and glomerular capillaries. In a mouse model of malaria, mRNA of

proinflammatory cytokines is found to be upregulated in the kidney [196]. This suggests that host immune activation, such as leukocyte localization, activation, or cytokine release, may play a role in malaria-associated AKI. This thesis will assess the role of sequestration, CFH-mediated lipid peroxidation and immune activation in the pathophysiology of malaria-associated AKI.

1.4.5 Pathology

Pathologically the mechanisms underlying malaria-associated AKI are poorly understood and disagreement exists regarding the role of glomerular injury. Renal histopathology in adult and paediatric AKI commonly shows ATI, which can be severe, with pathological evidence of interstitial immune cell infiltrates and a mild endocapillary proliferative glomerulonephritis [171, 197]. Individual pathology cases reporting various glomerulonephropathies, and microangiopathy in *P.falciparum*, may reflect concomitant medication use or preexisting latent kidney disease, which declares itself during acute malaria illness as opposed to malaria being directly causative (Table 1.4.4). This is important to distinguish, since an accurate understanding of the pathophysiology is needed to develop targeted therapies for prevention and treatment of this serious complication of malaria.

There are several arguments against a glomerulopathy being the main pathological injury in malaria infection. Firstly, the clinical presentation and outcome is not consistent with this diagnosis. As discussed above, patients rarely present with clinical findings of nephritic or nephrotic syndrome.

Table 1.4.4 Case reports of rare pathology associated with malaria complicated by AKI

Pathology reported	Cases	Country	Species	Alternative explanation	Reference
P. falciparum					
IgA Nephropathy	1	Korean ex. Uganda	PF	Undiagnosed, latent, pre-existing IgA	Yoo DE et al. J Korean Med Sci 2012;27:446-449.
IgA nephropathy	1	Isfahan, Iran	PF	Undiagnosed, pre-existing IgA, or infection ie. HIV	Rafieian-Kopaei, M et.al. Iran J Public Health 2013; 42:529-533.
Cortical necrosis	1	Pune, India	PF	Likely secondary to intravascular hemolysis/heme induced damage/prolonged ischemia	Baliga KV et al. Renal Failure 2008; 30:461-463.
Collapsing glomerulopathy	1	Dakar, Senegal	PF	Sickle cell disease	Niang, A et al. Nephrol. Dial Transplant 2008; 23:3359-3361
Immune complex glomerulonephritis	7	Orissa, India	PF	Undiagnosed other cause of ICGN (no other testing done); not correlate with endocapillary changes therefore IC not explained and not causal	Rath, RN et al. Indian J Med Res 1990; 91:129-132.
P vivax					
MPGN with crescentic GN	1	Gujarat, India	PV	No PCR PV confirmation, low C3/C4; no HIV, no SPEP, UPEP	Patel MP et al. Parasitol Res 2013; 112:427-430.
PIGN	1	Ahmedabad, India	PV	Only smear; low C3/C4, ASO neg, ANA/ANCA neg; not rule out any other infection	Kanodia KV et al. Renal Fail 2013; 35:1024-1026
Acute cortical necrosis	1	Ahmedabad, India	PV	Smear, neg HRP2; BC/UC ne, lepto, dengue, 'hepatitis' neg; resulting CKD at 5mo f/u	Kute VB et al. Parasitol Res 2012; 111:2213-16
Thrombotic microangiopathy/ Tubulointerstitial nephritis	9	New Delhi, India	PV	Slide dx; -ve dengue, lepto, salmonella, HUS; Ddx drug induced	Sinha A et al. Clin Exp Nephrol 2013; 17:66-72
Acute tubular necrosis	2	Uttarakhand, India	PV	ATN most plausible	Kaushik R et al. TRSTMH 2013; 107:188-194

Glomerulonephritis causes a large proportion of end stage renal disease in developing countries but data is limited [198]. AKI associated with malaria resolves in approximately 2 weeks [22]. Although long-term sequelae of malaria-associated AKI are not well described, one series reported chronic kidney disease (CKD) in only 5% (4/98) of patients admitted with AKI caused by malaria [199]. Gupta *et al.* reported that 65% (n=13/20) of malaria-associated AKI showed evidence of mesangioproliferative glomerulonephritis [160]. However, such definitions require strict immunohistochemical and electron microscopic proof of immune complex deposition, which was lacking in this report. There would likely be more CKD associated with malaria if glomerulonephritis was a cause. Secondly, more common causes of glomerulonephritis with similar geographic distribution as malaria are chronic viral infections such as HIV [200], hepatitis B or C [201-203], which are often not tested for in these studies. In a pre- and post-mortem study of 43 HIV patients, 12/43 (28%) had 'incidental' findings of acute malaria, of whom 73% had histopathological changes commonly seen in HIV patients [152]. Therefore, in the absence of assessing for other causes of glomerulonephritis in malaria patients, it is problematic to assign causality to malaria. Thirdly, it is unclear from several studies if only light microscopy was used, which limits the accuracy of these pathological diagnoses. Fourthly, there is a paucity of comprehensive studies of malaria-associated AKI pathology. Overall the evidence thus far does not support a glomerulonephropathy as a prominent underlying injury in *P. falciparum*-associated AKI.

The largest renal pathology study in 63 fatal cases of *P.falciparum* malaria in Thai and Vietnamese adults showed evidence for widespread ATI in 66% of cases, with a mild endocapillary proliferative glomerulonephritis in 22%. Parasitized erythrocyte sequestration was seen in glomerular and peritubular capillaries, and the degree of microvascular sequestration was associated with the risk of developing AKI, defined as a creatinine > 3mg/dl and urine output < 400 ml per day. Additionally, there were increased interstitial infiltrates by a mixed population of CD3+ T cells, CD20+ B cells and CD68+ve monocyte macrophages. Immunoperoxidase and electron microscopy did not indicate deposition of immune complexes or changes to the basement membrane. Tubular casts with PAS positive cellular material and epithelial changes reflecting ATI were seen but no gross red cell cast nephropathy. Extraction of microRNA from formalin-fixed, paraffin-embedded tissues allowed microarray analysis of microRNA signatures, which showed a significant difference in unclustered hierarchy analysis separating malaria case from controls. Ingenuity analysis indicated upregulation of microRNAs controlling gene transcription in families of mRNA controlling cell death and apoptosis, cellular metabolism and inflammatory pathways [204].

A clinical histopathological study of 35 patients with BWF complicated by kidney injury showed medullary capillary congestion with cortical ischaemia and limited tubular obstruction [205]. This suggests that the pathology of AKI associated with BWF is related to alterations of the renal circulation, which may be associated with peripheral circulation atony, as opposed to physical

obstruction of the tubules. Pathology of 16 Vietnamese soldiers with hyperparasitaemic falciparum malaria showed that the kidneys were enlarged with cortical pallor and hyperemic medulla with striking medullary capillary congestion in 25% of cases [206]. The glomeruli appeared normal in the majority and the patients, while 73% and 67% had tubular pathology and pigmented casts, respectively. Studies of three smaller pathology case series' of falciparum malaria complicated by AKI (total n=12) similarly show tubular necrosis or vacuolization, casts and normal glomeruli [207-209]. Overall, the predominant pathologic injury in malaria-associated AKI is focused in the tubules, often with necrosis and pigmented casts, as opposed to glomerular pathology, with the exception of glomerular capillary sequestration and mild endocapillary hypercellularity due to exogenous monocytes, often phagocytosing pigment and 'ghosted' residual RBC membranes.

While cytoadherance and sequestration contribute to AKI in *P. falciparum* infections, this phenomenon does not occur in non-falciparum infections. However, AKI does complicate these non-falciparum infections, particularly *P. knowlesi* [164]. Small series or individual case reports of pathology in other human malaria species infections have also been reported. A histopathology case report of a fatal *P. knowlesi* patient showed ATI, intratubular casts, and intravascular parasitized RBCs [166]. *P. vivax* cases have shown ATI, membranoproliferative GN, cortical necrosis, and thrombotic angiopathy [162, 210-213]. The range of pathologies reported is likely a function of the under-recognition of pre-existing renal disease and/or

alternative etiologies as observed in *P. falciparum* case reports. The predominant pathologic injury is difficult to describe in *P. vivax* without data from a larger prospective histological study. The absence of sequestration contributing to the pathology of AKI in malaria caused by non-falciparum species suggests that common alternative mechanism leads to the ATI observed across species.

These pathophysiological studies require further work, including analysis of mRNA transcriptomes from fresh material to study the mechanisms of tubular and glomerular injury during AKI in malaria. The development of an *ex vivo* perfused kidney model represents a potential way to study organ function in response to malarial infection, as has been done in the human spleen [214].

1.4.6 Clinical presentation

Depending on the clinical setting, patients will present at different stages of their malaria illness and kidney injury. Patients presenting early after infection to primary health care facilities will generally have less severe malaria and a mild degree of pre-renal kidney dysfunction. However, patients presenting to tertiary care centres, usually on a referral basis after several health facility visits, are typical more severely ill with multiple organ involvement and more established renal kidney injury. Patients can be generally categorised into 4 groups: (1) those with UM or SM with few severity criteria who present early with pre-renal kidney dysfunction that resolves with fluid resuscitation, (2) those with SM and several severity

criteria who present with AKI that resolves in the absence of RRT, (3) those with SM who develop progressive AKI that resolves with antimalarial treatment and RRT, (4) those with SM and MODS, often with established anuric AKI and cerebral malaria, who die prior to or during dialysis with haemodynamic shock and/or respiratory failure. The prognosis of patients in the first 3 groups is favorable and those in group 4 tend to have a higher creatinine on admission with a greater degree of anaemia, likely reflecting a long duration of illness prior to presentation [22].

In a detailed study of fluid resuscitation in SM patients, invasive monitoring showed that all patients were hypovolemic [177], which likely contributes to AKI. Patients often receive intravenous fluid prior to transfer or referral and therefore may not be dehydrated or fluid overloaded. Clinical assessment of volume status is generally difficult due to the lack of sensitivity and specificity of physical signs. The jugular venous pressure (JVP) is often inaccurate and challenging to assess in resource limited settings where 30° positioning is difficult and lighting may by insufficient. Furthermore, the previous WHO recommendation of targeting a CVP of 0-5 cmH2O has been rescinded, as CVP and invasive haemodynamic monitoring do not necessarily accurately reflect effective circulating blood volume, likely due to microvascular obstruction [177, 215]. However, ensuring that the JVP does not rise above 0 cm above the sternal angle (corresponding to a CVP of less than 5cm) will likely benefit the patient as a crude measure of keeping the patient slightly dry to avoid precipitating pulmonary oedema. Assessing the JVP to gauge hypovolaemia and fluid responsiveness in SM patients with AKI is unlikely to

be helpful since these patients are not necessarily more hypovolaemic and kidney function does not improve with fluid loading [177]. In general, the proportion of adult and paediatric SM patients presenting with oligo-anuric AKI (oliguria: < 400 ml/24 hours; anuria: <50 ml/24 hours) is 70-80% and non-oliguric in 20-30% [22, 87, 88, 111, 142, 145, 199, 216-219]. Therefore, urine output alone is insufficient to rely upon to identify patients with AKI but may be a better measure to gauge fluid responsiveness in non-oliguric patients.

AKI complicating SM develops in older patients, 6 to 7 (±2.6) days into the malaria illness [22, 220]. Clinical signs that are frequently found with malaria-associated AKI in adults include jaundice (65%), anaemia (40%), decreased level of consciousness (36%), haemoglobinuria (23%) and acute respiratory distress syndrome (20%). AKI complicating SM does not present with clinical or laboratory features of nephrotic or nephritic syndrome, such as hypertension, oedema or active sediment [22]. Blood pressure is usually stable, whereas hypotension usually reflects concomitant bacterial sepsis or terminal cardiovascular collapse as part of MODS. Mild proteinuria (<1 g/24 hours) is common and often disappears with resolution of fever [168]. Generalised peripheral oedema often develops following excess volume resuscitation, as opposed to nephrotic syndrome, which results in increased pulmonary oedema and death [177]. Urine sediment is typically unremarkable but has been reported to contain haemoglobin, few RBCs, leukocytes and occasional granular casts [22]. Laboratory abnormalities associated with AKI include elevated aminotransferases, bilirubin, lactate,

potassium and decreased albumin, bicarbonate, base excess and pH [220]. High parasitaemia and anaemia are inconsistently associated with AKI [22, 171, 180, 181, 220].

Patients with AKI complicating SM typically have a prolonged hospital stay compared to those without AKI (mean ± SD: 24 ± 8.8 days versus 11 ± 3.9 days; *P* <0.001)[22]. Time to kidney function recovery is slower in patients with anuric AKI compared to oliguric AKI, time to creatinine < 2 mg/dL is 19 ± 6 days versus 16 ± 6 days (*P* =0.04) and urine output > 20 ml/kg/day 6 ± 4 versus 3 ± 2 days (*P* <0.001). Duration until creatinine returns to normal is 17 ± 6 days. Patients with anuria have a higher mortality and often require prompt RRT [22].

There are limited studies describing the clinical features of AKI in paediatric malaria. Studies in African children with UM revealed that 15-17% had intrinsic renal impairment, and 85% had glomerular and tubular patterns of proteinuria [221, 222]. In a study in Gambian children, 25% of patients with cerebral malaria had an elevated creatinine (> 62 μmol/L) with excretion of non-nephrotic range protein suggestive of tubular damage compared to febrile controls (*P* = 0.004). A recent study of Congolese paediatric AKI, found that the most common cause of AKI was malaria with BWF in 43% of patients [88]. In a second study of 378 Congolese children with SM, 89 (24%) had AKI (creatinine ≥ 0.3 mg/dL, or ≥ 50% with or without oliguria (<12ml/kg/24 hours)); of whom 87 (98%) had BWF, which was more frequent in male children older than 5 years [86]. A similar age distribution

was found in a study of paediatric SM in Bikaner, India; among the 30% (24/79) of patients with AKI (creatinine >3 mg/dL) 13% were under 5 years, 58% were 5-10 years old, and 25% were over 10 years of age [149]. In this study more patients aged 5-10 years had haemoglobinuria, however it was not noted whether this was associated with kidney dysfunction. A detailed study of haemoglobinuria in Nigerian children with SM, did not find a high association with AKI (6.3%, 3/48), however the definition of AKI differed [83]. Another study of paediatric SM in Togo found that 100% of patients with AKI (11/361, 3%; creatinine>3 mg/dL) had haemoglobinuria, however the method used to determine haemoglobinuria was not specified. The challenge of generalising from these studies is that the definition and methods of haemoglobinuria detection are not consistent and various AKI definitions are used. The potential strong association of haemoglobinuria in paediatric malaria-associated AKI, warrants further studies using standardized definitions of haemoglobinuria and AKI.

In Congolese children with malaria-associated AKI and BWF, the average delay in admission was 6.1 (±1.4) days and duration of hospitalization 15.0 (±3.4) days [86]. The most frequent clinical signs associated with AKI were “coca cola” urine (97%), prostration (95%), oliguria (85%), altered consciousness (72%) and jaundice (66%). The most common laboratory associations were severe anaemia (haemoglobin < 5 g/dL) and metabolic acidosis (bicarbonate <1 5mmol/L) in 60% and 58% of patients, respectively. Dialysis was indicated in 23/89 (26%) and the most frequent indications were prolonged anuria, uraemia, refractory hyperkalaemia and volume

overload. 11/89 (12%) died while receiving peritoneal dialysis and two were unable to receive dialysis for financial reasons.

In 63 adults from northern India with *P. vivax* complicated by AKI, 70% of patients were from rural areas and mean time until AKI development from onset of fever was 9.1 ± 4.3 days, indirectly suggesting that a delayed presentation is associated with AKI [211]. Categorising these patients by RIFLE criteria showed 43% (27/63) in RIFLE-R, 37% (23/63) in RIFLE-I, and 21% (13/63) in RIFLE-F groups. As observed in falciparum malaria, AKI in vivax malaria was observed as part of MODS. Oligo-anuric AKI was observed in 48% (35% oliguria, 13% anuria) versus 52% non-oliguric. Urinalysis and sediment microscopy was similar to what is observed in falciparum malaria, with mild proteinuria, and granular casts. The most common signs associated with AKI were hepatomegaly (81%), splenomegaly (67%), pedal oedema (38%) pulmonary rales (24%) and impaired consciousness (195). Common laboratory complications were thrombocytopenia (84%), anaemia (70%), and hepatic dysfunction (48%). Pulmonary oedema and cerebral malaria were less frequently associated with AKI compared to falciparum malaria in the literature (14% and 11%, respectively). Interestingly, parasitaemia correlated with decreasing GFR. Since sequestration does not occur in vivax malaria, this indirectly suggests that CFH released during schizont rupture may contribute to AKI in vivax malaria. Time to renal recovery was 10.3 ± 4.6 days in the absence of RRT and 21.4 ± 10.5 days in those who received RRT. Mortality was associated

with worsening AKI by RIFLE classification anaemia, longer duration of illness, hepatocellular injury, ARDS and shock.

AKI in paediatric severe vivax patients showed a younger age distribution compared to that of falciparum malaria in the study by Kochar *et al.*; of the 15.4% (10/65) severe patients with AKI (creatinine >3 mg/dL), 80% were under 5 years, 10% were 5-10 years and 10% were over 10 years of age [149]. The mean urea and creatinine concentrations were 194 (±47) mg/dL and 3.7 (±0.7) mg/dL, respectively. Oliguric AKI was present in 70% (7/10) versus 30% (3/10) had non-oliguric AKI. Requirement for RRT has been reported to be as high as 15% [223].

1.4.7 Diagnosis

The recognition and diagnosis of AKI is a challenge not unique to malaria endemic regions with limited resources. A UK study showed that recognition of post-admission AKI was unacceptably delayed in 43% of patients and when diagnosed, only 50% of AKI care was considered ‘good’ [224]. The KDIGO recommendations stress the importance of early recognition and diagnosis in order to avoid the substantial morbidity and mortality associated with AKI [103, 224]. This recommendation is crucial in SM and must be better implemented, particularly in children, as it is likely the most cost effective strategy that can be successfully tackled in endemic countries. The mortality of adults with malaria-associated AKI is unacceptably high, reaching 75% in the absence of RRT [22]. The available literature of falciparum malaria AKI in African children cites the mortality ranges from 13

to 53% (Supplementary Table 1.4.2). While RRT reduces mortality in adult patients from 75% to 26% [22], this lifesaving therapy is frequently unavailable and/or unaffordable in countries most affected by SM.

While there is no robust prognostic risk model or biomarker that can predict the development of AKI or need for RRT in SM, all malaria patients should be considered at risk of developing AKI. A study of adults with falciparum malaria found that elevated total bilirubin and decreased haemoglobin were associated AKI (defined by creatinine >1.6 mg/dl) in a multivariate analysis [225]. A comparative analysis of receiver under the operating curves of various admission laboratory indices showed that creatinine best predicted RRT requirement during admission [220]. The majority of malaria patients will have at least one risk factor for developing AKI including: (1) dehydration/volume depletion due to fever, vomiting, diaphoresis, and decreased oral intake, (2) hypoalbuminaemia [226], (3) older age [227], (4) male gender [217], (5) previous AKI [103], and (6) concomitant bacterial sepsis. G6PD deficiency has been associated with AKI complicating various infections, such as typhoid and hepatitis A, due to intravascular haemolysis [103, 228]. Chronic co-morbidities frequently associated with risk of AKI, such as diabetes, cardiovascular disease and chronic liver disease, are commonly unrecognized or not diagnosed at the time of admission in patients with malaria. It is important to recognise that a significant proportion of patients present to hospital with established AKI that needs prompt diagnosis and potentially RRT.

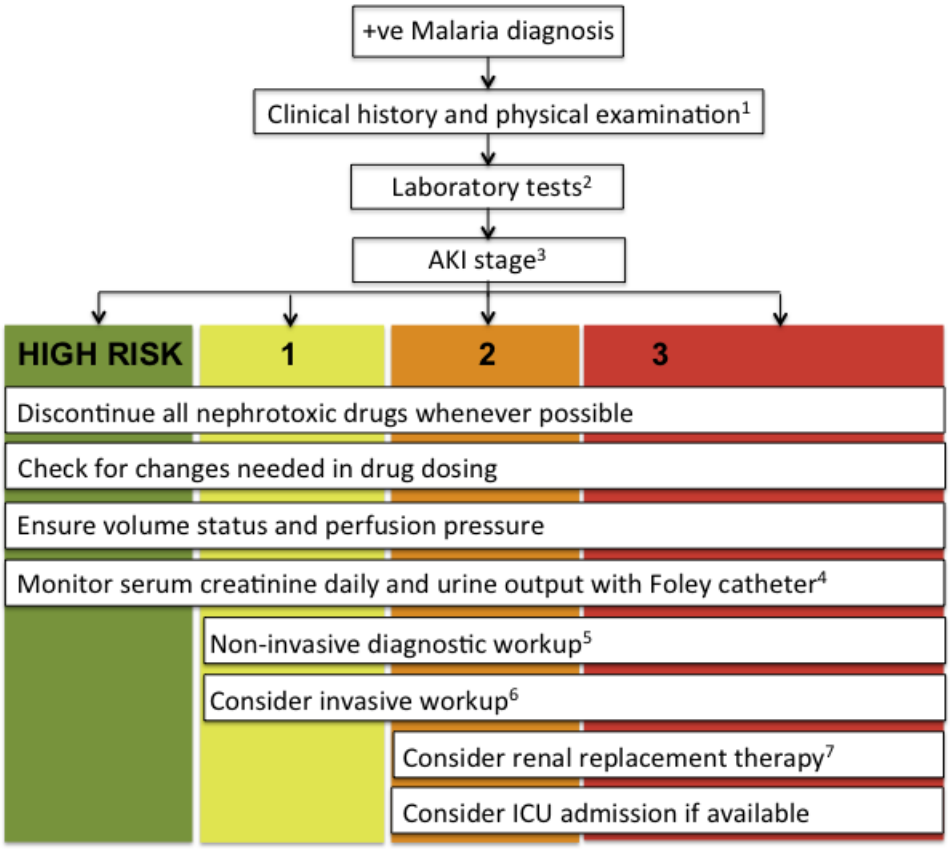


Figure 1.4.3 General management of AKI in malaria based on KDIGO staging (full legend on following page).

Figure 1.4.3 Legend.
[1] History including pre-admission medications, comorbidities, physical examination focusing on volume status and signs of concomitant sepsis;[2] Laboratory tests including serum creatinine, urea, electrolytes and full blood count. [3] Stage using KDIGO staging criteria. If baseline creatinine unknown, may need to use back-calculation of MDRD equation to estimate baseline creatinine. Urinary bladder catheterisation to monitor initial urine output if unconscious. If ambulating, urine should be collected (i.e. in water bottles) to monitor output. Patients should be managed according to AKI stage, since stage correlates with increased morbidity and mortality. [4]Daily creatinine and urine output to monitor change in AKI stage severity and guide management. [5]Additional investigations to assess AKI etiology: urine analysis, sediment microscopy, creatinine and sodium; renal ultrasound to assess kidney size, presence of pyelonephritis, and inferior vena cava filling as a gauge of volume status; AKI biomarkers if applicable. Nephrotoxic drugs, i.e. aminoglycosides, should be avoided when possible. Discontinuation of nephrotoxic drugs may assist with determining AKI etiology [6] If resources permit, monitor haemodynamic variables. Static central venous pressure is limited but recommended target is 0 to +5 cm H2O. Arterial pulse pressure as a dynamic variable may be more useful to gauge response to fluid administration. [7] Early referral to centre with RRT, particularly if one indication for RRT present. Patients with MODS are recommended to receive urgent dialysis within 2 hours [1]. Patients should be evaluated 3 months after AKI resolution to monitor of resolution of kidney function and/or development of chronic kidney disease. Adapted from [103].

At a minimum, all patients admitted with malaria should have kidney function and risks evaluated including: detailed past medical history, physical examination (particularly volume status), serum creatinine, estimated GFR, urine output (via urinary catheter), urinalysis, microscopy of urine sediment and ultrasound, if possible (Figure 1.4.3). While G6PD deficiency is important to screen for in malaria with respect to primaquine administration, it is also useful for AKI risk assessment as these patients are prone to intravascular haemolysis that can contribute to AKI. Urinary indices, including the fractional excretion of sodium, are imperfect in detecting AKI however are still helpful in differentiating the cause of AKI (Table 1.4.5). The aforementioned assessments are required to establish the diagnosis of AKI and assess the potential etiology. Concomitant causes that may contribute to and/or complicate AKI associated with SM are shown in Table 1.4.6. Based on their initial assessment and clinical course, patients should have repeat volume status examined, serum creatinine assessments and ongoing urine output monitoring to optimize patient management and stage patients as per the KDIGO criteria. Since small incremental increases of serum creatinine (≥ 0.3 mg/dl) have been associated with increased in-hospital mortality in both adults and children, creatinine should be monitored at least daily [4, 107]. Patients surviving AKI associated with malaria should be followed up on a long-term basis to monitor for sequelae including chronic kidney disease. Clinical classifications of SM relating to AKI include anuria and haemoglobinuria [1]. Anuria represents cessation of glomerular filtration; patients have a worse prognosis and require prompt RRT. While oliguria clinically indicates decreased GFR with a prerenal component, up to 80% of

Table 1.4.5 Urinary indices

Indices	**Prerenal**	**Renal**
Urine sediment	Hyaline casts	Abnormal
Specific gravity	> 1.020	~1.010
Urine osmolality (mOsm)	> 500	< 350
Urine sodium (mmol/l)	< 20	> 40
Fractional excretion of sodium (%)	< 1	> 2

Table 1.4.6 Common **c**auses complication and/or contributing to AKI in severe malaria

	Mechanism
Decreased kidney perfusion (prerenal)	
Decreased intravascular fluid volume	Extracellular fluid loss (diarrhea, vomiting, diaphoresis)
Peripheral vasodilation	Bacterial sepsis Hypoxia
Renal vasoconstriction	Bacterial sepsis Non-steroidal anti-inflammatory agents
Parenchymal or vascular diseases (renal)	
Renal vascular disorders	Disseminated intravascular coagulation
Interstitial nephritis	Drugs (ciprofloxacin, proton pump inhibitors
Co-infections	Bacterial sepsis Rickettsial infection (scrub or murine typhus) Leptospirosis Pyelonephritis
Tubular necrosis	Prolonged renal ischemia Nephrotoxins (aminoglycosides, tenofovir) Pigmenturia (haemoglobinuria)
Urinary tract obstruction (post-renal)	
Bladder	Prostate hypertrophy, stones
Urethral	Structure (post-sexually transmitted disease)

patients with malaria have non-oliguric AKI [160, 161]. Thus, the clinical diagnosis will capture patients with established AKI, but will under-diagnose mild AKI and diagnose patients late in their kidney disease precluding them from preventative management.

The use of urine microscopy in the clinical evaluation of AKI can provide vital information towards differentiating prerenal AKI and ATI, but unfortunately is not routinely utilized. Recently AKI scoring systems have shown good diagnostic performance in confirming ATI among hospitalized patients with AKI [117, 229]. Urine microscopy would be readily available in all malaria endemic areas where routine slide diagnostics are performed. Therefore, it is critical to develop a more robust and practical AKI diagnostic algorithm that includes clinical features, urine microscopy and colour to apply to patients with malaria. While recent research has intensively assessed the use of urine biomarkers in the diagnosis of AKI, there is still question regarding their utility in detecting early AKI and as predictors of AKI severity and reversibility. While these biomarkers are helpful to further our understanding of the pathophysiology, they have yet to be introduced into routine clinical practice for the diagnosis of AKI. These new potential tools are unfortunately not practical in most resource-limited settings due to high cost and unavailability given that even a simple test like creatinine is often difficult to routinely perform. Efforts should focus on developing an algorithm or scoring system that includes clinical and basic laboratory urine microscopy variables, which can be employed with ease and little cost in order to be effective for the timely diagnosis of malaria-associated AKI.

In general, AKI is often under-recognised and this can result in serious morbidity and mortality since there are no specific treatments available to reverse AKI other than RRT. To improve renal outcomes, early diagnosis and management is critical. All malaria patients should have a risk assessment for AKI and serum biochemistry on admission followed by frequent monitoring of renal function thereafter. Further studies are needed for the development of risk factor and prognostic models for the development of AKI, requirement for RRT and long-term outcomes of AKI.

1.4.8 Management

Fluid management is the cornerstone for treatment and prevention of malaria-associated AKI. However, fluid management in malaria is particularly challenging as patients are at increased risk of developing pulmonary capillary leak and subsequent pulmonary oedema if excess fluids are administered too rapidly. The WHO recommends individualized restrictive fluid management, keeping the patient slight dry, using isotonic crystalloids and not colloids [1, 21]. The general guidelines recommend fluid replacement according to clinical hydration status of the patient (Table 1.4.7). However caution must be exercised as in SM, patients with AKI are not necessarily more hypovolaemic and AKI often does not improve with fluid loading [177]. Further, 40-50% of SM patients with a CVP of 0 cmH2O or less are not fluid responsive [215, 230]. The critical aspect to these recommendations is that patients require reassessment of their respiratory status regularly (every 2 hours) in order to determine response or fluid overload and to further individualize fluid management. Urine output is

Table 1.4.7. General guidelines for fluid management in severe malaria

Clinical status	Adults	Children
1. No severe dehydration, no anuria, no shock	Initial: 0.9% saline IV 3-5 ml/kg/h for 6 hours Maintenance: 5% dextrose/0.9% saline IV 2-3 ml/kg/h Monitoring: every 2 hours for first 6 hours	Initial: 0.9% saline[#] IV 3-5 ml/kg/h for 3-4 hours Maintenance: 5% dextrose IV 2-3 ml/kg/h Monitoring: every 2 hours for first 6 hours
2. Severe dehydration, urine output <0.5 ml/kg/h*	Initial: 0.9% saline IV 10 ml/kg/h for 2 hours If no urine output (>0.5 ml/kg/h) response: Repeat: 5% dextrose/0.9% saline IV 5 ml/kg/h for 4 hours Monitoring: every 2 hours	--
3. Haemodynamic shock	Initial: 0.9% saline IV 20 ml/kg bolus If no blood pressure response: Repeat: 0.9% saline IV 20 ml/kg bolus Monitoring: every 30 minutes If no response after several liters of fluid start vasopressor	--

* Accurate urine output must be monitored with urinary catheter. # Alternate resuscitation fluid in children- 0.45% saline/5% dextrose. Adapted from WHO guidelines [1].

valuable to monitor fluid responsiveness to initial fluid administration however is an unreliable maker of kidney perfusion during AKI and may result in over- or under resuscitation if used to guide fluid therapy.

Patients with hypotensive haemodynamic shock should be cautiously bolused normal saline to restore intravascular volume and preserve renal perfusion [231]. Inadequate haemodynamic response after fluid resuscitation is an indication for vasopressors to be administered particularly in patients with, or at risk for, AKI [97]. Aggressive fluid resuscitation in paediatric patients with compensated haemodynamic shock is harmful. A large multi centre, multi-national study in 3,138 African children with severe febrile illness and compensated shock, showed a relative risk (95%CI) for death of 1.45 (1.13–1.86; P=0.003), with fluid bolus therapy (20 or 40 ml/kg saline or albumin) [232]. In this study, 1,793 (57%) of the children were malaria slide positive. In these children, the case fatality rate was 9.2% with fluid bolus therapy compared to 5.8% in the control group, corresponding to a relative risk (95%CI) for death of 1.59 (1.10-2.31). In paediatric non-malaria MODS patients with AKI, fluid overload was associated with increased mortality suggesting that early vasopressor support and RRT would be beneficial rather than ongoing fluid administration [233]. Alternative causes of shock must be considered since only 16% of SM is complicated by haemodynamic shock and broad-spectrum empiric non-nephrotoxic antibiotics should be initiated for concomitant bacterial sepsis. The best vasopressor to prevent or treat patients with malaria-associated AKI is unclear. A study of epinephrine in SM did not specifically report renal outcomes but showed the

development of worse lactic acidosis in the treatment group [178]. Studies in the general AKI literature have focused on dopamine, norepinephrine and vasopressin. A large clinical trial and subsequent meta-analysis of dopamine versus norepinephrine in septic patients shows that dopamine is associated with higher morality and adverse events [234, 235]. Vasopressin has shown potential for reducing progression of renal injury and mortality in patients with septic shock [236, 237]. However, this has not been studied in SM and the recommendation is to use norepinephrine as the preferred vasopressor agent [1]. The KDIGO guidelines recommend the use of vasoactive agents in volume replete patients with haemodynamic shock as a means to improve kidney perfusion [97].

As mentioned, previously low-dose dopamine was recommended for prevention and treatment of AKI on the basis of its potential to renal vasodilation and increase GFR. However, multiple studies now indicate that there is no benefit of low-dose dopamine in preventing or treating AKI and may cause harm [238]. In SM, low-dose dopamine was shown to increase renal blood flow but did not improve oxygen consumption, reduce peak creatinine or RRT requirement [239]. Low-dose dopamine is not recommended for prevention or treatment of malaria-associated AKI nor in other medical conditions [1, 97].

Loop diuretics have been recommended in the past for patients with oliguria to protect against AKI [97]. However, furosemide is ineffective in preventing and treating AKI and potentially may be harmful [240, 241]. Based on expert

opinion, an escalating dose regimen of furosemide over 1.5 hours is recommended to establish a diagnosis of malaria-associated AKI, guide fluid restriction and hasten triage for RRT [1]. Since furosemide may potentially cause kidney injury, future study of this indication and dosing would be valuable.

Mannitol was historically recommended for treatment of BWF and has been studied in rhabdomyolysis [206, 242, 243]. However, in patients with cerebral malaria, mannitol was shown to prolong coma and be potentially harmful [244]. Given the lack of prospective studies and potential harm, this therapy is not recommended by the KDIGO or WHO malaria guidelines.

Nephrotoxic drugs contribute to AKI in 20-30% of patients in general [97]. This may be overlooked in settings with limited drug formularies and standard empiric approaches to supportive medical treatment of malaria patients. Common nephrotoxic drugs administered empirically to malaria patients in Bangladesh include ciprofloxacin and proton pump inhibitors, which can cause interstitial nephritis. Given the serious consequences of developing malaria-associated AKI, it would be prudent to reduce the use of any nephrotoxic medication by considering alternative therapies i.e. paracetamol instead of non-steroidal anti-inflammatory antipyretic. The WHO malaria guidelines recommend administering broad-spectrum antibiotics, in addition to antimalarials, to children with SM in intermediate and high transmission areas [1]. In practice, adults with SM frequently receive empiric antibiotics concomitantly with antimalarials. The choice of antibiotics should

be based on local antimicrobial resistance patterns and available antibiotics. Aminoglycosides are particularly nephrotoxic in a dose-dependent manner. While the global trend of aminoglycoside use is decreasing, it is still used frequently and is a favored choice in several settings [245]. The KDIGO guidelines recommend against the use of aminoglycosides unless there is no other suitable alternative [97]. This message of limiting nephrotoxic medications for the treatment of malaria may be assisted by including a recommendation to limit the use of aminoglycosides. This is of particular importance since the potential combination effect of aminoglycosides with widely used cephalosporins is unknown.

While catheterisation is recommended and important in the management of SM for monitoring urine output and ruling out post-renal causes of AKI, their use increases the risk for catheter-associated urinary tract infections and pyelonephritis [246]. Even in the absence of catheterisation, up to 16% of children with malaria had concomitant urinary tract co-infections [247-250]. Parasitaemia was associated with higher number of colony forming units on urine culture [247]. In pregnant women, HIV, malaria and HIV-malaria co-infection was associated with increased risk of urinary tract infection [251]. Therefore, urinary catheters must be promptly removed if the patient has established oligo/anuric AKI, or when patients become ambulatory and the catheter is not clinically indicated to avoid iatrogenic upper and lower urinary tract infections.

BWF is associated with renal dysfunction in up to 64% of patients [33]. The proposed mechanisms of renal injury include tubular obstruction, direct proximal tubule cell injury and vasoconstriction. Similar to the treatment of rhabdomyolysis, the general principles of treatment include preventing AKI by prompt administration of isotonic saline to (1) correct volume depletion, and (2) reduce tubular cast formation. In general, the amount and rate of fluid administration for haem pigment-mediated AKI is unclear. Given the high risk of pulmonary oedema with aggressive fluid resuscitation in SM, intravenous saline should be administered as per the current recommendations in Table 1.6.6. Monitoring volume status and urine output is paramount. Haemoglobin and/or haematocrit should be monitored to guide intravenous fluid and requirement for blood transfusion to maintain a haematocrit above 20% [1]. Antimalarials should not be discontinued. In the absence of diuresis of at least 4 ml/kg/h with worsening renal function, or ongoing anuria, patients should be referred for potential RRT, which was shown to be life saving in a small case series of BWF patients [206]. Administration of bicarbonate to alkalinise the urine is recommended under specific circumstances in severe myoglobin-induced AKI (rhabdomyolysis) despite limited evidence supporting the benefit [97]. There are no studies addressing alkalization in haemoglobin-induced AKI (haemolysis) or in patients with malaria and in general is not recommended. A study of bicarbonate administration after cardiopulmonary bypass showed there was no association with lower incidence of AKI but did reduce AKI severity and need for RRT [252]. However, CFH was not measured in this study to specifically address if the mechanism of protection was associated with

haemoglobin-mediated injury. Mannitol and loop diuretics have not shown clear clinical benefit, largely in studies of rhabdomyolysis, and are not recommended.

Treatment of established malaria-associated AKI with RRT reduces mortality from 75% to 26% [22]. The decision of whether and when to start RRT are challenging issues when managing malaria-associated AKI, particularly in situations where the patient may require long and costly transfer to a tertiary centre with RRT facilities. In general, RRT is urgently indicated when biochemical disturbances and volume overload refractory to conventional therapy are present (Table 1.4.8). The specific threshold concentrations recommended in the malaria guidelines are not universal standards due to limited evidence [103]. In SM, additional indications for RRT are included in the guidelines based on the finding that anuria, and elevated or rapid rising creatinine concentrations are sensitive indicators for RRT [22, 253]. A threshold of urea as an indication for early dialysis is a focus of ongoing study in the AKI literature. In a large multicentre prospective study, the initiation of RRT at higher urea concentrations (> 27.1 mmol/L) was associated with a higher risk of mortality (RR 1.85; 95%CI 1.16-2.96) [254]. In light of this finding, assessing the urea threshold for RRT recommended in the WHO malaria guidelines of > 35 mmol/L would be a valuable focus of future study. The symptoms of uraemia are not specified in the indications for RRT. While cerebral malaria is often associated with AKI it is unclear if a component of the coma is due to uraemic encephalopathy. It is important to

Table 1.4.8 Indications for renal replacement therapy

Indications	Definition
1. Oliguria¶	Urine output < 200 ml/12 hours¶
2. Anuria¶	Urine output ≤ 50 ml /12 hours¶
3. Uraemia*	Urea > 35 mmol¶
4. Acidaemia*	pH < 7.1¶
5. Volume overload*	Pulmonary oedema unresponsive to diuretics
6. Electrolyte disorder*	K > 6.5 mM or rapidly rising¶ Na < 100 mmol and > 160 mmol¶
7. Renal dysfunction¶	Creatinine > 400 μmol¶

* general urgent indications for renal replacement therapy (RRT).
¶Recommendations in the WHO severe malaria guidelines [1]. If 1 criteria present, consider RRT. If 2 criteria present, strongly recommend RRT.

consider the broader clinical context as opposed to basing the decision to start RRT on a single creatinine or urea concentration [103].

In the absence of urgent indications, in general the decision to initiate RRT in resource-limited settings is delayed due to concerns of potential cost and logistics of patient transfer, procedure-associated risks, and renal recovery compromise [103]. However, since AKI in malaria progresses rapidly and is often compounded by multi-organ dysfunction syndrome, early RRT is recommended, based on expert opinion, as early as within 2 hours in the latter situation [1]. In critically ill non-malaria ICU patients, patients stratified into receiving 'late' dialysis (> 5 days from ICU admission) had a higher mortality, longer duration of RRT and greater dependence on RRT compared to those receiving 'early' dialysis (<2 days) [255]. In ICU patients post-operative AKI classified as receiving early (RIFLE score 0 or R) or late (RIFLE score I or F) RRT, late dialysis was an independent indicator of in-hospital mortality (HR 1.8, 95%CI 1.1-3.2; *P* =0.027) [256]. A study of Thai adults with SM showed an association of RRT with AKI severity, defined by the RIFLE criteria, where 0% RIFLE-R, 11.6% RIFLE-I and 44.9% RIFLE-F required RRT [112]. While definitive studies regarding early versus late initiation of RRT in SM are warranted, patients should still be referred as early as possible to a nephrology centre or ICU with RRT if AKI is suspected.

There is no prospective study specifically addressing need for RRT in children with SM complicated with AKI, but the above recommendations are relevant for paediatric patients. Extrapolating from the AKI literature suggests that timing of RRT in children is critical in volume overload [103]. Evidence suggests a reduction in mortality with less than 10-20% fluid overload and early initiation of RRT [233, 257, 258].

Prior to RRT and/or while arranging for RRT patients require close monitoring for complications of AKI and potential urgent management including: (1) fluid restriction, and/or diuretics to prevent and manage volume overload, (2) treating life-threatening hyperkalaemia (defined by some authors as potassium >7mmol/L with ECG change [1], but more conservatively treatment should be initiated if ECG changes or potassium ≥ 6.5 mmol/L) by promoting intracellular potassium shift (i.e. intravenous glucose and insulin, intravenous calcium) and (3) correcting acidosis (venous pH <7.1) with intravenous bicarbonate. If these complications are refractory to medical management then RRT should be administered promptly. Deterioration in mental status may represent uraemic encephalopathy and indication for RRT.

Intermittent haemodialysis (IHD) and continuous veno-venous haemofiltration (CVVHF) have been shown to be superior to peritoneal dialysis (PD) in adult patients with SM [253]. Haemofiltration was associated with faster correction of acidosis and azotaemia, shorter duration of therapy and was more cost effective. However, in resource-limited settings the

modality choice for both adults and children is usually based on availability and local expertise. In the absence of IHD, life-saving peritoneal dialysis should he initiated promptly if this is only modality available and may be more appropriate in haemodynamically unstable patients [259]. CVVHF has not been compared to IHD in SM but theoretically may be beneficial in patients with haemodynamic instability patients and/or cerebral malaria with brain oedema. In a large meta-analysis of critically ill patients treated with continuous RTT and IHD, outcomes including in-hospital mortality, length of hospital stay and renal recovery were similar [260]. Although continuous RRT is recommended in haemodynamically unstable patients with AKI, the cost and establishment may not be practical in resource-limited settings.

Renal dosing of artemisinins in patients with AKI is not necessary. Quinine does require dose adjustment according to renal dysfunction after 48 hours of full dosage. If the patient is on dialysis, the dose of quinine should be administered after haemodialysis as 6.5% clearance is achieved with 1 hour of haemodialysis. Concomitant medications should be dose adjusted according to renal function as needed.

A general approach to management of malaria-associated AKI starting from the time patients are first identified as high risk, or have established AKI is outlined in Figure 1.4.4, adapted from the KDIGO guidelines [103]. The overall goals of therapy are to reduce kidney injury and complications. Since all malaria patients are at risk for AKI, the outlined steps should be applied to

all patients as a strategy to promote early diagnosis and potential prevention of malaria-associated AKI.

Aside from RRT there is no other treatment modality clinically proven to reduce morbidity or mortality of AKI in malaria. Unfortunately, the capacity to deliver RRT in resource-limited settings is limited. Further, patients with hepatitis B, C and/or HIV co-infection are often excluded from receiving dialysis due to lack of dedicated dialysis machines. Further research into the timing, early prediction of RRT requirement, to prioritise use of scarce resources, and other possible treatment and/or prevention therapies is warranted.

1.4.9 Prognosis

The short-term outcomes of malaria-associated AKI are well reported. Renal dysfunction is an independent predictor of mortality in both adults and children [12, 19]. In a study of adult patients with *P. falciparum* and *P. vivax,* mild AKI (creatinine 1.6-3.0 mg/dl) was associated with higher malaria-associated organ complications than in patients with no AKI (P <0.05) [139]. In a study of Vietnamese adults, the mortality of SM patients with AKI reaches 75% in the absence of dialysis [22]. The mortality rate was reduced to approximately 25% when RRT is administered [22]. In the current systematic review (Supplementary Tables 1-3), the mortality of malaria-associated AKI among patients admitted to hospital with AKI was 22% in adults [87, 217-219, 261-264] and 21% in children [142, 145]. Among patients admitted with malaria-associated AKI in Asia and Africa, the

average mortality rate was 42% in adults [3, 22, 112, 124, 129-131, 133, 135, 139, 160, 161, 265-267] and 26% in children [125, 127, 140, 268]. The mortality rate of imported malaria-associated AKI remains elevated at 15-23% in Western settings [156, 157]. In a retrospective study of Thai adults in-hospital mortality was associated with severity of AKI defined by the RIFLE criteria, with mortalities of 10.3% RIFLE-R, 30.2% RIFLE-I, and 30.5% RIFLE-F [112]. AKI may contribute to multiple-organ dysfunction and complicate SM as a result. Renal recovery to baseline creatinine was shown to be 17 +/- 6 days [22].

The mortality rate of *P. vivax* associated with AKI is reported to be approximately 12% [146, 149, 160, 161, 211, 269-272]. Prognosis is worse in patients with anuria, multi-organ involvement and high parasitaemia [22]. In two small case series' of *P. knowlesi* associated AKI, the mortality rate was 50% (n=22; and 33% (n=8).

The long-term outcomes of malaria-associated AKI are less well characterised. Chronic kidney disease (CKD) and dialysis dependence are rarely described following acute malaria complicated by AKI, but has been reported to occur in up to 5% of patients [199]. Bonomini *et al*. found that among non-malaria patients with AKI due to ATI, confirmed on biopsy, 31% had partial recovery, while 6% and 11% had no recovery at 1 year and 5 years, respectively [273]. In non-malaria paediatric ICU patients with AKI who recovered kidney function, chronic kidney disease developed 1 to 3 years later in 10% (13/126) [274]. In non-malaria adult AKI requiring RRT,

long-term sequelae include a 28-fold increased risk in developing CKD and 2-fold risk of death [275]. Further long-term outcome studies are required to explore the impact of malaria-associated AKI, which may contribute to the hidden burden of CKD in resource-limited settings [276].

1.5 Paracetamol use in malaria

Fever is a cardinal symptom of malaria. Malaria fever exhibits a daily spiking pattern that corresponds to schizogony, where the intraerythrocytic contents released induce a pro-inflammatory response. The classic description of the tertiary periodic fevers (every 2 days) in falciparum malaria is seen mainly in untreated infection. Fever in malaria is proposed to have several negative effects. In adults, high fever can cause mild impairment of consciousness (delirium, confusion, obtundation) even in the absence of cerebral pathology [98]. In children, febrile convulsions and psychosis have been reported with isolated high fevers above 38.5°C [98, 277]. In pregnant women, fever has been linked to worsening foetal distress, uterine contractions and increased susceptibility to hypoglycaemia [277, 278]. In all populations, fevers are associated with increased nausea, vomiting and diaphoresis, which can exacerbate hypovolaemia and potentiate AKI and hyponatraemia.

Paracetamol is one of the most extensively administered analgesics/antipyretics worldwide. In 1990, paracetamol suppositories were first formally recommended for fever in malaria as an adjunct to mechanical antipyresis [98]. Subsequent guidelines continue to recommend regularly dosed paracetamol, administered orally or by suppository, to treat fever in

malaria however various temperature thresholds for treatment have been proposed, ranging from >38°C to >39°C [100, 101, 279, 280]. In practice, paracetamol is frequently administered on an 'as needed' basis for temperatures above a certain threshold, which often varies on local hospital prescribing practices. Studies showing conflicting therapeutic benefit have questioned the continued recommendation of paracetamol in the treatment of fever in malaria.

1.5.1 Paracetamol mechanism of action

Despite the widespread use of paracetamol for over 100 years, its mechanism of action is not completely understood. It is believed to have a principally central analgesic effect potentially mediated by: (1) a serotonergic pathway through 5-HT3 receptors, (2) an active metabolite that indirectly activates cannabinoid receptors, (3) inhibiting L-arginine nitric oxide pathway by interfering with N-methyl-D-aspartate receptor activation, and/or (4) a weak inhibition of cyclooxygenase (COX) mediated production of prostaglandins in the hypothalamus [281].

However, recent studies support that acts both centrally and peripherally in a tissue dependent manner as a reducing agent to inhibit COX mediated metabolism of arachidonic acid to unstable prostaglandin H2 endoperoxide and subsequent prostaglandin formation [63, 282]. It exhibits preferential COX-2 (inducible) over COX-1 (constitutive) inhibition in tissues by reducing the higher oxidation state of the COX enzyme [283]. Each COX enzyme is made up of 2 domains: a cyclooxygenase (COX) site and a peroxidase

(POX) site. The function of the COX domain is dependent on being oxidized by POX at its active site. The peroxidase site functions independently from the COX site. It contains haem in the active site that undergoes oxidation to form ferryl protoporphyrin IX, which is required to oxidize the COX active site via intermolecular electron transfer. Paracetamol indirectly reduces the amount of oxidized COX active site by its direct action on POX domain, where it acts as a reducing co-substrate to partially reduce the ferryl state resulting in less activity in the COX active site for conversion of arachidonic acid to prostaglandins. More recently, paracetamol has been shown to be potent inhibitor of haemoprotein-induced lipid peroxidation by reducing ferryl haemoglobin to ferric haemoglobin [66]

1.5.2 Pharmacokinetics of paracetamol in falciparum malaria

The pharmacokinetics of single-dose paracetamol in malaria have only been described in patients with uncomplicated disease [284]. Further, there are no studies reporting the pharmacokinetics in patients with malaria after multiple regular doses, which would be required to achieve therapeutic steady state concentrations. The pharmacokinetics of paracetamol in patients with severe or moderately severe malaria have not been described. Since artemisinins have become first line therapy for the treatment of malaria, paracetamol continues to be recommended as first line therapy for antipyresis. However, no drug interaction studies between artesminins and paracetamol have been conducted. This thesis will address the aforementioned knowledge gaps.

Oral paracetamol

In healthy volunteers, oral (PO) paracetamol is best described by a two-compartment open model, where it is rapidly absorbed and distributed, reaching peak concentration within 40 to 60 minutes with a bioavailability of approximately 75% [285]. The elimination half-life is 2 to 3 hours and thus time to reach steady-state concentration is achieved between 10 to 15 hours. It is extensively metabolized by conjugation, and a minor oxidation pathway, in the liver and excreted mainly as glucuronide and sulphate conjugates; only 2 to 5% is excreted unchanged in the urine [285]. Multiple dose studies in healthy volunteers do not suggest dose dependent pharmacokinetics for doses < 18 mg/kg but non-linear behavior was suggested in one patient who received doses higher than 18 mg/kg [286]. There are no conclusive data regarding dose dependency in doses >18 mg/kg in healthy volunteers or patients with liver dysfunction who may have impaired metabolism.

In the rat malaria model, the formation clearance of paracetamol glucuronide is decreased and paracetamol sulphate increased, resulting in no overall change in the metabolic clearance of paracetamol [287]. The pharmacokinetics of PO paracetamol (1 g) were shown to be similar in Thai patients with acute and convalescent UM and in healthy subjects [284, 288].

Rectal paracetamol

Rectal paracetamol (PR) is frequently administered to malaria patients who are unable to tolerate the drug via the oral or nasogastric (NG) route. Formularies still suggest that the rectal dose is the same as the oral dosage

(15 mg/kg every 6 hours). However, paracetamol administered by rectal suppositories has slower and incomplete absorption, resulting in a delay to reach a sub-therapeutic steady state [289]. In practice, patients rarely receive regular suppository administration and instead receive doses closer to 10mg/kg on an 'as needed' basis. Computer simulations of rectal administration suggests that a loading dose of 50mg/kg followed by 30mg/kg every 6 hours would achieve therapeutic plasma paracetamol steady state concentration of 10 to 20 µg/ml [290]. Birmingham *et al.* studied a new paracetamol rectal dosing recommendation, showing that administering a 40mg/kg loading dose followed by 20 mg/kg every 6 hours to children 2 to 12 years resulted in therapeutic paracetamol concentrations with no evidence of drug accumulation [291]. In children aged 9 weeks to 11 years, it was shown that rectal suppositories at 25mg/kg every 6hours for 5 days after surgery was safe and that a simulated loading dose of 35 mg/kg followed by 25 mg/kg every 6hours would produce therapeutic steady state levels [292]. In healthy adult volunteers, a dose finding study showed that a single dose of rectal paracetamol of 35 mg/kg and 40 mg/kg achieved sustained therapeutic concentrations while doses of 15 mg/kg and 25 mg/kg were sub-therapeutic [293]. Similarly, in adult women undergoing surgery, single dose 40 mg/kg paracetamol suppositories achieved therapeutic concentration whereas a 20 mg/kg suppository did not [294]. None of the studies using loading rectal doses have shown drug accumulation or hepatotoxicity. Despite widespread use, the pharmacokinetics of single or multiple dose rectal paracetamol have never been described in UM or SM.

Intramuscular and intravenous paracetamol

Adequate pharmacokinetic data for intramuscular (IM) paracetamol are lacking. In healthy volunteers, 600mg IM paracetamol resulted in a maximum plasma concentration of 7.52 μg/ml [295]. This bioavailability would be expected to be even lower in ill patients with poor muscle perfusion. IM pharmacokinetics of paracetamol have not been described in patients with malaria.

An intravenous (IV) formulation of paracetamol has recently become available, however the availability is limited in malaria endemic countries [296]. In healthy volunteers, the maximum concentration of IV paracetamol was shown to be 2-fold higher compared to oral paracetamol, whereas the area under the curve (AUC) and volume of distribution (Vd) were similar between these two routes of administration [297]. IV paracetamol has not been studied in malaria, however should studies of oral paracetamol show therapeutic benefit, then subsequent studies of the IV route may be valuable. Given the ease of IV administration, showing a safe, therapeutic benefit would give strength to advocate for its global distribution.

Interactions with antimalarials

Drug interactions between paracetamol and antimalarials have previously been described. In healthy Ghanaian volunteers, chloroquine reduced the time to reach peak plasma paracetamol concentrations (T_{MAX}), and increased paracetamol peak plasma concentration (C_{MAX}) and AUC [298]. This effect was explained by accelerated gastric emptying induced by

chloroquine. Primaquine can inhibit conjugative and oxidative metabolism via cytochrome P450, but a single dose has been shown to have no effect on paracetamol metabolism [299]. Ascorbic acid can exhibit haemoprotein-ferryl reductase activity and has been shown to reduce the in vitro activity of artemisinins [66, 300]. Since paracetamol exhibits a similar ferryl-reductase activity [66], there is potential concern for an antagonistic interaction with artemisinin antimalarials.

The mechanism of action of artemisinins is not completely understood. Various mechanisms have been proposed for its initial activation and subsequent target(s), which result in an antiparasitic effect [301]. Broadly, artesunate is proposed to exert its anti-malarial effect through an oxidative mechanism. Thus, the antioxidant ferryl-reductase activity of paracetamol may potentially decrease the efficacy of artesunate. Studies suggest that artemisinins are activated by ferrous haem and/or non-haem ferrous iron, which are proposed to cleave the endoperoxide bridge that is vital for artemisinin drug activity [302]. The activation is proposed to generate a ferryl-oxo intermediate and this intermediate may itself contribute the oxidative damage observed during treatment with artemisinins [303-305]. Thus paracetamol would potentially reduce the activation and/or action of artemisinins if the ferryl intermediate is essential to its mechanism of action.

There are three sources of ferrous haem and iron that could potentially activate artesunate, namely: (1) intravascular (extra-erythrocytic) space, (2) intra-erythrocytic cytoplasm, and (3) intra-erythrocytic parasite after host

haemoglobin digestion. Intravascularly, excessive haemolysis of infected and uninfected RBCs can deplete the haemoglobin scavenging systems and may result in a build up of free ferrous haemoglobin, ferric haemoglobin, ferric haem along with transferrin and non-transferrin bound iron. Potentially systemic redox cycling of free ferric haem may generate ferrous haem that could activate artesunate intravascularly [306]. Intravascular activation could lead to its deactivation and reduced antimalarial activity. Paracetamol could then potentially act synergistically by preventing intravascular artesunate activation.

Intraerythrocytic sources of iron include: (1) ferrous haemoglobin, (2) ferritin bound iron and (3) cytoplasmic labile iron pool [307]. The potential involvement of ferritin bound or non-haem iron activation of artesunate is seems likely considering that artesunate is active against other protozoan organisms, viruses and cancer cells that do not infect RBCs or digest host haemoglobin. All these organisms and tumor cells have been shown to possess transferrin receptors to obtain iron and many of the viruses that are susceptible to artemisinins are hepatotrophic, where hepatocytes are involved in ferritin iron recycling. Cancer cells have been shown to display increased transferrin receptors and synthetic transferrin-artesunate conjugates have been shown to mediate enhanced anti-tumor effects [308]. In cancer cells artesunate was found to accumulate in lysosomes and its anti-tumor effect shown to be mediated by the release of iron in the lysosomes, which results from the lysosomal degredation of the iron storage protein ferritin [309]. While parasite specific transferrin and non-transferrin

iron uptake has been proposed, the literature is inconclusive that the intraerythrocytic parasite can utilize host ferritin-bound iron [307, 310-314]. Recently, it was shown that the latent iron pools increases with parasite maturation and suggested that the intra-erythrocytic parasite may be able to access intraerythrocytic iron [307]. Artesunate is localized to the tubulovesicular membranous network in the RBC cytoplasm and parasite cytoplasm, and its intracellular delivery mediated by a carrier at the infected RBC membrane [315, 316]. Thus, the intraerythrocytic parasite uptake of host erythrocyte labile iron could lead to the activation of artesunate during the early ring stage, before host cell haemoglobin digestion by the parasite begins. Since paracetamol is not known to possess any reductive activity with iron in the absence of a haemoglobin moiety, it would not be expected to interfere with artesunate in this circumstance.

Studies of intraparasitic sources of ferrous haem and iron have focused on the parasite digestion of host haemoglobin. It was recently shown that artemisinin activity is highly dependent on host haemoglobin digestion by the intraerythrocytic parasite [317]. This would explain the potent activity of artemisinin against late stage rings and trophozoites since parasite host haemoglobin digestion begins 14 hours after parasite invasion. When host haemoglobin enters the acidic parasite digestive vacuole, the haem is oxidized to haematin and stored in haemazoin. It has been proposed that haematin may enter the parasite cytoplasm to be reduced to haem that could then activate artemisinins [318]. The recent finding that asexual blood stage parasites with ablated haem biosynthesis grow normally supports that

a host haem scavenging mechanism by the parasite exists [319]. The proposed host haem sources are the haem from the digestive vacuole or from the erythrocyte cytosol that is taken up by the parasite and transported within the parasite by haem-binding proteins. Alternatively, an unstable ferrous haem still bound to the denatured globin has been proposed which could activate artemisinins in the digestive vacuole [320]. However, the absence of artesunate localization to the digestive vacuole argues against this hypothesis [313]. It is unclear how the iron from host haemoglobin is released is released from haem since no functional parasite haem-oxygenase has been identified [321]. It has been proposed that the iron release may be mediated by a ferryl radical species [320]. In this case, paracetamol could potentially inhibit artemisinin activation.

The literature suggests that either intravascular or intraerythrocytic ferrous haem/ferrous iron may be involved in the early activation of artesunate. In the case of intravascular activation of artemisinins, paracetamol may act synergistically by preventing its activation whereas intra-erythrocytic activation may result in paracetamol antagonizing artemisinins.

The biological targets of artesminins are all still unclear. Studies support that artemisinins can lead to haem-mediated lipid peroxidation, which may lead to parasite autodigestion [322]. The general oxidative stress mechanism of artemisinins is supported by the transcriptomics of artemisinin resistant parasites, which show an upregulation of unfolded protein response pathways proposed to mitigate oxidative damage [323]. If paracetamol can

inhibit haem-mediated lipid peroxidation then it may antagonize the antiparasitic effects of artesunate.

1.5.3 Pharmacodynamics of paracetamol in falciparum malaria

Multiple dose oral and rectal paracetamol pharmacodynamic studies in severe and moderately severe malaria have not been conducted. The pharmacodynamic effects of oral and rectal paracetamol have only been studied in patients with UM. There is conflicting evidence regarding its clinical effects on fever and parasite clearance [324-327]. However, there are limitations in these studies including: low paracetamol dosage, lack of parallel paracetamol pharmacokinetics/drug concentration assessment, adequacy of endpoint measures, and unsupervised dosing. This thesis will address the conflicting pharmacodynamic effects of paracetamol on fever clearance and parasite clearance in addition to assessing its potential novel renoprotective effect in in severe and moderately severe falciparum malaria.

Acetaminophen antipyretic efficacy is dose dependent where the proposed effective antipyretic concentration is 10-20 μg/ml [328]. The clinical efficacy of maximum plasma concentration to temperature reduction is delayed by several hours as the drug must reach the hypocampus to induce antipyresis [329]. A pharmacodynamic study of single dose (15 mg/kg) oral paracetamol showed a rapid decrease in temperature by 3.9 hours post-dose and improved symptoms associated with fever in adults with UM [326]. In a study of multiple oral doses of paracetamol (50 mg/kg for 3 days) in children with UM, there was an insignificant temperature reduction on the first day of

treatment compared to the placebo group [325]. However, in this study paracetamol administration was not directly observed, paracetamol concentrations were not measured and temperature was monitored only twice daily. In Gabonese children with UM, multiple doses of rectal paracetamol reduced fever clearance time compared to mechanical antipyresis (fanning and tepid sponging) but this difference was not significant [324]. This study did not report actual temperature changes and administered rectal paracetamol at a dose of 50 mg/kg/day, which would not likely have achieved therapeutic antipyretic concentrations [330]. While loading doses and higher maintenance doses of rectal paracetamol have been proposed to achieve therapeutic concentration levels, these studies have not been performed in patients with malaria.

In the Gabonese study, those treated with paracetamol showed a prolonged parasite clearance time (difference of 16 hours (8-24 hours), p =0.004) compared to those treated with mechanical antipyresis alone [324]. This raised the hypothesis that antipyretics may attenuate the host response against malaria infection. An alternative hypothesis is that paracetamol may reduce fever-induced sequestration, which could manifest as prolonged parasite clearance since more infected RBCs would persist in the peripheral circulation [331]. However, since parasite clearance is dependent on pre-treatment parasitaemia, it is more accurate to assess the linear slope of parasite clearance curve to determine the parasite clearance rate, as oppose to parasite clearance time [332, 333]. A systematic review of paracetamol in UM reviewed three randomised controlled trials (RCT) and

found that there was insufficient evidence that antipyretic medicines prolonged illness [334]. The more recent study in Guinea-Bissau, found no difference in parasitologic response in children with UM treated with a multiple oral dose paracetamol regimen compared to placebo [325].

1.5.4 Paracetamol, AKI and survival

The novel mechanism of paracetamol recently demonstrated that it acts as a potent inhibitor of haemoprotein-catalyzed lipid peroxidation by reducing ferryl haem to its less toxic ferric state and quenching globin radicals [66] (Figure 1.4.5). In a proof of concept study, paracetamol at therapeutic levels was shown to significantly decrease oxidative kidney damage and improve kidney function by inhibiting the haemoprotein-catalyzed lipid peroxidation in a rat model of rhabdomyolysis-induced kidney injury [66]. Two RCTs of paracetamol in children and adults undergoing surgery with cardiopulmonary bypass, showed that paracetamol to attenuated CFH-mediated production of plasma isofurans [335, 336]. A trial in patients with severe sepsis and increased CFH showed that paracetamol had a protective association with in-hospital mortality [337]. The role of paracetamol in protecting the kidneys against CFH-mediated oxidative damage has not been studied in SM.

The main hypothesis of this thesis was that paracetamol may reduce kidney dysfunction in patients with severe falciparum malaria by inhibiting CFH-induced lipid peroxidation. The mortality rate from SM remains unacceptably high in patients with AKI who do not have access to dialysis [22]. Prevention of malaria-associated AKI is an important step in reducing mortality in SM.

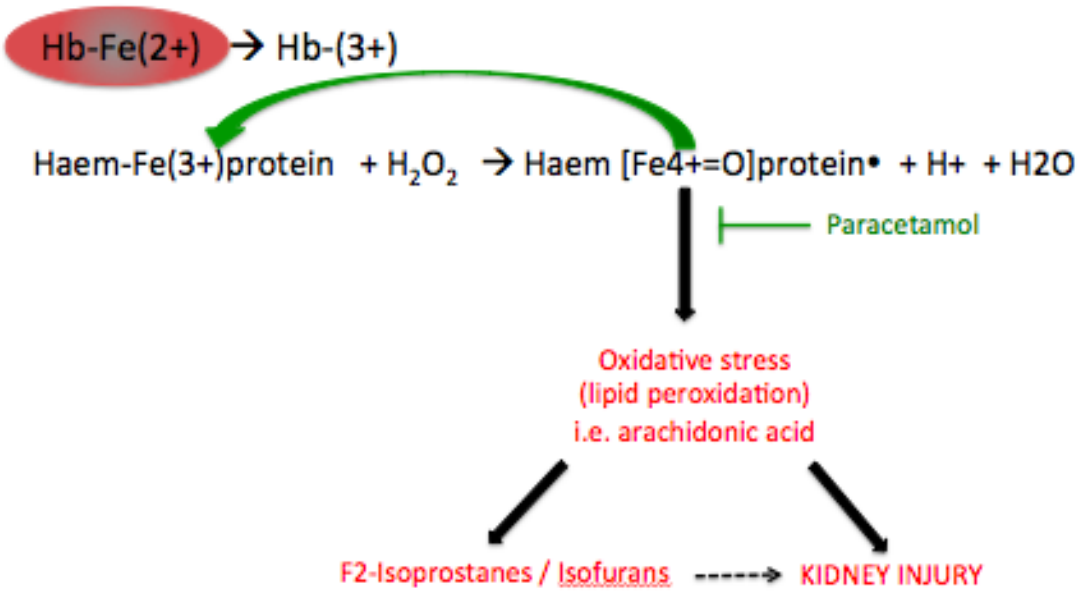


Figure 1.4.4. Inhibitory action of paracetamol on cell-free-haemoglobin mediated oxidative stress. Paracetamol has been shown to reduce ferryl (Fe^{4+}) haemoglobin to ferric (Fe^{3+}) haemoglobin and to reduce lipid peroxidation, as measured by F2-isoprostanes and isofurans [66].

2.0 Methods

The overall objectives of this thesis were to describe AKI in falciparum malaria, characterise its pathophysiology and to assess the role of paracetamol as an adjunctive therapy to reduce kidney dysfunction in this population. In 2001, a crossover RCT of intramuscular versus oral suspension paracetamol in patients with uncomplicated malaria was conducted in Mae Sot, Thailand. The population pharmacokinetic analysis investigating the preferred route of administration was conducted and included in this thesis. From 2004 to 2014, prospective observational pathophysiology studies in severe and uncomplicated malaria were conducted in Chittagong, Bangladesh. The characterisation of AKI, and the role of CFH-mediated oxidative stress, sequestration and immune activation in the pathophysiology of malaria-associated AKI were examined using this data. From 2012 to 2014, a randomized controlled trial of oral paracetamol in severe and moderately severe malaria was conducted in in Chittagong and Ramu, Bangladesh to assess the potential renoprotective effect of paracetamol. Three studies were nested in this latter RCT (1) characterisation of G6PD deficiency in Bangladesh, (2) in vitro assessment of possible drug interaction between artesunate and paracetamol from field isolates, and (3) population pharmacokinetic analysis of oral paracetamol in patients with severe and moderately malaria.

2.1 Study sites

2.1.1 Randomised controlled trial and observational studies

Bangladesh is a malaria endemic country with perennial transmission. Of the approximate 60,000 cases reported in 2012-2013, 80% were from the Chittagong Division in the southeast of Bangladesh and 91% were caused by *P. falciparum* [338]. The oral paracetamol RCT was conducted at two sites in southern Bangladesh: Ramu Upazilla Health Complex, Cox's Bazar District; and Chittagong Medical College Hospital (CMCH), Chittagong District (Figure 2.1.1). Recruitment took place from July 2012 to September 2014. The area surrounding both sites has a distribution of 10-50/1000 confirmed malaria cases.

Ramu Upazilla Health Complex is a rural, 31-bed government hospital with facility for blood transfusion but no advanced supportive therapies. Patients who required higher levels of care were transferred to the tertiary care centre in Chittagong (CMCH).

CMCH is a 1000-bed government tertiary care hospital with facilities for intensive care, RRT, and blood transfusion. From 2007- 2010, peritoneal dialysis was the main method of RRT. Thereafter, intermittent haemodialysis became the established method of RRT delivered in the Nephrology ward.

The observational studies were conducted at CMCH and the retrospective analysis was performed on patients recruited from this site. Observational study patients were frequently co-enrolled into interventional studies. From 2003 to 2005, patients were enrolled into a clinical trial assessing either intravenous artesunate (SEAQUAMAT) for the treatment of SM, or

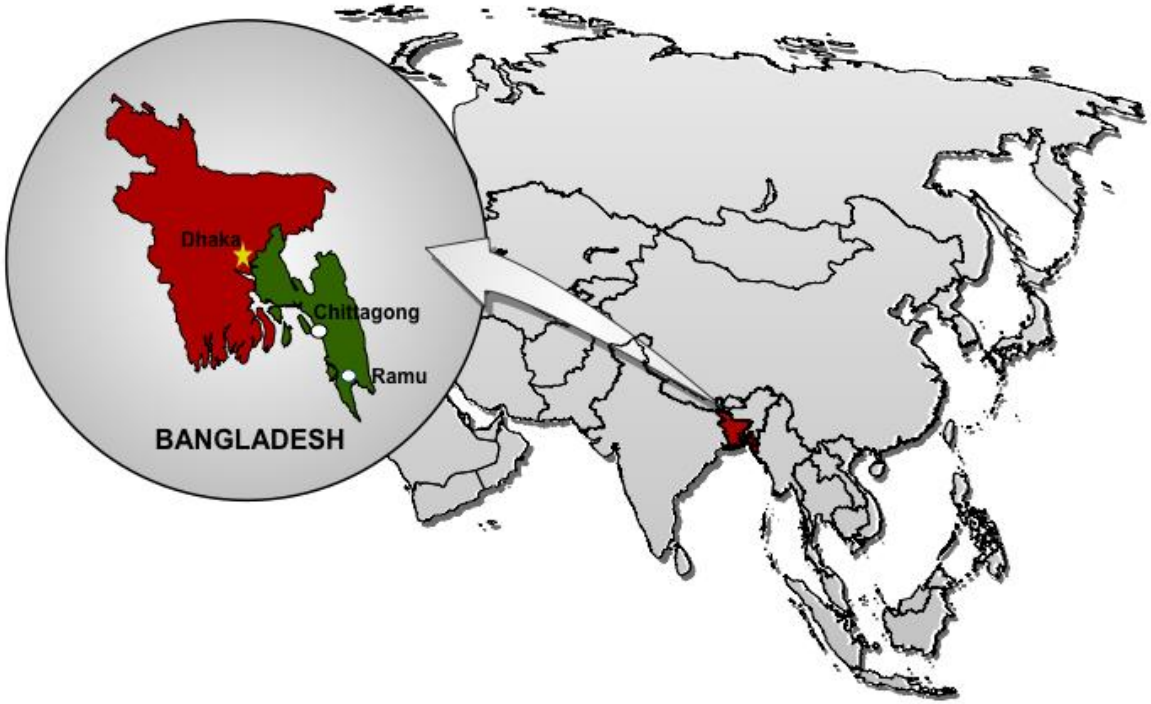


Figure 2.1.1. Map of Bangladesh indicating the location of the two study sites where the randomised controlled trial of paracetamol was conducted. Green area represents the Chittagong Division, which is the malaria endemic region within Bangladesh. White circles indicate study sites in Ramu and Chittagong. Yellow star indicates Dhaka (country capital).

N-acetylcysteine as an adjunctive treatment [19, 70]. From 2006 to 2010, a subset of patients was enrolled into an intervention study of levamisole as an adjunctive therapy [339]. From 2012 to 2014, patients were enrolled into either the oral paracetamol RCT or a study assessing L-arginine as adjunctive therapy in severe falciparum malaria. Patients were not co-enrolled into two interventional studies.

2.1.1 Crossover randomised controlled trial

This study was a randomised controlled trial of intramuscular versus oral paracetamol conducted at Mae Sot Hospital, Tak, Thailand from May to June 2001. Malaria transmission in this area is low and seasonal with peak transmission during the rainy season from May to August. All age groups are affected as few have naturally acquired immunity.

2.2 Summary of trial designs

2.2.1 Paracetamol randomised controlled trial

This was a randomised open labeled, parallel-group, controlled study to assess oxidative stress inhibitory effects of paracetamol measured by its ability to reduce renal dysfunction caused by CFH-induced oxidative damage in patients with severe and moderately severe falciparum malaria. The patients were randomised into two groups to receive either: (1) regular therapeutic dosing of oral paracetamol as an adjunctive therapy to intravenous artesunate versus (2) no paracetamol and intravenous artesunate, with allowance for antipyretics when temperature was elevated to greater than 40°C.

2.2.2 Crossover randomised controlled trial

This study was a randomised, open-label, two treatment crossover pharmacokinetic comparison of paracetamol given by intramuscular route versus oral/NG route in patients with uncomplicated malaria to determine the relative bioavailability of paracetamol. Consecutive patients were randomised into two groups to receive either; (1) a day 1 intramuscular dose followed by an oral dose on day 2, or (2) a day 1 oral/NG paracetamol dose followed by an intramuscular dose on day 2.

2.3 Trial participants

2.3.1 Overall description of trial participants

For the RCT of oral paracetamol, the target study population was male and non-pregnant female patients above 12 years old with severe or moderately severe falciparum malaria admitted to Chittagong Medical College Hospital and Ramu Upazilla Health Complex.

For the observational studies, the target population was consecutive patients with severe and uncomplicated *Plasmodium falciparum* malaria admitted to Chittagong Medicine College Hospital.

For the crossover RCT of intramuscular versus oral paracetamol, the target study population was consecutive non-pregnant adult patients older than 14 years admitted with slide confirmed UM and a temperature > 38°C requiring oral or intramuscular paracetamol.

All interventional and observational study patients must have met the applicable inclusion and exclusion criteria.

2.3.2 Inclusion criteria

2.3.2.1 Paracetamol randomised controlled trial

1. Patient age >12 years
2. Presence of severe or moderately severe *P. falciparum* malaria, with and without BWF, confirmed by positive blood smear with asexual forms of *P. falciparum*. Severe and moderately severe malaria are defined in sections 2.3.4 and 2.3.5.
3. Temperature >38°C on admission or fever during the preceding 24hours
4. Patient or attending relative able to and willing to give informed consent.

2.3.2.2 Observational studies

1. Patient or attending relative able and willing to give informed consent
2. Severe malaria (defined in Section 2.3.4)

2.3.2.3 Crossover randomised controlled trial

1. Patient age > 14 years
2. Temperature > 38°C requiring paracetamol
3. Uncomplicated malaria (defined in Section 2.3.6)
4. Patient or attending relative able and willing to give informed consent

2.3.3 Exclusion criteria

2.3.3.1 Paracetamol randomised controlled trial

Patients were excluded from the RCT of oral paracetamol if they met any of the following criteria:

1. Patient or relatives unable or unwilling to give informed consent
2. History of chronic liver disease
3. History of alcohol use (>3 drinks per day)
4. Contraindication or allergy to paracetamol or artesunate therapy
5. Contraindication to NG tube insertion i.e. facial fracture, bleeding diathesis
6. Pregnancy

2.3.3.2 Observational studies

Patients were excluded only for specific physiologic studies proposed in the observational studies if there were contraindications or no consent for these procedures.

2.3.3.3 Crossover randomised controlled trial

Patients were excluded from the randomised crossover controlled trial of oral paracetamol versus intramuscular paracetamol if they met any of the following criteria:

1. Paracetamol administered in previous 12 hours
2. Contraindication to paracetamol or requirement for interacting drugs

2.3.4 Criteria for severe malaria

2.3.4.1 Randomised controlled trial

Patients with *P. falciparum* asexual parasitaemia positive peripheral blood film and no other cause of their symptoms and a diagnosis of SM according to criteria below, or severe prostration (defined as extreme weakness, inability to walk or sit up without assistance), or impaired consciousness with behavioral changes, confusion or drowsiness.

Presence of one or more of the following:

1. Cerebral malaria (GCS<11 or >2 seizures within last 24 hours)
2. Severe anaemia (haematocrit <20% with parasite count >100,000/mm^3)
3. Jaundice + bilirubin >2.5 mg/dl with parasite count >100,000/mm3
4. AKI (serum creatinine >1.7 mg/dl (150.3 µmol/L))*
5. Hypoglycaemia with venous glucose <40 mg/dl (2.2 mmol/L)
6. Systolic blood pressure (SBP) <80 mmHg with cool extremities
7. Peripheral asexual stage parasitaemia > 5% or >200,000/µl
8. Peripheral venous lactate >4mmol/l
9. Peripheral venous bicarbonate <15mmol/l
10. Pulmonary oedema (clinical: oxygen saturation <90%, respiratory rate >40 breaths/minute + inspiratory crackles; or radiological: bilateral alveolar infiltrates)
11. Spontaneous bleeding
12. Haemoglobinuria (black/red colored urine with positive hemoglobin on urine dipstick and negative RBCs on urine sediment microscopy)

* creatinine of 1.7 mg/dl is the mean creatinine of patients with KDIGO stage 1 on admission (Plewes K, unpublished).

2.3.4.2 Observational studies

Patients ≥ 16 years with a positive peripheral blood film with asexual forms of *Plasmodium falciparum* plus any of the following:

1. GCS <11 (adults), BCS<3 (preverbal children)
2. Convulsions (≥ 2 in 24 hours)
3. Severe anaemia (haematocrit < 20 % with parasite count >100,000/µl)
4. Jaundice with bilirubin > 3.0 mg/dl with parasite count > 100,000/µl
5. AKI (serum creatinine > 3 mg/dl)
6. Hypoglycaemia with venous glucose < 40 mg/dl
7. Systolic blood pressure < 80 (adults), <70 (children) mmHg with cool extremities
8. Peripheral asexual stage parasitaemia > 10 %
9. Peripheral venous lactate > 4 mmol/l
10. Peripheral venous bicarbonate < 15 mmol/l
11. Pulmonary oedema

2.3.5 Criteria for moderately severe malaria

Moderately severe malaria was defined as fever within the preceding 24h, asexual *P. falciparum* positive parasitaemia, no WHO warning signs or SM criteria (section 2.3.4.1), and a requirement for inpatient parenteral therapy because of inability to tolerate oral treatment.

2.3.6 Criteria for uncomplicated malaria

Positive peripheral blood film with asexual forms of *Plasmodium falciparum* but not meeting any severity criteria as defined in section 2.3.4.2.

2.3.7 Withdrawal criteria

Every participant had the right to withdraw from the study at any time. The investigator may have withdrawn a participant if he/she considered it necessary for any reason including:

1. An adverse event that required discontinuation of the study medication or resulted in the inability to continue to comply with study procedures

If a participant was withdrawn, the study investigator continued patient follow up including physical exam, vital signs assessment and parasite count. The withdrawn patient was referred to the local physicians for continuation of their primary medical care. Any patient information gathered up to the point of withdrawal was relayed to the local physicians to assist in ongoing care. Reason for withdrawal was recorded in the case report form (CRF) and the data obtained from the participant up to time of withdrawal from study was still included in the analysis.

2.4 Informed consent

2.4.1 Informed Consent Form and Patient Information Sheet

The subject (or witness if illiterate), or the parent or guardian of a minor personally signed and dated the latest approved version of the informed consent form before any study specific procedures were performed. A dedicated research physician or nurse obtained consent in the local language. If the patient was unconscious, consent was obtained from a

relative. For participants under 18 years old, consent was obtained from a parent or guardian. The study description detailing the exact nature of the study, patient confidentiality, rights and study risks and benefits, was explained verbally in the local language and a translated 'Patient Information Sheet' paper copy was provided to the patient or patient representative. It was clearly stated that participation was voluntary and the patient or guardian had the right to withdraw from the study at any time, for any reason without prejudice to their medical care, and with no obligation to provide the reason for withdrawal.

The investigator ensured that the consent procedure was as simple and quick as possible as the patients were often severely ill. The subject or guardian had the opportunity to question the Investigator, or individual taking consent, to decide whether they would (or allow his/her relative to) participate in the study. The consent form was translated into the local language to be signed and a signed copy was provided to the patient. If the participant or representative was illiterate, a fingerprint was accepted as informed consent along with a witness signature. It was clearly stated and verbally expressed that the subject was free to withdraw from the study at any time, for any reason without prejudice to their medical care, even if consent had previously been given.

2.4.2 Screening, eligibility and assessments

Patients presenting to CMCH, Ramu Upazilla Health Complex and Mae Sot Hospital were screened to assess eligibility. Full consent was obtained

before any enrolment procedures were performed. It was explained that declining to participate in the study would not jeopardize treatment. A screening log was kept.

Basic demographic and epidemiological data (i.e. age, sex, address, previous malaria episodes, tobacco smoking, previous medications, dietary history) and a full medical history was obtained and recorded in the CRF by the research staff.

2.4.3 Screening physical exam and laboratory tests

A research physician conducted a physical examination upon enrolment and recorded all findings in the CRF. Daily examinations were performed during the participants' hospitalization period. Vital signs, aural temperature and urine colour were measured every 6 hours until parasite clearance, fever clearance, and recovery of Glasgow Come Score (GCS), defined as 2 consecutive negative smears, 2 temperatures 24 hours apart below 37.5°C, and GCS of 15, respectively. At each aural temperature measurement, the average temperature of left and right ears was recorded. In Bangladesh, the urine colour was assessed using standardised urine colour charts; haemoglobinuria was defined as dark urine with a colourimetric level of 6 or greater [340].

2.4.4 Randomisation and blinding

From 2012 to 2014, concurrent observational and interventional studies were conducted at CMCH. All consecutive malaria patients who gave consent were enrolled in observational studies upon diagnosis. Enrolment in

the observational studies coincided with antimalarial administration and all other urgent treatments, so there was no delay in treatment due to participation. Once antimalarials were administered and the patient was stabilized, the patients' eligibility for the RCTs was assessed.

There were two interventional trials conducted at CMCH from 2012 to 2014, namely "Paracetamol effect on oxidative stress and renal function in severe and moderately severe malaria" and **"**Randomised multi-centre phase 2a trial of safety, efficacy, pharmacokinetics, pharmacodynamics of L-Arginine in severe falciparum malaria (ARGISM-2 trial)". Patients were not co-enrolled into the two intervention studies. The technical constraints of the ARGISM-2 trial and the logistical constraints of the clinical setting required limiting screening for enrolment in ARGISM-2 to one patient each day. All patients not screened for ARGISM-2 enrolment were assessed for enrolment into the paracetamol study. Analysis from previous data at CMCH supported that this enrolment strategy would not lead to an imbalanced selection of participants into the paracetamol study.

Patients allocated to the paracetamol study were then randomised into two groups with randomisation stratified by severity using a computerized binary sequence number generator in a 1:1 ratio in blocks of 20. Research clinicians enrolling patients were prevented from knowing the last allocation of each block of 20 by performing a random swap of one treatment allocation between blocks. Consenting patients at Ramu Upazilla Health Complex

were enrolled and randomised consecutively starting from the second block of the severity stratum randomisation envelopes (numbers 20 to 40).

Treatment allocation was kept in opaque sealed envelopes prior to randomisation allocation. Study arms were as follows: (Arm 1) Adults >50kg: paracetamol 1000mg PO/NG every 6 hours for 72 hours and until afebrile for 24 hours (maximum total dose 4 g/24 hours) Children <50kg: paracetamol 12.5 - 15 mg/kg/dose every 6 hours (maximum total dose 5 doses/24 hours; 75 mg/kg), or (Arm 2) No paracetamol. If patients in arm 2 developed a temperature > 40°C, ibuprofen PO/PR was administered in the absence of renal impairment, dehydration and dengue infection. If ibuprofen was contraindicated, patients received rectal paracetamol as per local practice.

The allocation sequences were computer generated by a statistician from MORU who was not involved in performing the trial. The clinical investigator and patients were not blinded to the treatment randomisation. However, randomisation was masked from the laboratory staff performing investigations including: the oxidative stress markers, CFH, renal function and paracetamol measurements. Since administration of paracetamol by a third party, i.e. patient attendants, was difficult to control, paracetamol levels were expected in the control arm and were therefore measured. This was anticipated to contribute to the power of study to define a dose-effect relationship.

For the two-treatment crossover study of oral and intramuscular paracetamol, a computerised randomisation schedule generated treatment allocations that were implemented by drawing an individual pre-prepared, sealed and sequentially numbered opaque envelope for each enrolled participant. As an open-label study, blinding of investigators and patients was not applicable. However, the randomisation procedure allowed for adequate drug allocation concealment before envelopes were opened. All laboratory investigations were performed without knowledge of the treatment allocation.

2.4.5 Blood and urine sampling

Every enrolled patient had two peripheral venous cannulae placed, one for administration of IV medications and fluids, and a second in the opposite forearm for venous blood sampling. The cannulae were replaced every 3 to 4 days at a new site to reduce the risk of phlebitis. The blood sampling cannula was capped and filled with 2 mL of saline after blood draws to reduce clotting. Prior to subsequent blood draws the 2 mL saline was withdrawn and discarded.

After enrolment, a blood sample was taken for: haemoglobin, haematocrit, reticulocytes, mean corpuscular haemoglobin concentration, white blood cell count, platelets, peripheral blood smear, plasma CFH, haptoglobin, haemopexin, parasitaemia, *Plasmodium falciparum* histidine rich protein 2 (*Pf*HRP-2) lactate, glucose, electrolytes, phosphate, magnesium, calcium, albumin, serum creatinine, urea nitrogen, direct and indirect bilirubin,

aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase, lactate dehydrogenase (LDH), creatinine kinase, *in vitro* drug sensitivity testing, G6PD spot test, and G6PD genotyping. Complete blood cell counts were performed locally. In Bangladesh, basic biochemistry was performed using a handheld portable analyser (iSTAT, Abbott, USA).

Peripheral blood parasitaemia was assessed on thick and thin films prepared using standard techniques and stained with Field's modified Giemsa stain. Slides were examined by experienced laboratory technicians. Parasitaemia was measured at enrolment (T=0 hours) and then every 6 hours until parasite clearance. Plasma lactate was measured on admission, at 6 hours and then every 12 hours until lactate clearance (lactate < 2 mmol/L). Serum creatinine was measured every 12 hours for 72 hours, at discharge, then daily or weekly until creatinine returned to normal (< 1 mg/dL), in the absence of RRT. Haemoglobin, haematocrit, reticulocytes, LDH and peripheral blood film were performed daily for 72 hours and at discharge. Haemoglobin, LDH, and host factors reflecting and influencing haemolysis were performed at discharge, 1, 2 and 4-week follow up visits.

Plasma CFH, F2-Isoprostanes (F2-IsoPs) and isofurans (IsoFs) were assessed from samples collected at enrolment (T=0 hours) and then daily for 72 hours. The lithium heparin samples collected for F2-IsoPs and IsoFs were promptly cooled after collection, centrifuged at 3,000 rpm for 10 minutes, and frozen at –80°C. Plasma CFH was assessed from citrated plasma. Plasma paracetamol levels were assessed from EDTA plasma

samples on enrolment prior to and 0.5, 1.5, 2.5, 4 hours post-first dose paracetamol, then 6-hourly prior to each dose for 72 hours and then at 72.5, 73.5, 74.5, 76, 78, and 84 hours post-first dose paracetamol. In the control arm, paracetamol levels were assessed 6-hourly for 72 hours. The samples were processed in Bangladesh then stored in liquid nitrogen until transport on dry ice to Bangkok, Tennessee and Darwin for analysis.

In the crossover paracetamol trial, lithium heparin plasma samples for paracetamol concentration measurement were collected on enrolment (pre-dose), and then at 0.5, 1.0, 1.5, 2, 3, 4, 6, 8, 10, 12 hours post-dose.

A urine sample was obtained on admission for: urinalysis, microscopy, electrolytes, osmolarity, specific gravity, supernatant free haemoglobin, methaemoglobin, F2-IsoPs, IsoFs, and neutrophil gelatinase-associated lipocalin (NGAL). Urine for F2-IsoPs, IsoFs and creatinine measurement was collected 24-hourly for 72 hours. Urinalysis, microscopy and colour were performed 12-hourly for 72 hours then at 1, 2, and 4 weeks until creatinine returned to normal (<1 mg/dL), in the absence of RRT. Urine sediment microscopy was performed after centrifuging 10 mL of urine at 2,000 rpm for 5 minutes. Urine was collected from a voided specimen or urinary catheter and stored without preservative. Urine samples for F2-IsoPs and IsoFs were promptly stored in liquid nitrogen. All samples were shipped on dry ice to Bangkok, and Tennessee for specific analysis at the end of the study period.

2.4.6 Sample transport

Blood and urine samples were shipped from Bangladesh to Bangkok, Tennessee, Australia, and Hong Kong by 'World Courier', or other appropriately licensed courier, in compliance with: the UN committee of Experts/Technical Instructions, International Air Transport Association (IATA) Infectious Substances and Diagnostic Specimens Shipping Guidelines, the IATA Dangerous Goods Regulations and the Thailand Department of Civil Aviation. Samples were classified as Biological substances, Division 6.2, Category B (UN3373) with dry ice Class 9 (UN1845) and transported in packing, which met the UN specification of Packing Instruction 650 and 904, respectively.

2.4.7 Source documents and data

Source documents were original documents, data reports, and records from which participants' CRF data were obtained. These include, but are not limited to, hospital records (summarized into CRF), external clinic reports/pre-referral files, laboratory and pharmacy records, radiographs and CRFs. CRF entries were considered source data if the CRF was the original recording in the absence of a photocopy of written or electronic record of data. All CRFs were kept confidential and stored in a secure locked research office on site at CMCH and Ramu Upazilla Health Complex during the field research months. Following the research seasons at CMCH and Ramu, CRFs were stored securely by the data management team at MORU in Bangkok, Thailand. All study specific documents referred to the participant by subject number and initials with the exception of the signed consent form.

2.5 Study drug regimens

2.5.1 Antimalarials

All study subjects were promptly treated with intravenous artesunate (Guilin No.2 Pharmaceutical Factory, Guangxi, People's Republic of China) 2.4 mg/kg on admission, then at 12 hours, 24 hours, and once daily thereafter until oral medication was tolerated. When the subject recovered sufficiently to take tablets, they continued treatment with oral artemether/lumefantrine 20mg/120mg (Ramu: Coartem, Novartis Pharma AG, Basel, Switzerland; Chittagong: Lumartem, Cipla Ltd, Mumbai Central, India) 4 tablets twice daily for three days. This oral ACT was given with a suitable source of fat in accordance with package insert to ensure adequate drug absorption. In the crossover paracetamol study, oral step down therapy included oral artesunate (2 mg/kg; Guilin No. 2 Pharmaceuticals, China) and doxycycline (4mg/kg per day in 2 doses, Vibramycin, Pfizer) for a total of 7 days. At the time of this study, this antimalarial regimen was proven to be effective in this area of highly resistant *Plasmodium falciparum*.

2.5.2 Paracetamol

2.5.2.1 Paracetamol randomised controlled trial

Oral paracetamol was administered as either:

ARM 1: <u>> 50 kg</u>: 1,000mg PO/NG 6-hourly for 72 hours and afebrile for 24 h (maximum total dose 4 g/24 hours)

<u>< 50 kg:</u> 12.5-15 mg/kg PO/NG 6-hourly for 72 hours and afebrile for 24 h (maximum total dose: 5 doses/24 h; 75 mg/kg)

<u>Note</u>: Unconscious patients received paracetamol via NG tube

ARM 2: > 50 kg: No paracetamol

< 50 kg: No paracetamol

In arm 1, each dose of paracetamol was observed and recorded in the CRF. UM was shown to have no effect on the metabolism of paracetamol [284]. Study drug dosages were in keeping with the recommended dosing according to the Bangladesh National Drug Formulary. There have been no toxicities reported from its use in treating fever in patients with malaria. No drug interactions between paracetamol and artesunate or artemether have been reported.

In arm 2, if subjects developed a temperature >40°C ibuprofen PO/PR was administered in the absence of renal impairment, dehydration or dengue infection. Ibuprofen was shown to be a safe and effective antipyretic agent with no renal adverse events when administered to adults and children with UM [326, 341, 342]. If ibuprofen was indicated, dengue testing was performed prior to drug administration. In pyrexic subjects with the aforementioned contraindications to ibuprofen, 500 mg paracetamol PO/PR was administered in accordance with local paracetamol dosing practice and recorded in the CRF.

2.5.2.2 Crossover randomised controlled trial

Study participants were randomly assigned to receive on the first day of enrolment, either 600 mg paracetamol oral suspension (Tylenol ®; Janssen), followed by 100 mL of water, or 600 mg intramuscular paracetamol (Partamol ®; Atlantic) divided in 2 doses into the upper legs. After a washout

period of 24 hours, the patients received paracetamol by the route they did not receive on the first day.

Study participants whose aural temperature exceeded 40°C despite the study dose of paracetamol, received mechanical antipyresis including tepid sponging and a bedside fan. If the aural temperature exceeded 40°C after 6 hours from the first dose of paracetamol, further doses were administered to a daily maximum of 4 g. The dosage and time of each additional paracetamol dose were recorded.

2.5.3 Study drug administration

Artesunate was administered by a research physician or nurse. If the subject vomited within one hour after intake of paracetamol, the dose was repeated. Repeat doses were recorded in the CRF. Unconscious patients in Arm 1 who were unable to swallow received paracetamol suspension via NG tube. The NG tube was inserted using universal precautions by an experienced research physician following the standard operating procedure. Following insertion, placement was assessed by aspiration of gastric contents using a syringe. The pH of the aspirated contents was then assessed with litmus paper to ensure contents were acidic (pH<5.5). A chest x-ray was also performed to confirm placement whenever possible. To prevent aspiration, patients were positioned upright during medication instillation down the NG tube and for one hour afterwards. Patient attendants were also instructed not to feed the patient through the NG tube at any time. Contraindications to NG tubes include the presence of severe facial fracture, basilar skull fracture,

oesophageal varices and bleeding diatheses. Standard management of patients with SM includes the insertion of a NG tube [101]. NG tubes were removed as soon as patients were able to tolerate oral paracetamol.

2.5.4 Storage of drugs

Study drugs were stored on site at CMCH and Ramu Upazilla Health Complex in accordance with the manufacturers' recommendations in a secure room and with temperature monitoring. The artesunate and artemether/lumefantrine were stored between 15°C and 30°C. Paracetamol is stable to temperature and humidity and was stored at 4°C.

2.5.5 Compliance with drugs

In arm 1, all scheduled paracetamol doses were administered orally or, if unconscious, via NG tube by a research physician or nurse. All doses were recorded in the CRF.

2.5.6 Concomitant medications

Research physicians and local physicians may have prescribed concomitant mediations as clinically indicated (e.g. anti-emetics, antibiotics) to provide appropriate supportive care. Any other medications taken during the study were recorded in the CRF.

2.6 Follow up procedures

Subjects were assessed daily during hospital admission until discharge or death. All surviving subjects were requested to return for follow up at 1, 2, 3, and 4 weeks post discharge for a repeat serum creatinine, CBC, LDH and urine dipstick. Repeat G6PD testing was performed at 4 weeks for all

subjects who attended follow up. Subjects were notified of their screening G6PD status once testing is completed during their hospital admission and at 4 weeks if repeated. The G6PD screening test result was explained to all subjects. All patients received a wallet sized medical card indicating their G6PD status in Bengali for their own records.

2.7 Adjunctive supportive treatments

All subjects were managed according to WHO treatment guidelines [101]. In particular, subjects with evidence of AKI (creatinine 2 to 3 mg/dl, BUN > 60 mg/dl, and/or anuria) were referred to nephrology for a formal consultation including assessment for the need of RRT. Subjects requiring dialysis were admitted to the CMCH nephrology ward and RRT delivered at the discretion of the local nephrologists. Local indications for RRT include: (1) anuria for more than 24 hours, (2) severe electrolyte and acid base disturbance, (3) serum creatinine > 3 mg/dL with urine output < 0.5 ml/kg/h for 12 hours, or (4) gradual rise in creatinine despite normal urine output. Subjects with respiratory failure and/or haemodynamic shock were referred to the Intensive Care Unit at CMCH for mechanical ventilation and/or inotropic support at the discretion of the local intensivists. Research physicians continued protocol specific follow up of subjects admitted to nephrology or intensive care wards. Since dialysis and mechanical ventilation were not available at Ramu, a CMCH nephrologist or intensivist was consulted by telephone to evaluate if transfer to CMCH was indicated.

2.8 Safety reporting

These trials involved the administration of drugs that have been registered and evaluated extensively. Therefore, the safety aspects of this trial were limited to close observation, including laboratory investigations, during hospital admission for any drug-related side effects and recording and reporting serious adverse events.

2.8.1 Definitions

2.8.1.1 Adverse event

An adverse event is any untoward medical outcome in a patient undergoing a study related procedure or intervention that is believed to be caused by that study related procedure or intervention. A medical condition or abnormal laboratory value that is present at the time of enrolment will be considered as baseline and not reported as an AE.

2.8.1.2 Serious adverse event (SAE)

A serious adverse event is an AE that:

1. Results in death*
2. Is life threatening i.e. patient was at risk of death at the time of the AE
3. Requires inpatient hospitalization or prolongation of existing hospitalization
4. Results in persistent or significant disability/incapacity
5. Requires acute medical or surgical care to prevent one of the outcomes listed below i.e. hepatotoxicity

* In the RCT of artesunate versus quinine for SM in adults (SEAQUAMAT), 23 to 32% of patients with SM died in Chittagong [19]. Therefore, death from SM as the primary etiology is likely in this study.

Studies evaluating paracetamol in UM have not reported serious adverse events or hepatotoxicity attributable to paracetamol [284, 326]. Toxicity is possible to develop with single ingestions in excess of 250 mg/kg or greater than 12 g over a 24-hour period [343]. The doses prescribed in this study are well below these amounts and in accordance with the Bangladesh National Drug Formulary.

The early symptoms of paracetamol toxicity are non-specific and are not predictive for paracetamol-induced hepatic toxicity. Between 24 and 72 hours of supra-therapeutic ingestions, clinical and laboratory evidence of hepatotoxicity may become evident. All patients receiving paracetamol were monitored daily for right upper quadrant pain, liver enlargement with tenderness and AST/ALT levels were measured on admission, at 72 hours and on recovery. Local resources did not permit plasma paracetamol concentrations to be monitored during hospital admission. If clinical or laboratory investigations were consistent with potential hepatotoxicity, additional investigations including total bilirubin, International normalisation ratio (INR), and creatinine were promptly performed. If there was evidence of severe hepatic toxicity, defined as a peak AST or ALT level greater than 1,000 IU/L, paracetamol dosing was stopped and the patient was referred to

the gastroenterology consultation service for consideration of N-acetylcysteine administration.

2.8.2 Reporting of serious adverse events

All SAEs were recorded and reported by the site investigator to the Medical Monitor, Professor Arjen Dondorp, within one day of awareness of the SAE. The Medical Monitor then reported the SAE to the Data Safety Monitoring Board and Bengali ethics committee in accordance with local requirements.

2.8.3 Monitoring

In addition to the CMCH and Ramu Upazilla Health Complex system for internal monitoring, a MORU Clinical Trials Support Group (CTSG) representative performed annual external monitoring according to International Conference on Harmonization Good Clinical Practice (ICH GCP). Data was evaluated for compliance with the protocol and accuracy in relation to source documents. The monitors checked whether the clinical trial was conducted and data generated, documented and reported in compliance with the protocol and GCP.

2.8.4 Ethics

The study was conducted in accordance with the current approved protocol, ICH GCP and national regulations that applied to this study. The MORU CTSG was engaged in assuring QA/QC of study execution.

2.8.4.1 Declaration of Helsinki

The Investigator ensured that the studies were conducted in compliance with the revision of the Declaration of Helsinki, Seoul 2008.

2.8.4.2 ICH guidelines for Good Clinical Practice

The investigator ensured that the studies were conducted according to any National Regulations and that they followed the principles of the ICH Guidelines for Good Clinical Practice 1996.

2.8.4.3 Approvals

The study protocols and associated documents were submitted to the Oxford Tropical Research Ethics Committee (Reference 07-12) and the Chittagong Medical College, Ethical Review Committee (Reference CMC/PG/2012/6). The Investigator submitted and, where necessary, obtained approval from the above parties for all substantial amendments to the original approved documents. The RCT of oral paracetamol in severe and moderately severe malaria patients was registered with ClinicalTrials.gov (Identifier NCT01641289).

2.8.4.6 Alternatives to study participation

All participants had the right to decline or withdraw from the study. If so, they received standard of care for the treatment of malaria.

2.8.4.7 Incentives and compensation

Study participants were compensated for the costs of their hospitalization including: medications, cannulae, catheters, intravenous fluids, haemodialysis, and ICU admission. The study did not pay for long term care for disability resulting from complications of the illness.

2.8.4.8 Confidentiality

The research physicians ensured that all participants' anonymity was maintained. Their initials and a study number on the CRF identified the subjects. All CRFs were stored securely at the field sites and were accessible to research staff and authorized personnel only. Following the field research, CRFs were stored securely at MORU in Bangkok, Thailand.

2.9 Definitions

2.9.1 Kidney function

Assessment of the KDIGO criteria for the diagnosis of AKI requires baseline creatinine. However, since these were not available the MDRD back-calculation was applied as recommended by the KDIGO guidelines: [103]

Cr (mg/dL) = (GFR /((sex) x (race) x 186 x (age^-0.203))^(-1/1.154)

For the pathophysiology studies, AKI was defined by creatinine clearance (CrCl) using the Cockcroft-Gault formula [344] since the MDRD has not been validated in this population. The Cockcroft-Gault estimated CrCl rate was calculated as follows:

$$\text{CrCl} = \frac{(140 - \text{Age}) \times \text{Mass (in kilograms)} \times (0.85 \text{ if female})}{72 \times \text{serum Creatinine (in mg/dL)}}$$

2.10 Analysis

2.10.1 Paracetamol drug assay

For paracetamol quantification from EDTA plasma samples, a liquid chromatography-tandem mass spectrometry method was developed and validated following the United States Food and Drug administration (FDA) and China FDA standards on bioanalytical method validation standards [345, 346]. The Pharmacokinetics/Pharmacodynamics Laboratory, Department of

Chemical Pathology/Phase 1 Clinical Trial Centre at the Chinese University of Hong Kong, performed the method validation and analysis of the plasma paracetamol levels in both treatment arms. All samples were stored at -80°C until analysis.

Analytical grade paracetamol and stable isotope-labeled paracetamol-d4 (internal standard) were used for method development (Sigma-Aldrich, PHR1005-1g and 80317-5mg). Drug free plasma was pooled from anonymous patients samples in the Prince of Wales Hospital, Shatin, Hong Kong. Pooled plasma was analysed without additional of internal standard to ensure it did not contain endogenous analyte and internal standard. All analysis was performed on a Waters ACQUITY I-Class UPLC liquid chromatographer coupled online to Waters Xevo TQ-S triple quadrupole mass spectrometer. A 6-point series of calibrators and quality control (QC) samples at low, middle and high concentration were prepared for method development.

Plasma samples were simply extracted once with organic solvent followed by dilution then used directly for LC-MS/MS analysis. Samples were separated on a Waters ACQUITY BEH C18 UPLC column, 2.1 x 50 mm, 1.7 μm, from 5% mobile phase A 90.1% formic acid in MilliQ water) to 35% mobile phase B (100% HPLC grade methanol) in 3.5 minutes, followed by 0.5 minutes washing at 90% mobile phase B and 1 minute re-equilibration. Paracetamol and internal standard paracetamol-d4 were eluted at 2.25 ± 0.2

minutes and monitored by MRM transition m/z 152 > 110 and m/z 156 > 114, respectively. MRM transition 152 > 65 was used for paracetamol as a qualifier for increased specificity. The method covered a concentration range from 125 to 50,000 μg/L with less than 15% deviation from nominal concentration. The imprecision was less than 2% and inaccuracy less than 15% throughout the concentration range, which are within the acceptance criteria of the FDA and CFDA guidelines. The imprecision and inaccuracy of LLOQ were 18% and 13.4%, respectively, both of which are below the requirement by FDA and CFDA (± 20%). Recovery and matrix interference were both within acceptance criteria of 15%. Compared to the currently available LC-MS/MS method for paracetamol concentration analysis, the method developed has a larger linear range, improved precision, extraction efficiency, and process efficiency [347].

2.10.2 Population pharmacokinetic analysis

Paracetamol plasma concentrations after intramuscular and oral administration were transformed into their natural logarithms and modeled simultaneously using NONMEM, version 7.2 (Icon Development Solution, Ellicott City, MD). Model diagnostics and automation were performed using Xpose version 4.0 [348], Pirana [349], and Pearl-speaks-NONMEM (PsN; version 3.5.3). The first-order conditional estimation method with interaction was used throughout the model development. The difference in objective function value (ΔOFV; calculated by NONMEM as proportional to -2 x the log-likelihood of data) was used as a statistical criterion for discrimination of hierarchical models. ΔOFV of > 3.84 and > 10.83 were considered

statistically significant at p-value of < 0.05 and < 0.001, respectively, with one degree of freedom. Goodness-of-fit and simulation-based diagnostics were used for assessing the descriptive and predictive performances of the model. There were no observed data below the limit of quantification reported in the oral versus intramuscular paracetamol dataset.

One-, two-, and three-compartment disposition models were evaluated to describe the pharmacokinetic properties of paracetamol. Different absorption models were investigated to describe the absorption process after intramuscular and oral administration, including zero-order absorption, first-order absorption with and without lag time, and a flexible transit-absorption model [350]. For patients who had measurable pre-dose concentrations of paracetamol, baseline estimation was implemented for these patients. Inter-individual variability was introduced exponentially; $\theta_i = \theta \cdot \exp(\eta_{i,\theta})$, where θ_i is the individual "i" parameter estimate, θ is the population mean parameter estimate, and $\eta_{i,\theta}$ is the inter-individual variability with zero mean and variance ω^2 . Variability components with an estimated coefficient of variation (%CV) of less than 1% were fixed to zero. The residual unexplained variability was assumed to be additive on a logarithmic scale, essentially equivalent to an exponential error on an arithmetic scale.

Body weight was evaluated as an allometric function on all clearance and volume of distribution parameters using; $\theta_i = \theta \times \exp(\eta_{i,\theta}) \times (\mathrm{BW}_i / \mathrm{BW}_{median})^{0.75}$ for all clearance parameters and $\theta_i = \theta \times \exp(\eta_{i,\theta}) \times (\mathrm{BW}_i / \mathrm{BW}_{median})^{1.00}$ for all

volume of distribution parameters, where BW_i represents individual body weight and BW_{median} represents median body weight of the study population.

The influences of demographic patient characteristics were investigated using a stepwise forward inclusion (*P*-value < 0.05) and stepwise backward deletion (*P*-value < 0.001) approach. The admission covariates evaluated in the crossover study included: age (years), AST, ALT, bilirubin, BUN, creatinine level, gender, haemoglobin, parasitaemia, SBP, and temperature. The covariates assessed in the oral paracetamol RCT analysis included: age, INR, AST, ALT, serum creatinine, parasite count, total bilirubin, direct bilirubin, indirect bilirubin, CrCl, haemoglobin, parasitaemia, *Pf*HRP2, gender, haemodialysis status, severity of malaria disease, route of paracetamol administration.

Bootstrapping (n=1,000), stratified for administration route, was used to assess the robustness of pharmacokinetic parameter estimates from the final model, and to compute relative standard errors and nonparametric confidence intervals for population mean estimates. Numerical and visual predictive checks (n=2,000) were used to evaluate the predictive performance of the final model.

Final pharmacokinetic population parameter estimates from NONMEM were used to simulate different dosing scenarios in Berkeley Madonna [351]. The therapeutic target level of paracetamol was assumed to be 10 - 20 mg/L [328, 329]. Different dosage regimens were investigated based on a

maximum dose of 4 g paracetamol per day and the available paracetamol products (i.e. 500 mg oral tablet and 300 mg/2 mL for injection).

2.10.3 Pharmacodynamic parameters

To assess the pharmacodynamic effect of paracetamol on fever control, the relationship between C_{MAX} and temperature reduction 2 hours post-dose (Δtemperature $_{0\text{-}2h}$, AUC $_{\text{temperature >37.5 °C at 0-2h}}$) was assessed using ordinary linear regression. This temperature endpoint was chosen because the nadir median temperature drop occurred at 2 hours. The effect of paracetamol on parasite clearance half-life was assessed by two methods. First, by linear regression to assess the relationship between paracetamol C_{MAX} and parasite clearance half-life. Second, using a simplified population parasite clearance model based on all available parasite count data, i.e. an estimated baseline parasite biomass and an estimated first-order parasite clearance rate, including inter-individual variability on both parameters. In order to investigate the relationship between plasma concentration of paracetamol and the relative change in parasite clearance rate, individually predicted paracetamol concentrations were derived from the final pharmacokinetic model and then evaluated in the model as a time-varying covariate on the parasite clearance rate using a linear relationship. An indirect paracetamol concentration-effect model (i.e. using a hypothetical effect-compartment) was also assessed. The significance level was defined at *P* value = 0.05.

2.10.4 Oxidative stress assay

Plasma and urine F2-isoprostane and isofuran concentrations were determined in lithium heparin plasma and urine samples by gas

chromatography-mass spectrometry at the Vanderbilt University Eicosanoid Core Laboratory, Nashville Tennessee, USA, as previously described [62, 352]. Urine F2-isoprostane and isofuran concentrations (ng/ml) were normalized to urinary creatinine and expressed as ng/mg of creatinine. All samples were stored at -80°C until analysis.

2.10.5 Cell-free haemoglobin assay

Plasma CFH concentrations were measured by enzyme-linked immunosorbent assay (ELISA) as per manufacturer's instruction (Bethyl Laboratories) at Menzies School of Health Research and Charles Darwin University, Darwin, Northern Territory, Australia.

2.10.6 *P. falciparum* histidine rich protein 2

Plasma *Pf*HRP2 was assessed by commercial sandwich ELISA (Celisa, Cellabs, Sydney, Australia), according to the manufacturer**'** s instructions with minor modifications [353]. Pooled reference plasma from 20 subjects with parasitaemia >200,0000/**µ**l was calibrated to purified *Pf*HRP2 standard curves (kindly provided by D Sullivan, John Hopkins School of Public Health, Baltimore, USA). Concentrations in duplicate plasma dilutions (1/25 to 1/3,125 in PBS/ 0.01%Tween) were determined according to the linear component of the standard curve. Cases where duplicates differed by more than 50% were re-assayed. Analysis was performed at MORU, Thailand.

2.10.7 Soluble urokinase-type plasminogen activator receptor

Plasma suPAR concentrations were measured using suPARnostic™ ELISA (ViroGates, Copenhagen, Denmark), according to the manufacturer' s

instructions. Specimens were diluted to read within the calibration curve defined by quantitative standards. Reported results are the mean suPAR concentration (ng/ml) of duplicate wells for each specimen. Analysis was performed at the Department of Intensive Care Medicine & Laboratory for Experimental Intensive Care and Anesthesiology (LEICA), Academic Medical Centre, University of Amsterdam, Amsterdam, the Netherlands.

2.10.8 Neutrophil gelatinase-associated lipocalin

Urine NGAL concentrations were measured using Human Lipocalin-2/NGAL ELISA (R&D Systems, Abingdon, UK) according to the manufacturer' s instructions. Multiple dilutions were tested in duplicate. The final urine NGAL concentration (pg/ml) was normalized to urinary creatinine and expressed as pg/mg of creatinine (uNGAL/Ucr). Analysis was performed at the LEICA, Academic Medical Centre, University of Amsterdam, the Netherlands.

2.10.9 Glucose-6-phosphate dehydrogenase

G6PD activity was assessed on admission whole blood by the G6PD qualitative rapid fluorescent spot test (FST) (R&D Diagnostics, Greece) in Bangladesh. This assay has been validated against the gold standard spectrophotometry quantitative method and compared with other point-of-care tests [354-357]. Whole blood samples were incubated with the reagents for 10 minutes, spotted on filter paper, air dried and interpreted after 15-20 minutes. Interpretation of the test was done in a darkened room using long wave UV lamp to observe fluorescence. Samples with normal or slightly reduced G6PD activity showed strong fluorescence. Samples with total or marked G6PD deficiency did not fluoresce after the 10-minute incubation.

Photographs were taken of each result for a permanent record. This qualitative method allows fluorescent samples to be classified as ‘normal’ and non-fluorescent samples to be classified as ‘G6PD deficient’. Normal and deficient controls were performed along with study samples. Patients had repeat G6PD testing performed at 4 weeks from admission. Filter papers were stored at 4°C and sent back to Bangkok for quality control.

2.10.10 Molecular methods

Genomic deoxyribonucleic acid (DNA) extraction

DNA for G6PD and Kelch13 (K13) genotyping was extracted from EDTA blood samples using the QIAamp® DNA Mini Kit (QIAGEN, Germany) as per manufacturer’s instructions. Eluted genomic DNA from all samples was stored at -20°C until polymerase chain amplification (PCR).

G6PD genotyping

G6PD gene sequencing strategy is shown in Figure 2.1.2. Primers used to amplify all G6PD coding regions for direct DNA sequencing were constructed based on previously published G6PD primer sequences [358-360]. PCR- restriction fragment length polymorphism-based analysis was used to assess the Mediterranean variant located in exon 6 using primers as indicated in Figure 2.1.2 as previously described [361, 362]. The two silent polymorphic markers, 1311C>T [363] and IVSXI C93T [364], were assessed by amplification of exon 11 and 12 along with introns 11 and 12, as previously described [360, 361]. DNA sequences were assessed by direct sequencing of the PCR product (Macrogen, Seoul, Korea) and analyzed with

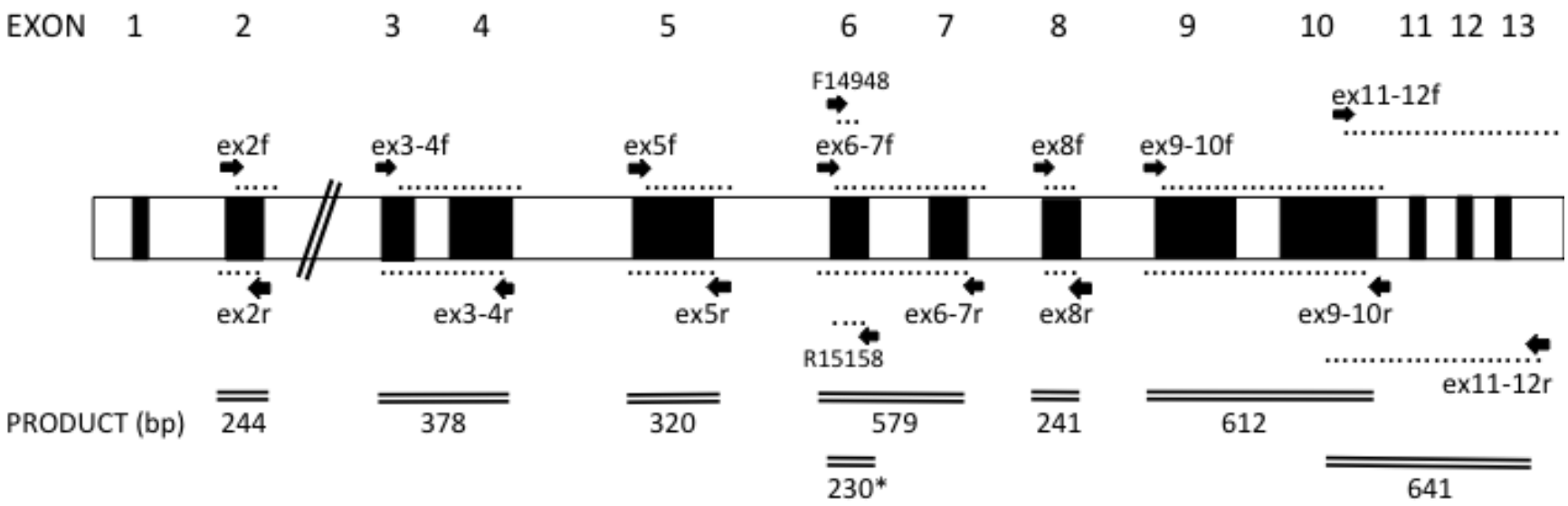


Figure 2.1.2. Diagram of G6PD gene and sequencing strategy. Solid boxes represent coding regions (exons (ex)). Open boxes represent non-coding regions (introns) and untranslated regions. Arrows represent forward (f) and reverse (r) primers and dotted lines represent areas amplified by polymerase chain reaction that were subsequently sequenced. Primers used for restriction fragment length polymorphism analysis of Mediterranean variant in exon 6 are indicated by F (forward) and R (reverse). Parallel lines below gene diagram represent amplified products and their respective lengths in base pairs (bp). Asterisk represents amplified product for restriction fragment length polymorphism analysis of G6PD Mediterranean mutation.

BioEdit bioinformatics programme [365] using NCBI Reference Sequence X55448.1 as G6PD reference.

All molecular testing was performed by the Molecular laboratory (Dr. Mallika Imwong) at MORU, Bangkok, Thailand.

2.10.11 Parasite clearance

Peripheral blood films were assessed 6-hourly for the presence of asexual parasitaemia until negative on 2 consecutive blood films by MORU laboratory staff. Parasite clearance time was defined as the interval between the start of treatment and the first of two consecutive negative blood smears. Parasite clearance half-life was assessed using the WorldWide Antimalarial Resistance Network parasite clearance estimator based on the linear portion of the slope of log-parasitaemia versus time relationship [332]. All slides were stored and transported to MORU laboratories in Bangkok.

2.10.12 Fever clearance time

Aural temperature was assessed every 6 hours and at each pharmacokinetic sampling time point. The average temperature of left and right ears was recorded. Fever clearance time (FCT) was defined as: (i) FCTA, the time taken for the tympanic temperature to fall below 37.5°C and (ii) FCTB, the time taken for the tympanic temperature to fall below 37.5°C and remain there for at least 24hours. Fever time was defined as the duration in hours of an individuals temperature above 37.5°C. Area under the temperature-time curve above the temperature versus time curve (AUC $37.5_{0\text{-}72}$) from admission to 72 hours, above 37.5°C was also calculated.

2.10.13 *In vitro* drug susceptibility assays

2.10.13.1 Parasite culture and field strains

Plasmodium falciparum Thai laboratory strain TM267 was cultured in malaria culture medium (MCM) supplemented with 0.5% Albumax II (Gibco, New Zealand), as previously described [366]. Field isolates for the nested drug susceptibility testing were collected in lithium heparinized whole blood samples collected from patients with severe and moderately severe falciparum malaria enrolled in the RCT of paracetamol in Ramu, Bangladesh (Chapter 6). Blood samples were centrifuged at 3,000 rpm for 7 minutes then plasma and buffy coat removed. The remaining packed RBCs were washed three times with RPMI 1640 (Gibco, Grand Island, N.Y., U.S.A). Parasites in a 1ml aliquot of packed RBCs were mixed with 25 ml MCM supplemented with 0.5% Albumax II to make up a 4% haematocrit. The parasites were then incubated at 37°C a 5% CO_2 airflow chamber before drug susceptibility assays were performed in Bangaldesh. The remaining packed RBCs were cryopreserved in liquid nitrogen. An additional 6 isolated were cryopreserved for drug susceptibility testing at the Mahidol Oxford Research Unit Malaria Laboratory in Bangkok, Thailand. Cryopreserved isolated were thawed and returned to culture.

2.10.13.2 Drug preparation

Drug stock solutions were initially prepared in appropriate solvents and further diluted with RPMI 1640 medium to prepare the desired concentration of working solutions.

Artesunate (Guilin No.2 Pharmaceutical Co., Ltd., Guangxi, China), dispensed as artesunic acid powder at 60 mg per ampoule, was prepared as 60 mg/ml by dissolving in 1 ml of 5% sodium bicarbonate. Working solution 'A' of artesunate at a concentration of 1 mg/ml was prepared by diluting 25 μl artesunate stock solution in 1.5 ml MCM. To prepare the 10 μg/ml artesunate working solution 'B', 10 μl of artesunate working solution 'A' (1 mg/ml) was diluted in 1 ml MCM. Eleven concentrations ranging from 0.039 to 400 ng/ml were prepared by serial dilution in duplicate in 96-well plates.

Paracetamol (APAP; Sigma A7085) was dissolved in 0.5M sodium hydroxide and titrated to pH 7.4 and made up with PBS to a final concentration of 25 mg/ml. A working solution at a concentration of 5 mg/ml was prepared by diluting 200μl stock solution in 1ml MCM. Eleven concentrations of paracetamol ranging from sub-therapeutic (0.1562 μg/ml) to toxic concentrations (160 μg/ml) were prepared by serial dilution in 96-well plates.

2.10.13.3 Drug susceptibility assays

A synchronized TM267 culture of ring stages at a 2% parasitaemia and a haematocrit of 2% was prepared in in MCM and 100 μl was exposed to artesunate concentrations ranging from 0.39 to 400 ng/ml and paracetamol concentrations ranging from 0.1562 to 160 μg/ml. Each concentration was assayed in duplicate and wells containing no drug were used as negative controls. After incubation at 37°C, 5% CO_2 for 24 hours, 4 μl of cell pellet per well was removed and thick and thin blood smears were prepared. The total number of trophozoites per 100 infected RBCs was counted on each thin

smear. If the parasitaemia was less than 1 per 1000 RBCs on the thin film, then the number of trophozoites per 100 high power fields of the thick film was counted. The percentage of maturation and inhibition were calculated by the following formulae:

$$\% \text{ trophozoite maturation} = \frac{\text{number of trophozoites with drug}}{\text{number of trophozoites without drug}} \times 100$$

$$\% \text{ trophozoite maturation inhibition} = 100 - \% \text{ trophozoite maturation}$$

After assessing the trophozoites inhibition at 24 hours, the plates were re-incubated at 37°C, 5% CO_2 for an additional 24 hours (total 48-hour incubation) to determine the schizont inhibition. At 48 hours of incubation, the control wells were sampled and thin films assessed to ensure that parasites (>70% of infected RBCs) had developed into mature schizonts (≥ 8 merozoites). If schizonts had not reached maturity then the plates were incubated another 24 hours (total of 72 hours) until assessment. After 48 hours (or 72 hours as needed), 4 μl of cell pellet per well was removed and blood smears (thick and thin) were prepared. The total number of schizonts (≥ 8 merozoites) per 100 infected RBCs was counted on each thin smear. If the parasitaemia was less than 1 per 1000 RBCs on thin film, then the number of schizonts per 100 high power fields of the thick film was counted. The percentage of maturation and inhibition were calculated by the following formulae:

$$\% \text{ schizont maturation} = \frac{\text{number of schizont with drug}}{\text{number of schizont without drug}} \times 100$$

% schizont maturation inhibition = 100 - % schizont maturation

2.10.13.4 Effect of paracetamol on anti-malarial activity of artesunate

The potential modulating effects of paracetamol on the anti-malarial activity of artesunate were assessed in seven *P. falciparum* isolates from Bangladeshi patients. Since there was no effect of paracetamol on schizont maturation at the maximum concentrations tested in the drug sensitivity assay (see result section) and the goal was determine the existence of any effect of paracetamol on artesunate, the highest paracetamol concentration of 160 μg/ml, which would be toxic in humans [367, 368], was assessed. The concentrations of artesunate ranged from 0.39 to 400 ng/ml based on those used for the artesunate sensitivity assay. Each test was done in duplicate. Parasites were incubated with paracetamol and different concentrations of artesunate for 24 hours, after which trophozoites maturation was assessed, and for 48 hours, after which schizont maturation was assessed, as described above. Control wells run in parallel, containing the either paracetamol (0.1562-160 μg/ml or artesunate (0.139-400 ng/ml) were also assessed under the same conditions.

All *in vitro* testing was performed by the Malaria laboratory (Dr. Kesinee Chotivanich) at MORU, Bangkok, Thailand.

2.10.14 Statistical analysis

Non-normally distributed data were compared by Mann-U Whitney test, unless otherwise specified. Normally distributed data were compared using

the Student's *t*- test. Categorical gender variable was compared using Fischer's exact test. *P*–values of less than 0.05 were considered significant. Statistical software used were STATA14 (STATA, USA) and Prism 6 (Graphpad Software, USA).

3.0 Clinical aspects of acute kidney injury and haemolysis in falciparum malaria

3.1 A prospective descriptive study of acute kidney injury in adult patients with severe malaria

3.1.1 Background

The annual global burden of AKI in low- and middle-income (LMI) countries is estimated to be 11.3 million cases with an associated mortality of 1.4 million [369]. Determining the actual incidence and AKI-associated mortality in LMI countries is hindered by the limited availability of data and heterogeneous definitions of AKI [109]. A recent meta-analysis of the world incidence of AKI, as defined by the KDIGO consensus criteria, identified only two studies in adults and zero studies in children from low- and lower-middle income countries, representing 1.5% and 0% of the 154 studies assessed [370]. Malaria-associated AKI is recognized to contribute to this global burden [371], however the precise contribution is unclear due to differences in AKI diagnostic criteria applied to this population. Underestimating the true magnitude of the problem prevents research and implementation of preventative and treatment measures, as the relevance of the problem may not be appreciated.

The ‘0 by 25’ human rights case initiative is a global effort led by the International Society of Nephrology that aims to eliminate preventable deaths due to AKI by 2025 [109]. Within the overall strategies, to establish

prompt diagnosis and treatment of potentially reversible AKI, are goals to fill the information gaps of the global incidence of AKI and its risk factors, management and outcomes, particularly in LMI countries. To avoid misinterpretation of AKI incidence and permit systematic analyses and programmatic interventions, it is important to report AKI among populations from LMI countries using the international consensus KDIGO (or KDIGO-equivalent) (Table 1.4.2). This is evidenced in the malaria literature, where the reported incidence of falciparum malaria-associated AKI ranges from 0.4 - 60% in adults and 2.5 - 51% in children [86, 125, 128, 132, 146, 149] due to variable diagnostic definitions and denominator populations.

There are only two publications that describe AKI among severe falciparum malaria patients using KDIGO-equivalent [370] criteria [86, 112]. In a retrospective study of Thai adults, the incidence of AKI using the RIFLE criteria was 74% compared to 45% using the 2000 WHO criteria (creatinine > 3mg/dl) and both definitions of AKI were associated with in-hospital mortality and requirement for RRT [112]. One prospective multicentre study of Congolese children with SM reported an AKI incidence of 23.5% using the AKIN criteria; of whom 25.8% required dialysis and 12.4% died [86]. The 2012 KDIGO group consensus definition and classification system [103], which harmonised the RIFLE and AKIN criteria, has not been applied to describe AKI in patients with falciparum malaria.

Although the WHO definition for malaria-associated AKI has been largely consistent for 25 years, the clinical diagnosis is often delayed and by the

time the creatinine reaches 3 mg/dl the GFR has already declined by 50% [103]. By this point, the window for preventative intervention has past [372], which impedes the ability to improve outcomes, particularly in countries where RRT is unavailable. As kidney dysfunction is an independent predictor for mortality in adults and children with falciparum malaria [12, 373], it is important to assess the KDIGO criteria and its prognostic accuracy in this population. In this study, the characteristics, incidence and outcomes of AKI in adults with SM using the different definitions are described.

3.1.2 Study aim

The first aim was to characterise severe malaria-associated AKI according to the WHO guidelines and the KDIGO international classification system. The second aim was to assess the incidence of AKI in adults according to both classification systems and compare outcomes including mortality, RRT requirement and length of hospital stay.

3.1.3 Study design

Characterisation of malaria-associated AKI was assessed from a detailed prospective observational study of consecutive adult Bangladeshi patients (n=138) diagnosed with *P. falciparum* malaria and admitted to CMCH and Ramu Upazilla Health Complex, Bangladesh from June to September in 2010 - 2014. SM patients were included as defined in chapter 2.3. Patients were excluded from the descriptive analysis of SM if aged less than 19 years old, were missing enrolment creatinine values or were diagnosed with UM. Patients less than 19 year were excluded since the MDRD formula is not valid for this younger population. The association of AKI and mortality was

assessed from SM and UM patients admitted to CMCH from 2010 to 2014 and SM patients enrolled in interventional studies from 2004 to 2007, as described in Chapter 4.1.

AKI diagnosis was defined by only the creatinine criteria of the WHO, and KDIGO classification systems as shown in Table 3.1.1. Baseline creatinine values were not available for any of the patients, therefore were calculated using the MDRD formula assuming a GFR of 75 ml/min per 1.73m^2. This methodology is recommended by the KDIGO guidelines to determine baseline creatinine in the absence of a reliable baseline creatinine on record [103]. Since RRT and urine output were not available for all patients these criteria were not incorporated. Patients with a KDIGO AKI diagnosis on admission were staged using only the enrolment serum creatinine concentrations with reference to the MDRD baseline creatinine in order to characterise outcomes by admission AKI stage.

Continuous and categorical data were analysed as described in Chapter 2.10.14. To assess the relationship of mortality with AKI diagnosed by WHO criteria and KDIGO criteria, univariate logistic regression analysis was performed.

3.1.4 Results

Characterisation of malaria-associated AKI

During the 2010 to 2014 study period, 303 patients with severe and uncomplicated malaria were enrolled. A total of 165 patients were excluded

Table 3.1.1 WHO and KDIGO definitions and classification for AKI on enrolment using serum creatinine

Criteria	WHO	KDIGO
Diagnosis	Serum creatinine > 3 mg/dl	Increase in SCr to ≥ 1.5 times baseline, known or presumed to have occurred within the prior 7 days
Staging*	--	1. Increase in SCr to 1.5-1.9 times baseline 2. Increase in SCr to 2.0-2.9 times baseline 3. Increase in SCr to 3.0 times baseline, or increase in SCr to ≥ 4 mg/dl

*Staging was performed only on admission creatinine. *Abbreviations:* WHO, World Health Organization; KDIGO, Kidney Disease: Improving Global Outcomes; AKI, acute kidney injury; Scr, serum creatinine

from the descriptive analysis; 39 patients were under the age of 19 years, 31 patients were missing enrolment creatinine values and 95 were diagnosed with UM. The remaining 138 patients were assessed for a detailed characterisation of severe malaria-associated AKI as classified by the WHO and KDIGO criteria.

Demographic characteristics of all SM patients are described in Table 3.1.2. On enrolment, 36 (26%) patients had AKI, as defined by the WHO, and 70 (51%) had AKI, as defined by KDIGO diagnostic criteria (Table 3.1.2). Patients diagnosed with AKI by KDIGO criteria were older (P =0.045), female (P =0.002), and had a longer duration of vomiting and/or diarrhea (P = 0.041). On physical examination, patients diagnosed with AKI by either definition had a lower temperature and were more often jaundiced compared to those without AKI (Table 3.1.3). Dehydration on physical exam was associated with AKI only when diagnosed by KDIGO criteria (P = 0.001). Patients diagnosed with AKI by either definition had a higher number of malaria severity criteria (P < 0.001) (Table 3.1.4).

Haematologic investigations showed that patients diagnosed with AKI by WHO criteria had higher leukocyte counts (P =0.025), lower haemoglobin (P = 0.013) and higher *Pf*HRP2 concentrations (P < 0.001) compared to those without AKI (Table 3.1.5). The same trends were observed in patients diagnosed with AKI by KDIGO criteria (P = 0.017; P = 0.008 and P < 0.001,

Table 3.1.2 Demographics by enrolment AKI according to WHO and KDIGO definitions

Characteristic	WHO			KDIGO		
	No AKI (n=102)	AKI (n=36)	*P* value	No AKI (n=68)	AKI (n=70)	*P* value
Sex, male (%)	65 (64)	21 (58)	0.35	51 (75)	35 (50)	**0.002**
Age, years	30 (24-45; 19,75)	36 (25-40; 20,55)	0.64	29 (24-41; 19,75)	36 (26-45; 20,70)	**0.045**
Ramu enrolled	6 (6)	0 (0)	0.14	4 (6)	2 (3)	0.38
Chittagong enrolled	96 (94)	36 (100)	0.14	64 (94)	68 (97)	0.38
Comorbidities (%)	7 (16)	4 (20)	0.72	3 (4)	8 (11)	0.42
Hypertension	2 (2)	3 (8)	0.18	1 (1)	4 (6)	0.36
Coronary artery disease	4 (4)	1 (3)	0.56	1 (1)	4 (6)	0.38
Type 2 diabetes	3 (3)	1 (3)	0.77	2 (3)	2 (3)	0.58
Days of fever	7 (5-8; 2,20)	7 (6-9; 3,15)	0.83	7 (5-8; 2,20)	7 (6-9; 3,20)	0.44
Days vomiting/diarrhea	2 (105; 1,10)	3 (2-7; 1,15)	0.06	2 (1-5; 1,10)	3 (2-5; 1,15)	**0.041**
Previous malaria	24 (27)	9 (26)	1.00	16 (27)	17 (26)	1.00

All values are median (IQR; range) or number (%). *Abbreviations*: WHO, World Health Organization; KDIGO, Kidney Diseases: Improving Global Outcomes; AKI, acute kidney injury.

Table 3.1.3 Physical examination characteristics on enrolment by AKI according to WHO and KDIGO definitions

Characteristic	WHO			KDIGO		
	No AKI (n=102)	AKI (n=36)	*P* value	No AKI (n=68)	AKI (n=70)	*P* value
Glasgow coma score	9 (8-14)	9 (8-14)	0.81	10 (8-14)	9 (6-14)	0.17
Temperature (°C)	38.6 (37.7-39.5)	37.4 (36.8-38.5)	**<0.001**	38.5 (37.6-39.5)	38.1 (37.0-39.0)	**0.047**
SBP (mmHg)	111 (101-121)	115 (106-124)	0.32	115 (102-125)	111 (101-121)	0.31
DBP (mmHg)	67 (57-7)	70 (59-78)	0.53	67 (57-75)	68 (58-78)	0.68
MAP (mmHg)	81 (73-90)	83 (74-96)	0.45	81 (73-90)	82 (73-96)	0.97
Heart rate (per min)	117 (101-134)	108 (89-123)	**0.028**	114 (102-132)	117 (95-130)	0.80
Breaths (per min)	32 (28-40)	29 (23-40)	0.17	32 (28-39)	32 (26-40)	0.83
Oxygen saturation (%)	96 (94-97)	95 (94-97)	0.47	96 (94-97)	95 (93-97)	0.13
Very sick condition	55 (54)	23 (66)	0.15	39 (57)	39 (56)	1.00
Dehydration	25 (25)	7 (20)	0.42	8 (12)	24 (35)	**0.001**
Jaundice	33 (34)	26 (74)	**<0.001**	20 (31)	39 (58)	**0.002**
Haemoglobinuria	19 (19)	10 (28)	0.18	12 (18)	17 (24)	0.23
Pregnant	11 (11)	2 (6)	0.29	4 (6)	9 (13)	0.13

All values are median (IQR) or number (%). *Abbreviations*: WHO, World Health Organization; KDIGO, Kidney Diseases: Improving Global Outcomes; AKI, acute kidney injury; SBP, systolic blood pressure; DBP, diastolic blood pressure.

Table 3.1.4 Severity criteria on enrolment by AKI according to WHO and KDIGO definitions

Characteristic	WHO			KDIGO		
	No AKI (n=102)	AKI (n=36)	*P* value	No AKI (n=68)	AKI (n=70)	*P* value
Cerebral malaria, GCS<11	68 (67)	22 (61)	0.34	45 (66)	45 (64)	0.48
Convulsions, >2/24h	21 (21)	3 (8)	0.07	15 (22)	9 (13)	0.12
Severe anaemia, <20%	7 (7)	4 (11)	0.31	4 (6)	7 (10)	0.28
AKI, SCr >3mg/dl	0 (0)	36 (100)	**<0.001**	0 (0)	36 (51)	**<0.001**
Bicarbonate < 15mmol/L	16 (16)	23 (64)	**<0.001**	6 (9)	33 (47)	**<0.001**
Lactate >4mg/dl	47 (46)	17 (47)	1.00	30 (44)	34 (49)	0.36
Jaundice + bilirubin >2.5mg/dl	18 (18)	9 (25)	0.24	13 (19)	14 (20)	0.53
SBP <80mmHg	1 (1)	0 (0)	0.74	0 (0)	1 (1)	0.51
Hyperparasitaemia >10%	19 (19)	8 (22)	0.40	12 (18)	15 (21)	0.37
Pulmonary oedema	1 (1)	0 (0)	0.74	1 (1)	0 (0)	0.49
Spontaneous bleeding	4 (4)	2 (6)	0.49	4 (6)	2 (3)	0.33
Hypoglycaemia	1 (1)	0 (0)	0.74	0 (0)	1 (1)	0.51
Number of severity criteria	2 (1-3; 0,7)	4 (2-5; 1,6)	**<0.001**	2 (1-3; 0,7)	3 (2-4; 1,6)	**<0.001**

All values are median (IQR; range) or number (%). *Abbreviations*: WHO, World Health Organization; KDIGO, Kidney Diseases: Improving Global Outcomes; AKI, acute kidney injury; Scr, serum creatinine.

Table 3.1.5 Haematology on enrolment by AKI according to WHO and KDIGO definitions

Characteristic	WHO		*P* value	KDIGO		*P* value
	No AKI (n=102)	AKI (n=36)		No AKI (n=68)	AKI (n=70)	
WBC (mm3)	9.1 (6.5-12.6)	10.2 (8.0-22.0)	**0.025**	9.0 (5.8-11.7)	10.5 (7.7-16.6)	**0.017**
Neutrophils (%)	74.7 (69.3-81.3)	77.4 (71.5-81.4)	0.44	74.0 (69.6-80.3)	75.9 (68.6-81.4)	0.61
Haemoglobin (g/dl)	10 (7.2-11.6)	8.3 (6.2-9.6)	**0.013**	10.6 (7.6-12.0)	8.3 (6.5-10.6)	**0.008**
Platelets (mm3)	33 (19-51)	28 (16-42)	0.09	37 (22-53)	28 (18-40)	**0.013**
CFH (ng/ml)[a]	64 482 (43,252-96,131)	125 779 (49,586-319,052)	0.12	47 155 (29,369-75,713)	111 685 (63,584-196,174)	**0.033**
Parasitaemia (/µl)[a]	80 070 (12,560-284,107)	30,395 (12,437-212,452)	0.38	96 586 (18,212-285,739)	36 801 (10,048-271,296)	0.30
*Pf*HRP2 (ng/ml)	1,752 (854-3,234)	7,784 (2 780-12,925)	**<0.001**	1,664 (778-3,094)	4,090 (1,513-10,877)	**<0.001**

All values are median (IQR) or number (%). [a]Geometric mean (95% CI). *Abbreviations*: WHO, World Health Organization; KDIGO, Kidney Diseases: Improving Global Outcomes; AKI, acute kidney injury; WBC, white blood cells, CFH; cell-free haemoglobin; *Pf*HRP2, *Plasmodium falciparum* histidine rich protein 2

respectively). However, lower platelet levels and higher CFH concentrations were only associated with AKI when diagnosed by KDIGO criteria (P = 0.013 and P = 0.033, respectively). Biochemical parameters showed expected associations in patients with AKI when diagnosed by either definition. Specifically, those with AKI had lower pH, bicarbonate and base excess compared to patients without AKI (Table 3.1.6). Phosphate, magnesium, AST, total bilirubin, indirect bilirubin and LDH were all higher in patients with AKI compared to those without AKI by either definition. Patients with AKI defined by WHO had lower urine pH, urine osmolality, BUN-to-creatinine ratio and higher fractional excretion of sodium (FeNa) (Table 3.1.7). However, KDIGO diagnosed AKI patients only had lower urinary pH and osmolality compared to those without AKI but similar trends in FeNa and BUN:creatinine ratio.

Outcomes of malaria-associated AKI

26/36 (70%) of patients with AKI on enrolment by WHO criteria received dialysis, compared to 30/70 (43%) when diagnosed by KDIGO (Table 3.1.8A). Notably, when enrolment AKI was staged by KDIGO criteria, 9 of 30 (30%) patients who received dialysis were KDIGO stage 0 to 2 on admission, and were not identified on enrolment by WHO AKI criteria (Table 3.1.8B). AKI by either definition was associated with a longer duration of hospital stay compared to patients without AKI.

The WHO definition missed diagnosing 34 patients with KDIGO AKI on enrolment, of whom 12% (4/34) received RRT and 48% (16/34) died (Table

Table 3.1.6 Biochemistry on enrolment by AKI according to WHO and KDIGO definitions

Characteristic	WHO			KDIGO		
	No AKI (n=102)	AKI (n=36)	*P* value	No AKI (n=68)	AKI (n=70)	*P* value
Creatinine (mg/dl)	1.2 (0.9-1.5)	4.5 (3.6-5.4)	**<0.001**	1.0 (0.8-1.2)	3.2 (1.8-4.5)	**<0.001**
BUN (mg/dl)	29 (19-44)	99 (77-118)	**<0.001**	24 (17-32)	63 (48-101)	**<0.001**
Sodium (mmol/l)	138 (133-143)	134 (129-143)	0.10	138 (132-142)	138 (131-145)	0.86
Potassium (mmol/l)	4.0 (3.7-4.6)	4.9 (4.2-5.4)	**<0.001**	4.0 (3.8-4.6)	4.5 (3.8-5.3)	**0.046**
Chloride (mmol/l)	102 (72-122)	91 (72-111)	0.48	100 (72-131)	98 (73-112)	0.55
pH	7.42 (7.37-7.47)	7.33 (7.26-7.37)	**<0.001**	7.43 (7.39-7.47)	7.36 (7.31-7.41)	**<0.001**
Bicarbonate (mmol/l)	19.2 (16.2-21.9)	13.7 (10.1-16.35)	**<0.001**	20.4 (17.7-23.1)	15.3 (11.7-18.6)	**<0.001**
Base excess (mmol/l)	-2 (-7 to -3)	-10 (-14 to -9)	**0.017**	-2 (-5 to -3)	-7 (-12 to -6)	**0.015**
Lactate (mg/dl)	3.35 (2.50-5.70)	3.55 (2.20-5.09)	0.76	3.28 (2.50-5.53)	3.80 (2.40-5.82)	0.74
Glucose (mmol/l)	6.3 (5.0-8.2)	6.7 (4.7-9.4)	0.76	6.2 (5.0-8.5)	6.5 (4.5-8.2)	0.66
Phosphate (mg/dl)	2.3 (1.6-3.1)	3.5 (1.9-4.6)	**0.010**	2.2 (1.6-2.7)	3.1 (1.8-3.9)	**0.018**
Magnesium (mg/dl)	2.1 (1.8-2.5)	2.7 (2.3-3.2)	**<0.001**	2.1 (1.7-2.3)	2.5 (2.1-2.9)	**<0.001**
Calcium (mg/dl)	8 (7.5-8.6)	7.8 (7.3-8.4)	0.25	7.9 (7.6-8.4)	7.9 (7.3-8.5)	0.57
Albumin (mg/dl)	2.8 (2.5-3.1)	2.5 (2.3-2.75)	**0.003**	2.9 (2.6-3.1)	2.6 (2.2-3.0)	**<0.001**
ALT (U/l)	24 (14-39)	18 (9-33)	0.20	25 (15-40)	19 (11-34)	0.09
AST (U/l)	64 (42-118)	100 (62-193)	**0.014**	58 (37-110)	99 (54-191)	**0.005**
Total bilirubin (mg/dl)	1.7 (0.9-3.3)	8.0 (2.1-23.3)	**<0.001**	1.7 (1.0-3.3)	2.9 (1.2-11.5)	**0.008**
Indirect bilirubin (mg/dl)	0.7 (0.4-1.6)	5.3 (1.1-14.1)	**<0.001**	0.7 (0.4-1.4)	1.4 (0.6-8.3)	**<0.001**
LDH (U/l)	567 (436-765)	827 (714-1214)	**<0.001**	540 (437-745)	748 (580-1 062)	**<0.001**

All values are median (IQR) or number (%). *Abbreviations*: WHO, World Health Organization; KDIGO, Kidney Diseases: Improving Global Outcomes; AKI, acute kidney injury; BUN, blood urea nitrogen; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase.

Table 3.1.7 Urinary indices on enrolment by AKI according to WHO and KDIGO definitions

Characteristic	WHO			KDIGO		
	No AKI (n=102)	AKI (n=36)	*P* value	No AKI (n=68)	AKI (n=70)	*P* value
Urine specific gravity	1.015 (1.010-1.020)	1.020 (1.015-1.020)	0.26	1.015 (1.010-1.002)	1.020 (1.015-1.020)	0.22
Urine pH	6 (5-6)	5 (5-5)	**<0.001**	6 (5-6)	5 (5-6)	**<0.001**
Urine pH meter	5.69 (5.47-6.14)	5.15 (5.09-5.64)	**0.001**	5.70 (5.51-6.14)	5.38 (5.12-5.65)	**0.004**
Urine protein	30 (30-100)	30 (30-100)	0.35	30 (30-100)	30 (30-100)	0.88
Urine haemoglobin	4 (2-4)	4 (4-4)	0.35	4 (2-4)	4 (3-4)	0.93
Urine urobilinogen	1 (0-2)	0 (0-1)	**0.012**	1 (1-2)	1 (0-1)	**0.001**
Urine bilirubin	1 (0.5-2)	1 (0-2)	0.84	1 (1-2)	1 (0-2)	0.17
Urine osmolality	650 (539-911)	389 (333-496)	**0.001**	828 (611-1024)	502 (379-646)	**0.001**
Urine sodium	52 (33-97.6)	41 (26-83)	0.40	65 (33-101)	45 (33-82)	0.12
FeNa	1.4 (0.5-47.2)	2.4 (1.1-244)	**0.044**	1.3 (0.4-44.6)	1.8 (0.8-87.2)	0.11
BUN:Cr ratio	26 (20-31)	21 (17-26)	**0.006**	26 (20-30)	23 (18-29)	0.47

All values are median (IQR; range) or number (%). All measures are from urine dipstick except pH meter, osmolality, sodium, FeNa and BUN to creatinine ratio. *Abbreviations*: WHO, World Health Organization; KDIGO, Kidney Diseases: Improving Global Outcomes; AKI, acute kidney injury; FeNa, fractional excretion of urine; BUN:Cr; blood urea nitrogen to creatinine ratio.

Table 3.1.8A Outcomes based on enrolment by AKI according to WHO and KDIGO definitions

Characteristic	WHO			KDIGO		
	No AKI (n=102)	AKI (n=36)	*P* value	No AKI (n=68)	AKI (n=70)	*P* value
RRT	8 (8)	26 (72)	**<0.001**	4 (6)	30 (43)	**<0.001**
Duration admission, days	5.5 (5.0-7.5)	15.0 (9.0-21.0)	**<0.001**	5 (4-7)	9 (6.5-16.5)	**<0.001**
Outcome, died	33 (32)	13 (36)	0.42	17 (25)	29 (41)	**0.031**
Time to death, hours	30 (19-64)	17 (7-37)	**0.022**	39 (19-92)	24 (15-43)	0.31

All values are median (IQR; range) or number (%). *Abbreviations*: WHO, World Health Organization; KDIGO, Kidney Diseases: Improving Global Outcomes; AKI, acute kidney injury; RRT, renal replacement therapy

Table 3.1.8B Outcomes based on enrolment by AKI according to KDIGO stage

Characteristic	KDIGO				*P* for trend
	0 (n=68)	1 (n=17)	2 (n=15)	3 (n=38)	
RRT	4 (6)	1 (6)	4 (27)	25 (66)	**<0.001**
Duration admission, days	5 (4-7)	6 (5-8)	7 (6-8)	15.5 (9-19.5)	**<0.001**
Outcome, died	17 (25)	6 (35)	9 (60)	14 (37)	0.07
Time to death, hours	39 (19-92)	30 (27-46)	24 (15-38)	18 (7-43)	0.09

All values are median (IQR; range) or number (%). *Abbreviations*: WHO, World Health Organization; KDIGO, Kidney Diseases: Improving Global Outcomes; AKI, acute kidney injury; RRT, renal replacement therapy

Table 3.1.9 Morality outcomes based on enrolment AKI according to WHO and KDIGO definitions

KDIGO	Mortality	AKI Missed by WHO criteria	Mortality of AKI missed by WHO criteria
Stage 0	17/68 (25%)	N/A	N/A
Stage 1	6/17 (35%)	17/17 (100%)	6/17 (35%)
Stage 2	9/15 (60%)	14/15 (93%)	9/14 (64%)
Stage 3	14/38 (37%)	3/38 (8%)	1/3 (33%)
Total	**29/70 (41%)**	**34/70 (48%)**	**16/34 (47%)**

Abbreviations: WHO, World Health Organization; KDIGO, Kidney Diseases: Improving Global Outcomes; AKI, acute kidney injury.

3.1.9). Pooled data analysis including the 2004-2007 cohort, showed that the incidence of AKI by WHO criteria was 16% (55/337) compared to 44% (147/337) using the KDIGO diagnostic criteria. In patients with SM, univariate analysis showed a strong association between AKI defined by KDIGO and fatal outcome with an odds ratio (95%CI) of 2.26 (1.34-3.78) (*P* = 0.002; N=256), whereas AKI defined by WHO criteria did not show this association (Table 3.1.10A). AKI as diagnosed by KDIGO criteria in severe and uncomplicated malaria was also significantly associated with mortality (OR = 2.89 (1.76-4.73), *P* < 0.001; N=337) (Table 3.1.10B).

3.1.5 Discussion

Kidney dysfunction has been shown to be a prognostic factor for poor outcomes in adults and children with severe falciparum malaria [12, 373]. However, the true incidence of malaria-associated AKI is likely underestimated in both populations due to the high creatinine threshold used to define AKI in the WHO malaria guidelines [1, 21]. Early recognition of this serious complication is critical since even mild AKI associated with small incremental increases in creatinine (≥ 0.3 mg/dl) are associated with higher mortality [374] and is preventable and potentially treatable [103]. The KDIGO consensus definition for AKI includes *changes* in creatinine that form the basis of AKI diagnostic criteria [103]. In the current study, the factors associated with AKI in severe falciparum malaria patients using the KDIGO criteria for diagnosis were comparable to those identified using the WHO AKI definition. In the pooled cohort of 337 hospitalised falciparum malaria patients, 16% were diagnosed with AKI at enrolment by the WHO criteria

Table 3.1.10A Logistic regression of enrolment AKI on outcome in patients with severe malaria

	Univariate model			
Variable	OR (95% CI)[b]	X^2	N	*P*
2010-2014 cohort				
WHO AKI	1.18 (0.53-2.62)	0.41	138	0.68
KDIGO AKI	2.12 (1.02-4.39)	2.03	138	**0.042**
2004-2007 & 2010-2014				
WHO AKI	1.09 (0.59-2.02)	0.29	262	0.78
KDIGO AKI	2.26 (1.34-3.78)	3.09	262	**0.002**

Table 3.1.10B Logistic regression of enrolment AKI on outcome in patients with severe and uncomplicated malaria

	Univariate model			
Variable	OR (95% CI)[b]	X^2	N	*P*
2010-2014 cohort				
WHO AKI	2.47 (1.14-5.38)	2.29	219	**0.022**
KDIGO AKI	2.93 (1.51-5.71)	3.17	219	**0.002**
2004-2007 & 2010-2014				
WHO AKI	1.80 (0.98-3.30)	1.90	337	0.06
KDIGO AKI	2.89 (1.76-4.73)	4.21	337	**<0.001**

[b]Odds ratios (OR) with 95% CIs and Wald X^2 values were determined for AKI associated with 1-U changes in the longitudinal independent variables. *Abbreviations*: OR= odds ratio; CI= confidence intervals.

compared to 44% using the KDIGO criteria. AKI staging on admission using the KDIGO criteria showed that more patients with mild AKI were diagnosed and 47% of AKI diagnoses missed by the WHO criteria died. Diagnosis ofAKI using the KDIGO criteria was superior in predicting hospital mortality among severe and uncomplicated malaria patients compared to the WHO criteria.

To stratify the risk of AKI in patients with malaria, exposures (referring to illnesses, therapies or procedures with AKI-inducing potential) and susceptibilities (referring to shared factors common to different AKI settings) in this population must be determined [103]. SM with multiple severity criteria has been identified as an exposure that increases the risk for AKI [219, 264], further supported by the findings in this study. The diagnosis of malaria-associated AKI on enrolment using the KDIGO criteria identified known demographic susceptibility factors for AKI including older age and hypovolaemia [135, 139, 375], as implied by longer duration of vomiting/diarrhea and dehydration on physical exam, whereas the WHO criteria did not. While male gender is more commonly reported as a risk factor [375], female gender was identified as a susceptibility for AKI [103]. The lower temperatures associated with WHO and KDIGO stage 3 AKI is difficult to interpret given the fluctuating nature of fever in malaria. This may be related to earlier presentation of patients with less severe disease, since schizogony mediates fever and symptomatic illness [353]. Lower temperatures were also observed in patients presenting with established uraemic AKI after pre-referral treatment compared to patients with acute AKI

[22]. Establishing susceptibilities for AKI in the malaria guidelines is critical so that patients can be systematically stratified and managed according to their AKI risk.

After risk stratification, early diagnosis and staging of AKI, patients should be managed according to cause [103]. AKI diagnosed by either definition was associated with jaundice, which agrees with previous studies [22, 133, 139]. While this observational study is unable to show causality, the KDIGO AKI patients had higher CFH, indirect bilirubin, LDH and AST and low haemoglobin compared to those without AKI, suggesting that this association is potentially related to intravascular haemolysis. These associations have also been reported previously [264]. CFH released during haemolysis is suggested to be involved in the pathophysiology of AKI in falciparum malaria (Chapter 4.2) and other haemolytic conditions [65, 376]. Plasma *Pf*HRP2, as an estimate of sequestered parasite biomass, and white blood cell count were elevated in patients diagnosed with AKI using either AKI definition. These observations are consistent with studies supporting that sequestration and immune activation contribute to AKI in severe falciparum malaria [171, 179]. The urinary indices of patients with AKI by the WHO and KDIGO stage 3 criteria were suggestive of ATI, consistent with previously reported histopathological findings [171]. Thrombocytopenia observed in patients with AKI by both definitions may likely be a general marker of disease severity and endothelial activation [377] as opposed to a specific marker for AKI. The acid-base and electrolyte abnormalities associated with AKI by either definition, are consequences of the kidney

dysfunction. Thus, targeted interventions to reduce renal dysfunction in malaria include prompt antimalarial treatment with artemisinins to reduce sequestration and prompt nephrology referral for close monitoring and potential RRT in those with severe AKI and established ATI. Further research into establishing a treatment to reduce renal injury secondary to effects of haemolysis is needed.

The consensus KDIGO criteria have been shown to have high discriminating ability for in-hospital mortality in both adults and children in high-income settings [378, 379]. This is the first study to report outcomes of malaria-associated AKI as defined by the KDIGO criteria, and also showed that KDIGO AKI is predictive of in-hospital mortality. Compared to the WHO definition the KDIGO criteria were less restrictive in diagnosing AKI, which resulted in a higher incidence of malaria-associated AKI and identified more patients who died with mild AKI on enrolment. This suggests that the KDIGO criteria would be beneficial towards earlier diagnosis AKI in patients with SM, which could therefore permit prompt preventative and therapeutic intervention. As the KDIGO criteria are also appropriate for diagnosing AKI in children [379], further study in children with malaria could very likely assist in accurately determining the AKI burden in this population. This would also support the incorporation of the KDIGO definition for AKI diagnosis that is appropriate for all age groups in the WHO malaria guidelines.

Potential limitations to this study include that the MDRD back-calculation to determine baseline creatinine has not been validated in this population.

However, this methodology is recommended by the KDIGO guidelines [103]. Also, the KDIGO criteria identified all patients diagnosed by the WHO criteria, which suggests that MDRD back-calculation resulted in appropriate diagnosis. No urine output or RRT criteria were incorporated into the definition for diagnosis or staging since this data and treatment was largely unavailable in the earlier cohort. The association of AKI, according to KDIGO, with mortality in the absence of RRT and urine output criteria is important since in practice both of these variables are frequently unavailable in resource limited settings. Only the admission creatinine was used for the KDIGO AKI diagnosis, however the characterisation and outcome analysis suggests that this is valid. This may be more valuable to allow for prompt recognition and management of AKI since early fluid administration after admission may decrease serum creatinine, which would be falsely reassuring and delay the diagnosis of AKI [380, 381]. While studies have demonstrated the utility of fluid-adjusted serum creatinine to circumvent this issue, this strategy is practically difficult in resource-limited settings [380]. Thus the ability to diagnose and stage using a single admission creatinine that is prognostic for outcomes would be advantageous in LMI countries.

3.1.6 Conclusions

The incidence of malaria-associated AKI was 44% compared to 16% using the KDIGO and WHO criteria, respectively. This suggests that AKI in falciparum malaria is under-reported and potentially under-diagnosed. The KDIGO diagnosis identified susceptibilities for AKI and was associated with factors proposed to be involved in the pathophysiology of AKI complicating

malaria. AKI as diagnosed by KDIGO criteria identified patients with mild AKI who subsequently died and predicted mortality in severe and uncomplicated malaria better than the WHO definition for AKI. The applicability of KDIGO to both paediatric and adult populations may provide a harmonised age-appropriate definition of AKI for the WHO malaria guidelines that is aligned with the ‘0 by 25’ initiative to reduce preventable deaths due to AKI by 2025. KDIGO should be considered to replace the current criteria used to define malaria-associated AKI in the WHO malaria guidelines.

3.2 Genotypic and phenotypic characterisation of G6PD deficiency in Bengali adults with severe and uncomplicated malaria

3.2.1 Background

CFH is associated with disease severity in falciparum malaria [50], however the mechanisms of haemolysis are not completely understood. The contribution of CFH from the three main populations of RBCs, including infected, uninfected and once-infected cells, must also be considered in the context of inherited RBC defects and haemoglobinopathies.

G6PD deficiency is an X-linked genetic trait resulting in an enzyme defect characterised by varying degrees of haemolysis depending on the mutation [382]. The similar global distribution of G6PD deficiency and malaria endemicity led to the hypothesis that this mutation confers protection against malaria [383, 384]. Clinical studies support that the African variant protects against SM [385, 386] and against high parasitaemias in heterozygous females [387]. The mechanism of protection and the fate of infected G6PD deficient RBCs are unclear.

Despite a potential advantage of G6PD deficiency in malaria infection, the 8-aminoquinolones, such as primaquine, used to treat malaria can cause severe haemolysis in G6PD deficient individuals [30]. Thus, determining the G6PD deficiency genotypes and diagnosing deficiency in malaria endemic

countries is critical with respect to malaria control and elimination programs in which primaquine, the only drug active against hypnozoites, is vital [388].

There is a paucity of literature on G6PD deficiency in Bangladesh. Among 150 Bengali males attending an outpatient clinic or admitted to hospital in Dhaka, 4% were found to have enzymatic deficiency [39]. A recent quantitative G6PD study among Marma and Khyang ethnic groups living in the Chittagong Hill Tracts of Bangladesh (n=220), found that 59% had normal G6PD activity, 35% had mild deficiency and 6.5% had severe deficiency [389]. However, the G6PD deficiency variants and effects on falciparum malaria infection have not been described in Bangladesh.

3.2.2 Study aim

To describe the G6PD deficiency genotype and polymorphic markers in addition to the G6PD deficiency phenotype with respect to intravascular haemolysis and disease severity in adults with falciparum malaria living in southern Bangladesh.

3.2.3 Study design

The study was undertaken in one urban and one rural centre in the southern Chittagong Division of Bangladesh as part of an observational study of the pathophysiology of falciparum malaria and a randomised controlled study of paracetamol in severe and moderately severe falciparum malaria from 2012 to 2014. Ramu Upazilla Health Complex is a rural centre in the District of Cox's Bazar close to the Chittagong Hill Tracts and Myanmar border. CMCH is an urban centre located District of Chittagong (Figure 2.1.1). Since there is

a paucity of literature describing the prevalence of G6PD deficiency in Bangladesh, the study was designed as a pilot and the sample size was chosen pragmatically. Of 141 falciparum malaria patients enrolled into the observational study, 137 malaria patients and 27 healthy controls were tested for G6PD deficiency using the qualitative fluorescent spot test (FST) upon enrolment and at 4-week follow up (Chapter 2.10.9).

All the patients who were enrolled into the randomised controlled study in addition to the two healthy controls that were deficient by qualitative screening had their G6PD genotype analysed (n=64). Four of these patients were missing FST results. Since the G6PD deficiency genotypes in Bangladesh have not been previously published, whole gene sequencing was performed using DNA extracted from EDTA blood samples, as described in Chapter 2.10.10. A literature review was performed to assess the G6PD deficiency genotypes commonly found in neighboring populations in India and Myanmar to assist with identification of G6PD mutations.

3.2.4 Results

137 of 141 falciparum malaria patients were included in the FST analysis as four were missing FST results. The mean (SD) age was 31 (13) and all subjects were of Bengali ethnicity. The FST on enrolment showed that 111/137 (81.0%) were not G6PD deficient, 2/137 (1.5%) were G6PD deficient (males) and 24/137 (17.5%) had indeterminate results (11 females,

Table 3.2.1. Results of G6PD fluorescent spot test

Result	SM (n=75)	UM (n=62)	Healthy (n=27)
Deficient	1 (1%) 1M*	1 (2%) 1M	2 (7%) 1M/1F
Indeterminate	10 (13%) 5M/5F	14 (23%) 8M/6F	3 (11%) 3M/0F
Normal	64 (85%) 42M/22F	47 (76%) 33M/14F	22 (82%) 13M/9F

*Genotype not performed in this individual. *Abbreviations*: SM, severe malaria; UM, uncomplicated malaria; M, male; F, female.

13 males) (Table 3.2.1). Of the healthy controls, 2/27 (7%) were G6PD deficient (one male and one female. Only 17/137 (12%) patients returned for a repeat FST at 4 weeks; 11/17 results were the same at both time points except for 6 indeterminate results on enrolment that had a normal G6PD result at follow up (2 males, 3 females). The frequency of G6PD deficiency by FST was similar between sites; 1/32 (3%) in Ramu and 1/105 (1%) in Chittagong. The enrolment site did not necessarily reflect area of residence.

A G6PD deficiency mutation was found in 4.8% (3/62) of the severe and moderately severe malaria patients in the randomised study. Two patients had the Orissa variant (131C>G); of whom one was a heterozygous female (FST not performed) and one was a hemizygous male (FST deficient). The third patient had the Kerala-Kalyan variant (949G>A) and was female with an indeterminate FST on enrolment but normal FST at 4-week follow up (Table 3.2.2) (Figure 3.2.1). A fourth male patient had a synonymous mutation in exon 7 (690C>T) and an indeterminate FST. This mutation has not been published in the literature or the www.LOVD.nl/MR database [390] and thus may be unique to Bangladesh. Of the two healthy controls with a deficient FST result, one male patient was found to have the Mahidol mutation (487G>A), while the other female patient had no mutation identified. Since only coding regions were sequenced this discrepancy may be have been due to a polymorphism in the promoter or a splicing site [391]. The genotype of the SM male patient with a deficient FST was not performed.

Table 3.2.2. G6PD genotypes and markers identified in the study

Mutation	Variant	Location	Ancestral allele	Derived allele	Amino acid effect	Enzyme activity
c.131C>G	Orissa	Exon 3	C	G	A44G	~10% normal activity [392]
c.949G>A	Kerala-Kalyan	Exon 9	G	A	E317K	~20% normal activity [392]
c.487G>A	Mahidol	Exon 6	G	A	G163S	<10% normal activity [356]
c.690C>T	Unknown	Exon 7	C	T	I230*	Unknown
c.1311C>T	--	Exon11	C	T	Y437Y	--
IVSXI 93C>T	--	Intron 11	C	T	Non-coding	--

* Synonymous mutation.

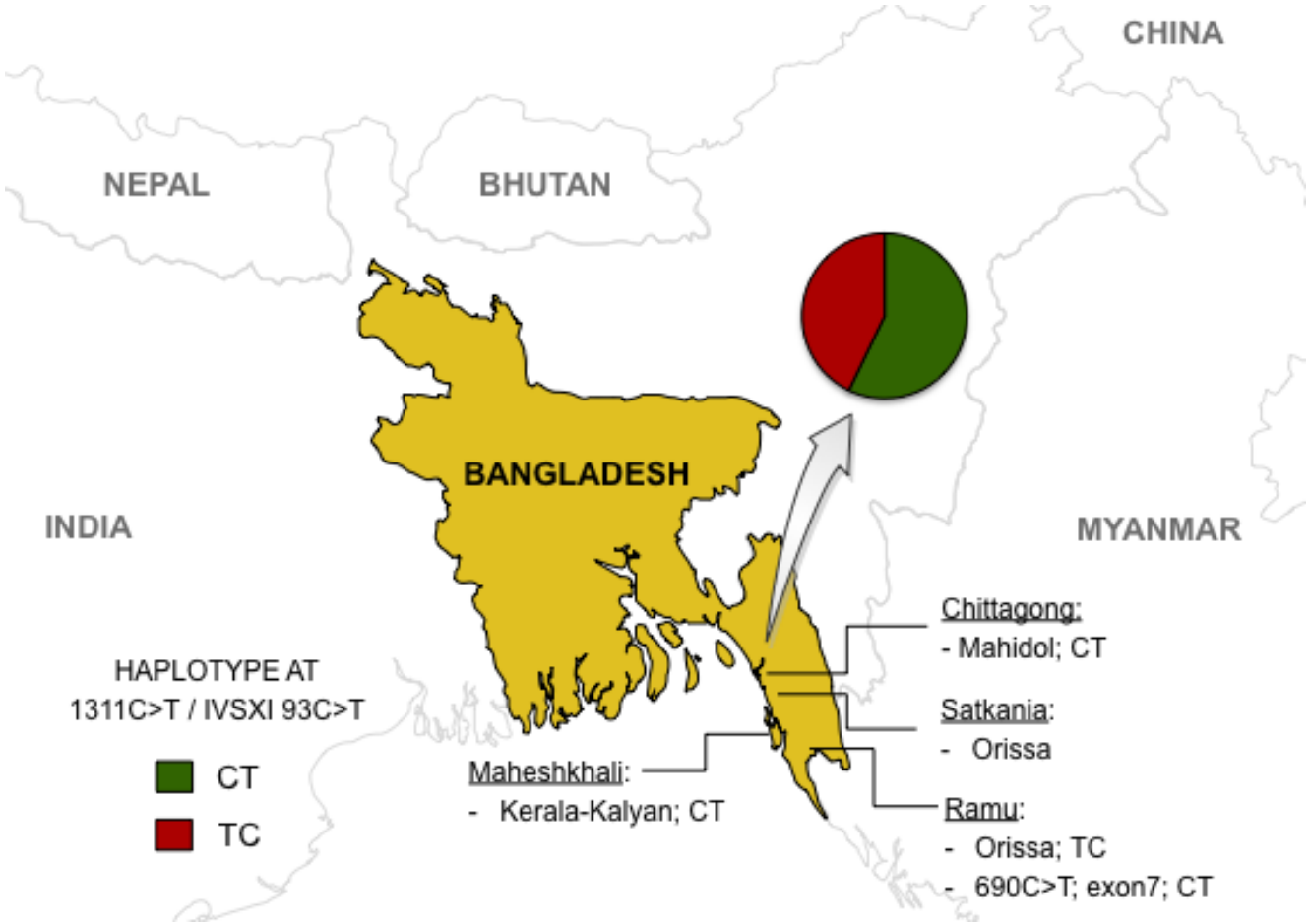


Figure 3.2.1 Location of residence of G6PD variants. All variants were identified in severe and uncomplicated malaria patients, except for the Mahidol variant (healthy control). Haplotypes at 1311C>T / IVSXI 93C>T are indicated beside each G6PD variant except the one Orissa variant identified in a female with heterozygosity at both markers. Overall proportions of haplotypes shown in the pie graph were assessed from G6PD normal individual expressed as total chromosomes.

The two polymorphic markers examined were the synonymous coding sequence 1311C>T [363, 393] and a non-coding polymorphism in exon 11, IVSXI C93T [364], both of which are thought to be of no functional importance. The C1311/IVSXI C93, CC haplotype was considered as the ancestral haplotype as published by Jamornthanyawat *et al.* [361]. 60 of 62 patients had sequencing done of both polymorphic markers; 29/60 (48%) had the CT haplotype, 25/60 (42%) had the TC haplotype and 6/60 (10%) were females heterozygous at both polymorphic markers (Table 3.2.3 and 3.2.4). No TT or CC haplotypes were detected. Excluding the 6 females heterozygous at both markers, 36 patients were wild type at the Orissa, Kerala-Kalyan and Mahidol loci and these patients had similar proportions of the CT haplotype (22/36, 58%) and TC haplotype (15/36, 42%) (Figure 3.2.1). The Kerala-Kalyan and Mahidol variants were associated with the CT haplotype and the Orissa variant was associated with the TC haplotype (Figure 3.2.1; Table 3.2.3 and Table 3.2.4). The second patient with the Orissa variant was female and heterozygous at both polymorphic markers.

Baseline characteristics of falciparum malaria patients with identified G6PD deficiency or mutation are shown in Table 3.2.5 and Figure 3.2.2. The parasite density is difficult to interpret in relation to wild-type G6PD patients since all patients except case 1 received antimalarials prior to enrolment. The duration of illness (days of fever) among G6PD deficient patients was longer than the median duration in G6PD normal patients (Table 3.2.6). There was a trend to lower *Pf*HRP2 in G6PD deficient patients compared to

Table 3.2.3. Frequency of 1311C>T / IVSXI C93T haplotypes according to allele at 131C>G (Orissa), 949G>A (Kerala-Kalyan), and 487G>A (Mahidol)

Allele at 131 Exon 3	**1311 Exon 11**	**IVSXI 93 Intron 11**	**# of Individuals**
C (wild-type)	C	C	0
	C	T	24
	T	C	18
	T	T	0
G (Orissa)*****	C	C	0
	C	T	0
	T	C	1
	T	T	0
Allele at 949 Exon 9	**1311 Exon 11**	**IVSXI 93 Intron 11**	**# of Individuals**
G (wild-type)	C	C	0
	C	T	29
	T	C	25
	T	T	0
A (Kerala-Kalyan[¶]	C	C	0
	C	T	1
	T	C	0
	T	T	0
Allele at 487 Exon 6	**1311 Exon 11**	**IVSXI 93 Intron 11**	**# of Individuals**
G (wild-type)	C	C	0
	C	T	28
	T	C	21
	T	T	0
A (Mahidol)[§]	C	C	0
	C	T	1
	T	C	0
	T	T	0

All females heterozygous at both polymorphic markers were excluded from this table. *****Excluding second female subject with heterozygous Orissa mutation and heterozygous at exon11 and intron11; [¶] Kerala-Kalyan mutation was heterozygous in female subject with homozygous 1311C>T / IVSXI C93T. [§]Includes one male subject with novel synonymous 690C>T mutation.

Table 3.2.4. Summary of G6PD variants and haplotypes

Haplotype	1311 Exon 11	IVSXI 93 Intron 11	131	487	949	# of Individuals
1	C	C	C	G	G	0
2	C	***T***	C	G	G	22
3	***T***	C	C	G	G	15
4	***T***	***T***	C	G	G	0
5	***T***	C	***G***	G	G	1 (Case 1)
6	C	***T***	C	***A***	G	1 (healthy control)
7	C	***T***	C	G	***A***	1 (Case 3)
8	C/***T***	C/***T***	C/***G***	G	G	1 (Case 2)

Italicised nucleotides in bold, underlined represent mutation from wild-type. Haplotypes 1-4 include patients homozygous at 1311 Exon 11 and IVSXI 93 Intron 11 and wild-type at exons 3, 6 and 9

Table 3.2.5. Characteristics of falciparum malaria patients by G6PD mutation

	Case 1	Case 2	Case 3	G6PD normal (wild type at exon 3,6,9)	
				SM (n=21)	UM (n=19)
G6PD variant	Orissa	Orissa¶	Kerala-Kalyan¶	--	
1311C>T/ IVSXI 93C>T	TC	¶	CT	10 CT/10 TC	11 CT/5 TC
Florescent spot test	Deficient	ND	Ind	17 Norm/2 Ind	14 Norm/4 Ind
Demographics					
Age (years)	27	32	55	38 (25-45)	28 (20-35)
Sex (%, M)	M	F	F	13 (62%)	11 (58%)
Previous malaria	Y	N	N	7 (33%)	10 (53%)
Days of fever	10	13	30	7 (6-8)	4 (2-7)
Haemoglobinuria	N	N	N	7 (33%)	1 (5%)
Severity of disease	UM	SM	UM	21 (100%)	0 (0%)
Physical examination					
Temperature (°C)	40.0	36.8	37.0	38.6 (37.5-39.0)	39.4 (38.2-40.0)
Glasgow coma score	15	15	15	9 (8-10)	15 (15-15)
Respiratory rate (per min)	40	32	28	36 (30-47)	28 (22-32)
Laboratory parameters					
Parasite count per µl[a]	171,821	10,048	2,520	29,474 (9,749-89,109)	41,686 (12,670-137,156)
*Pf*HRP2 (mg/ml)	384	1,297	172	3,397 (1,293-12,298)	93 (34-531)
CFH (ng/ml)	23,136	23,562	12,818	94,086 (43,092-620,560)	38,307 (24,525-71,708)
Haemoglobin (g/dL)	10.9	5.3	7.8	9.4 (8.0-11.2)	13.5 (11.0-14.3)
LDH (U/L)	141	967	275	635 (545-886)	257 (180-372)
Total bilirubin (mg/dL)[a]	1.4	6.0	0.6	1.7 (0.9-4.9)	1.1 (0.8-1.5)
Creatinine (mg/dL)	0.9	9.2	0.8	1.5 (0.9-379)	0.9 (0.8-1.1)
Urine pH	5.06	5.00	6.45	5.39 (5.23-6.14)	5.94 (5.60-6.65)
pH	7.51	7.41	7.43	7.42 (7.39-7.46)	7.50 (7.45-7.51)
Bicarbonate (mmol/L)	17.8	11.9	20.6	14.8 (12.8-16.5)	17.8 (16.4-19.6)
Lactate (mmol/L)	2.11	0.7	1.94	4.3 (2.7-6.0)	2.0 (1.6-2.4)

Data are number (%) or median (IQR, range), unless otherwise indicated. a geometric mean (95% CI, range). ¶Heterozygous.
Abbreviations: M, male; F, female; Y, yes; N, no; Ind, indeterminate; UM, uncomplicated malaria; SM, severe malaria

Table 3.2.6. Plasma *P. falciparum* histidine rich protein 2, cell-free haemoglobin and serum haemoglobin concentrations in G6PD normal patients stratified by duration and severity of illness as measured by fever days

				*Pf*HRP2 (ng/ml)		
Days of fever		G6PD normal (n=55)		SM (n=28)		UM (n=27)
0-4	n=16	127 (34-1,252)	n=3	3,184 (28-10,885)	n=13	128 (34-747)
4-8	n=19	1,996 (297-6,530)	n=17	3,775 (1,512-12,119)	n=11	404 (46-1,221)
>8	n=11	2,328 (1,356-9,946)	n=8	6,429 (1,733-10,128)	n=3	2,138 (35-2,328)
				Parasitaemia (/μl)[a]		
Days of fever		G6PD normal (n=55)		SM (n=28)		UM (n=27)
0-4	n=16	55,385 (16,057-191,041)	n=3	38,533 (474-3,133,537)	n=13	60,222 (13,397-270,704)
4-8	n=28	19,148 (6,384-57,433)	n=17	22,739 (4,861-106,364)	n=11	14,682 (2,441-88,291)
>8	n=14	63,003 (16,453-241,257)	n=10	86,632 (17,557-427,465)	n=4	28,416 (384-2,101,198)
				Haemoglobin (g/dL)		
Days of fever		G6PD normal (n=55)		SM (n=28)		UM (n=27)
0-4	n=16	13.1 (11.5-14.2)	n=3	11.2 (10.6-13.2)	n=13	13.5 (12.1-14.3)
4-8	n=26	10.9 (9-12.6)	n=15	9.3 (8.0-10.8)	n=11	12.3 (11.5-13.9)
>8	n=14	8.7 (5.4-10.3)	n=10	7.2 (5.2-10.3)	n=4	9.5 (8.45-12.1)
				Cell-free haemoglobin (ng/ml)		
Days of fever		G6PD normal (n=55)		SM (n=28)		UM (n=27)
0-4	n=16	32,568 (18,383-65,007)	n=3	94,086 (19,452-620,560)	n=13	29,372 (17,315-44,404)
4-8	n=28	46,234 (27,372-159,741)	n=17	50,839 (38,853-202,349)	n=11	37,241 (14,517-52,495)
>8	n=14	139,585 (71,708-260,424)	n=10	177,161 (74,047-316,552)	n=4	85,174 (48,116-179,532)

Data are median (IQR, range); [a]Geometric mean (95% CI). *Abbreviations:* UM, uncomplicated malaria; SM, severe malaria; *Pf*HRP2, *Plasmodium falciparum* histidine rich protein 2.

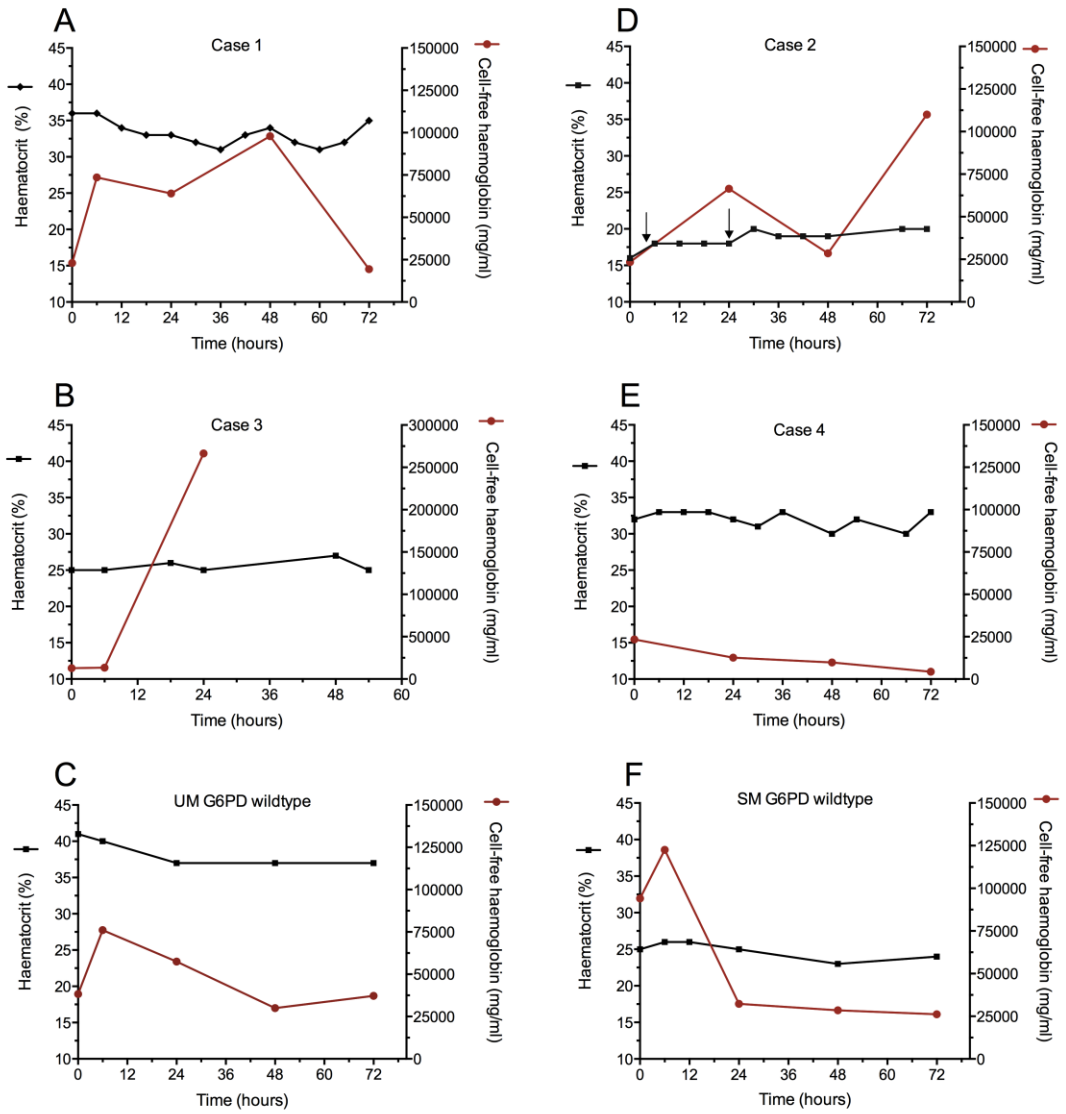


Figure 3.2.2. Plasma cell-free haemoglobin and whole blood haematocrit profiles during hospital admission of (A) G6PD Orissa hemizygous male with uncomplicated malaria, (B) G6PD Kerala-Kalyan heterozygous female with uncomplicated malaria, (C) G6PD normal patients with uncomplicated malaria, (D) G6PD Orissa heterozygous female with severe malaria, (E) G6PD exon 7, 690C>T synonymous hemizygous male with severe malaria, and (F) G6PD normal patients with severe malaria.

G6PD normal patients with similarly long durations of illness prior to enrolment. There was also a trend to lower CFH on enrolment in the G6PD deficient compared to normal patients (median, range: 23,062 (12,758-23,388) versus 47,927 (23,307-176,201), *P* =0.09, respectively), which peaked 24 to 72 hours after enrolment (Figure 3.2.2). None of the G6PD deficient patients had visible haemoglobinuria on enrolment or during admission. Of the G6PD normal patients that had haemoglobinuria (n=8), the median CFH concentration on admission was 644,843 (190,890-1,872,645).

Case 1 was a 27-year old male with G6PD deficiency Orissa variant (FST deficient) and UM. Haemoglobinuria was absent on admission and did not develop during admission. In the absence of prior antimalarial treatment, his parasitaemia was above the 75% percentile of G6PD normal patients, which was accompanied by an elevated *Pf*HRP2 concentration. The CFH was lower on enrolment compared to G6PD normal UM patients and exhibited a bimodal pattern with a peak of 97,952 ng/ml 42 hours after G6PD normal patients. The creatinine was normal on admission and he did not develop kidney dysfunction. His recovery was unremarkable however, he re-presented at 6 weeks, febrile with *P. vivax* infection.

Case 2 was a 32-year old female heterozygous for the Orissa mutation (FST not done) diagnosed as SM with AKI (creatinine > 4.6 mg/dL), and acidosis (bicarbonate <15 mmol/L). She did not present with or develop BWF but gave a history of red urine prior to admission and had haemoglobin

in her urine by dipstick on enrolment and at 76 hours. Anaemia was present on enrolment that required three blood transfusions during admission. The fractional excretion of sodium was >2% suggesting of acute tubular injury, while the urine pH (<5.3) and urine anion gap suggestive of a Type 2 renal tubular acidosis. CFH on enrolment was below the 75% percentile compared to G6PD normal SM patients but showed a bimodal increased to a CFH of 110,001 ng/ml at 72 hours. Dialysis was initiated 6 hours after enrolment and required an additional 5 cycles. She was discharged three weeks after enrolment and kidney function returned to baseline at 6 weeks.

Case 3 was a 55 year-old female heterozygous for the Kerala-Kalyan mutation (FST indeterminate) diagnosed with UM. Anaemia was present at enrolment but no blood transfusion was required. Haemoglobin was absent from the urine throughout. CFH was low on admission and increased to a CFH of 266,497 ng/ml at 24 hours. Creatinine was normal on enrolment and AKI did not develop during admission. Follow-up and complete blood collection was not possible as the patient withdrew from the study.

3.2.5 Discussion

The prevalence of G6PD deficiency and associated variants has not been well described in Bangladesh. This study shows that a G6PD deficiency mutation was present in 4.8% (95% CI: 1.5 – 14.4) of Bengali *P. falciparum* malaria patients admitted to two centres in southern Bangladesh. These genotypic data are in the same range as the phenotypic data exclusively from the same centre in Ramu, which found a frequency of G6PD deficiency

among male and female malaria patients to be 5.6% (9/162), as detected by FST (unpublished). A recent study of non-malaria subjects from Marma and Khyang ethnic groups, found that 34.7% had mild G6PD deficiency (1.2-7.1 U/g Hb) and 6.5% had severe G6PD deficiency (>0.12-1.2 U/g Hb) An older study found 4% of Bengali males in Dhaka to be G6PD deficient [39]. These frequencies must be considered in the context of the different sample populations and methods of G6PD deficiency detection between all of these studies. The FST correctly identifies patients as deficient if the activity level is below 0.83 g/Hb but does not reliably diagnose female heterozygotes [394]. Thus, the 5.6% identified in Ramu by FST would fall into the severe deficiency category as outlined above, which is a similar range to the 6.5% severe deficiency found among the Marma and Khyang ethnic groups.

This is the first study to report G6PD genotypes found in Bangladesh. The three variants identified were Orissa, Kerala-Kalyan, and Mahidol. While this study did not quantitatively assess G6PD deficiency, other studies have found these mutation to be associated with ~10% [392], ~20% [392] and <10% [356] normal enzymatic activity, respectively. In India, the most frequent G6PD variants are Mediterranean followed by Orissa and Kerala-Kalyan, the latter both found predominantly in eastern and southern India [392, 395-400]. The Orissa and Kerala-Kalyan variants are thought to have originated in India [398]. Given the history and location of Bangladesh, identifying these two variants supports the migration of individuals from eastern India to Bangladesh. The few Kerala-Kalyan and Orissa variants now reported outside India further support a southern migration pattern. The

Kerala-Kalyan variant has been reported in individuals in southern Myanmar [359], Thailand (Phuket) and Mauritius [400]. The Kerala-Kalyan haplotype background (1311C>T and IVSXI C93T) has been reported to be CT or TC (3:1) in India [398], whereas the Myanmar [359] and Bengali haplotype were both CT. The Orissa variant has been reported in Malaysian Malays [401, 402], Mauritius [400] and southern Italy [403]. Other than Italy, the distribution of this G6PD variant, originally reported among tribal, cultivator communities [404], seems to closely follow British colonization of South and Southeast Asia. The haplotype background in Malays and in the current study was TC, whereas in India, the Orissa variant has been reported with both haplotypes CT (n=6) and TC (n=7). While the Mahidol variant has not been reported in India, it is the predominant variant across Myanmar [359, 396, 405, 406]. This suggests a migration pattern of individuals westward from Myanmar to Bangladesh. The proportion polymorphic TC haplotypes in G6PD normal individuals was similar to that of India supporting the genetic similarity between these populations and influence from the Middle East [361].

Due to the low number of G6PD deficient malaria patients detected in this study, inferences on potential mechanisms cannot be made. However, male hemizygous case 1 had a long duration of fever before admission, high admission ring stage parasitaemia, but with low plasma *Pf*HRP2 concentration. This implies a high recent multiplication rate of the parasite but a low total parasite burden on admission despite the long duration of illness. G6PD deficiency is thought to confer protection against *P. falciparum*

malaria infection. *In vitro* falciparum parasite invasion [407] and growth [408] is reduced in G6PD deficient cells but parasites can adapt to continue development within deficient cells at a rate comparable to G6PD normal cells [408, 409] and have been shown to express its own *P. falciparum* specific G6PD in both G6PD deficient and normal RBCs [410]. *In vivo,* heterozygous G6PD deficient females are shown to have lower parasite densities [387] and hemizygous deficient males have been shown to develop less severe disease, in particular cerebral malaria [385]. In the current study, the observations suggest that parasites may take a longer duration to establish exponential growth, as observed *in vitro* [408], since higher *Pf*HRP2 concentrations would be expected after these long durations of illness (Table 3.2.5). Female heterozygous case 2 and case 3 also had a long duration of illness before presentation, and low plasma *Pf*HRP2, denoting a small total parasite biomass despite the long duration of illness. Case 2, however, presented with severe disease with renal failure, acidic urine, haemoglobinuria and metabolic acidosis with low plasma lactate). G6PD deficiency has been associated with haemoglobinuria and kidney dysfunction in adult and paediatric patients with and without malaria [33, 83, 411-416]. Experimental models suggest the urine free haemoglobin is nephrotoxic especially in the setting of dehydration [417], and aciduric urine [418], which converts haemoglobin to methaemoglobin [418]. Further, renal haptoglobin [419] and haemopexin [420] expression are upregulated with resultant higher urinary concentrations in response to various models of haemoprotein-mediated AKI.

Limitations of this descriptive study include the small sample size. While not designed to determine the prevalence of G6PD in Bangladesh, the number of deficient patients detected by FST and genotyping was similar to the frequencies of severe deficiency reported thus far in the literature. Further, this study was focused on assessing the phenotype of G6PD deficiency in malaria and to determine the genotypes present given the lack of literature available in Bangladesh. Quantitative assessment of G6PD activity was not assessed due to logistics of performing the assay in the field and the cost of performing the assay. However, based on the known enzymatic activity levels in the literature the level of deficiency can be extrapolated from the knowledge of the genotypes.

3.2.6 Conclusions

This the first report of G6PD deficiency variants in Bangladesh. The Orissa (131C>G), Kerala-Kalyan (949G>A), and Mahidol (487 G>A) variants were identified in the Bengali population. The polymorphic haplotypes (1311C>T and IVSXI C93T) associated with variant and G6PD normal genotypes were consistent with those reported from India. Further studies are required to characterise if these variants have a protective effect against malaria infection and determine the haemolytic risk in response to malaria infection and 8-aminoquinolones.

4.0 Pathophysiology of AKI in Bangladeshi adults

4.1 Sequestration and immune activation

4.1.1 Background

The histopathology of AKI in malaria predominantly shows ATI, hypothesized to be caused by the sequestration of parasitized RBCs (PRBCs) and accumulation of host mononuclear lymphocytes in both glomerular and peritubular capillaries [171, 209, 421]. However, the relative contribution of each of these mechanisms cannot be assessed from *post-mortem* studies.

*Pf*HRP2 is released in distinct amounts at the moment of schizont rupture and is quantitatively related to parasite density [353, 422]. The plasma concentration of *Pf*HRP2 estimates total body *P. falciparum* burden, including the sequestered biomass that causes obstructed microcirculatory flow in vital organs. Unlike the peripheral parasitaemia level, plasma *Pf*HRP2 concentration correlates strongly with disease severity and outcome [423-425].

Plasma soluble urokinase–type plasminogen activator receptor (suPAR) reflects the systemic level of cell surface urokinase-type plasminogen activator receptor (uPAR) expression, and has been proposed as a marker of immune activation via modulation of mononuclear cell adhesion and migration [426-428]. Cell-bound uPAR is expressed on several cell types including activated lymphocytes, monocytes, neutrophils, macrophages, vascular endothelial cells, and kidney podocytes [427, 428]. In African

children with acute malaria, plasma suPAR concentrations are elevated and associated with higher mortality [429, 430]. suPAR has not been assessed previously in adults with severe falciparum malaria.

Neutrophil gelatinase-associated lipocalin (NGAL) is secreted predominantly in damaged distal tubular renal epithelial cells prior to neutrophil activation, and is an early marker of renal tubular cell damage [431-433]. NGAL concentration is more reliable as a measure of kidney injury than creatinine as it is less dependent on pre-renal factors [431-433]. Upregulation of NGAL in renal tubule cells may be induced by local release of cytokines from monocytes in the microcirculation after ischemic injury [432].

4.1.2 Study aim

The relationship between plasma suPAR, as a proxy-measure of mononuclear cell immune activation, and plasma *Pf*HRP2, as a measure of sequestered parasite burden, was assessed in adult Bangladeshi patients with falciparum malaria either with or without associated AKI. Severity of kidney dysfunction and damage was measured quantitatively by creatinine clearance and urine NGAL (a marker of acute tubular necrosis), respectively. Since kidney failure in SM is a consequence of severe ATI, tubular damage was also assessed qualitatively by the requirement for RRT.

4.1.3 Study design

The study was a prospective analysis of a subset of adult patients (>16 years) enrolled in two clinical trials assessing N-acetylcysteine (2003–2005) and levamisole (2006–2010) as adjuvant therapy in severe falciparum

malaria, conducted at CMCH, Bangladesh. [70, 339]. Criteria for severe falciparum malaria included as defined in methods section 2.3.4.2. Of the 141 SM patients enrolled in these two studies, all patients who had suPAR levels measured were included in this analysis (N = 137 out of 141).

AKI was defined by the estimated CrCl, as described in section 2.9.1. Severity of AKI was classified as mild, moderate or severe dysfunction based on admission CrCl: mild (CrCl = 60–89.9 ml/min), moderate (CrCl = 30–59.9 ml/min) and severe (CrCl <30 ml/min). No AKI was defined as CrCl ≥90 ml/min. No patient included in the analysis had a known history of renal disease. Assays are described in Chapter 2.10. Urine NGAL normalised to urine creatinine was used in this analysis as a biomarker for kidney damage, specifically ATI.

Continuous and categorical data were analysed as described in Chapter 2. Data were transformed to achieve a normal distribution where possible. A non-parametric test-for-trend, which is an extension of the Wilcoxon rank-sum test, was used to identify increasing or decreasing associations with AKI severity. Correlations between variables were assessed using Pearson's correlation coefficient. A robust regression model was constructed to assess the contributions of suPAR, *Pf*HRP2, suPAR x *Pf*HRP2 interaction, age and SBP to the variation in uNGAL/Ucr. Age and SBP were included in the model as they are well-established risk factors for kidney injury. There were no interactions between CrCl and suPAR, nor CrCl and *Pf*HRP2, therefore these terms were not included in the models. To assess the relationship of the same independent variables with the indication for RRT, a logistic model

with stepwise exclusion of non-significant covariates was used. Improved model fit with the inclusion of the interaction term was confirmed using the likelihood ratio test.

4.1.4 Results

One hundred and thirty-seven adults with severe falciparum malaria were included in this analysis (Figure 4.1.1).

Baseline characteristics and patient outcomes are shown in Table 4.1.1. AKI was present in 106 patients (77%), of whom 32 (23%), 42 (31%) and 32 (23%) were classified as having mild, moderate and severe AKI, respectively. AKI classification using the WHO definition (creatinine >3 mg/dL) (24) rather than estimated CrCl, failed to identify 11 (34%) patients in the severe AKI group. These 11 patients had a geometric mean CrCl of 25 ml/min, and 5/11 (45%) required RRT. The WHO definition of AKI failed to identify 41/42 (98%) patients with moderate AKI (geometric mean CrCl 42 ml/min), of those 13/41 (32%) required RRT. There was unanimous consensus among three independent physicians on 39 patients (28%) who should have received RRT; 20 (51%) did not receive RRT for logistical reasons. Two patients received haemodialysis and 17 patients received peritoneal dialysis within 48 hours of admission. The most frequent indication for RRT was acidosis (Table 4.1.2). Of the 19 patients who received RRT, eight (42%) patients died; of the 20 patients who met criteria for RRT but could not receive it, 15 patients died (75%). The overall case fatality rate was 53/137 (39%); 50/53 (94%) had cerebral malaria, 33/53

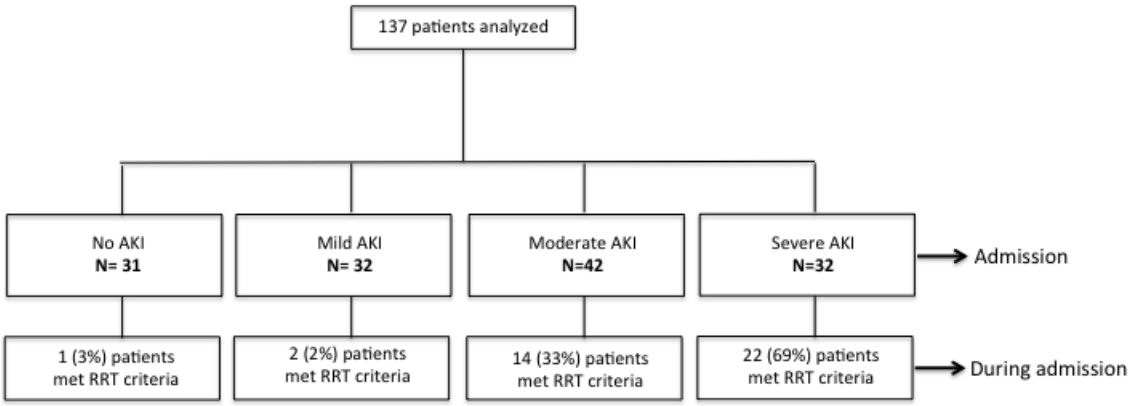


Figure 4.1.1 Consort diagram**.** After enrolment patients had blood and urine samples collected. Plasma and urine biomarkers were measured and correlated with the renal diagnosis and the subsequent hospital course. One patient with no AKI diagnosis on admission developed progressive renal impairment despite conservative measures and required RRT; this patient presented in deep coma with hyperlactataemia. AKI = acute kidney injury; RRT = renal replacement therapy.

Table 4.1.1 Baseline characteristics by acute kidney injury severity and clinical outcome.

Variable	No AKI (n = 31)	Mild AKI (n = 32)	Moderate AKI (n = 42)	Severe AKI (n = 32)	Test-for-trend
Age (years)[a]	26 (18–35)	33 (25–40)	35 (21–50)	40 (28–48)	**0.003**
Males[c]	27 (87)	27 (84)	30 (71)	26 (81)	0.301
Clinical					
SBP (mmHg)	116 (15)	113 (20)	108 (20)	107 (21)	**0.048**
RR (breaths/min)[b]	29 (27–30)	34 (31–37)	32 (29–34)	32 (28–35)	0.239
GCS (#/15)[a]	8 (7–10)	8 (5–10)	9 (6–11)	9 (5–12)	0.937
Laboratory					
HCT (%)	34 (7)	30 (9)	29 (8)	29 (8)	**0.011**
Parasitaemia (#/µL)[b]	46723 (19688–110884)	33560 (11984–93981)	174140 (120635–251377)	78018 (36814–165340)	0.070
Creatinine (mg/dL)[b]	0.69 (0.63-0.76)	1.11 (1.03-1.21)	1.75 (1.60-1.91)	3.80 (3.26-4.43)	**<0.001**
BUN (mg/dL)[b]	21 (18–25)	27 (23–32)	57 (50–65)	88 (73–105)	**<0.001**
CrCl (ml/min)[b]	123.3 (112.8-134.8)	72.5 (69.8-75.3)	41.7 (39.0-44.5)	19.3 (17.0-21.8)	**<0.001**
Potassium (mmol/L)	4.0 (0.7)	4.4 (1.1)	4.4 (0.7)	4.6 (1.4)	**0.031**
Sodium (mmol/L)	129 (7)	134 (5)	133 (8)	133 (6)	0.069
Glucose (mmol/L)[b]	7.4 (5.8-10.5)	7.6 (5.7-12.0)	6.3 (4.6-8.8)	7.0 (5.3-11.3)	0.223
Albumin (g/L)	31 (5)	30 (4)	28 (5)	27 (6)	**<0.001**
Total bilirubin (mg/dL)[b]	4.1 (2.8-5.9)	3.1 (2.3-4.1)	4.9 (3.5-6.7)	6.7 (4.7-9.6)	**0.011**
Direct bilirubin (mg/dL)[b]	1.2 (0.8-2.0)	1.1 (0.8-1.5)	2.2 (1.5-3.2)	3.4 (2.2-5.2)	**<0.001**
ALT (U/L)[b]	22 (17–28)	30 (21–43)	36 (25–51)	34 (23–51)	0.064
Base excess (mmol/L)	−4 (4)	−5 (4)	−11 (7)	−13 (7)	**<0.001**
Bicarbonate (mmol/L)	21 (4)	19 (4)	14 (5)	14 (4)	**<0.001**
pH	7.42 (0.08)	7.42 (0.09)	7.35 (0.12)	7.30 (0.17)	**<0.001**
pCO2 (mmHg)	32 (6)	29 (7)	25 (8)	26 (7)	**<0.001**
Lactate (mmol/L)[b]	4.3 (3.7-5.1)	3.9 (3.2-4.7)	7.1 (6.0-8.3)	4.9 (3.8-6.4)	**0.029**
Biomarkers					
HRP2 (ng/ml)[b]	678 (397–1156)	1413 (967–2064)	2419 (1715–3413)	2833 (1648–4872)	**<0.001**
uNGAL/Ucr (pg/mg cr)[b]	750 (589–956)	1,012 (745–1376)	1281 (1066–1541)	2549 (1943–3345)	**<0.001**
Outcomes					
RRT indicated[c]	1 (3)	2 (6)	14 (33)	22 (69)	**<0.001**
Death[c]	12 (39)	8 (25)	19 (45)	14 (44)	0.333

All values are mean (SD) unless otherwise specified; [a]median (IQR), [b]geometric mean (95% CI), [c]number (%). *P* <0.05 using test-for-trend; significant in bold. AKI = acute kidney injury; SBP = systolic blood pressure; RR = respiratory rate; GCS = Glasgow Coma Scale; HCT = haematocrit; BUN = blood urea nitrogen; CrCl = creatinine clearance; ALT = alanine transaminase; pCO2 = venous partial pressure carbon dioxide; HRP2 = *Plasmodium falciparum* histidine rich protein 2; suPAR = soluble urokinase-type plasminogen activator receptor; uNGAL/Ucr = urine neutrophil gelatinase-associated lipocalin corrected for urine creatinine; RRT = renal replacement therapy.

Table 4.1.2 Clinical indications for renal replacement therapy

Indication	**Number**
Renal impairment and acidosis	20
Renal impairment and hyperkalaemia	0
Renal impairment, acidosis and hyperkalaemia	6
Renal impairment, acidosis and convulsions	2
Renal impairment, acidosis, hyperkalaemia and convulsions	3
Worsening renal function despite conservative measures	5
Worsening renal function and convulsions	1
Renal impairment and pulmonary oedema	1
Renal impairment, acidosis and pulmonary oedema	1
Total	**39**

(62%) had moderate or severe AKI, 35/53 (66%) had hyperlactataemia, and 33/53 (62%) had severe acidosis.

Increasing severity of AKI correlated with increasing age, and decreasing SBP and haematocrit (test-for-trend *p* = 0.003, 0.048 and 0.011, respectively). Direct and indirect bilirubin increased and all measures of acid–base status (lactate, base deficit, bicarbonate, pH, pCO2) worsened with increasing kidney injury severity (test-for-trend *p* <0.001).

Plasma soluble urokinase-type plasminogen activator receptor, Plasmodium falciparum histidine rich protein-2 and AKI

Unlike peripheral parasitaemia, admission plasma *Pf*HRP2 increased with worsening kidney function (test-for-trend *p* <0.001, Figure 4.1.2). Plasma *Pf*HRP2 increased with both increasing creatinine (*r* = 0.49, *P* <0.001) and increasing uNGAL/Ucr (*r* = 0.29, *P* <0.002) (Figure 4.1.3 A-B). Peripheral parasitaemia had a borderline correlation with serum creatinine (*r* = 0.17, *P* = 0.05). Admission plasma suPAR increased with increasing AKI severity (test-for-trend *p* <0.001, Figure 4.1.2). There was a strong positive correlation between suPAR and both creatinine (*r* = 0.51, *P* <0.001) and uNGAL/Ucr (*r* = 0.38, *P* <0.001) (Figure 4.1.3 C-D).

Plasma suPAR strongly correlated with *Pf*HRP2 (*r* = 0.54, *P* <0.001), the interaction term (suPAR x *Pf*HRP2) also increased with both increasing creatinine (*r* = 0.58, *P* <0.0001), increasing uNGAL/Ucr (*r* = 0.38, *P* <0.0001) and AKI severity (test-for-trend *p* <0.0001). This was further evaluated using linear regression with uNGAL/Ucr as the dependent variable, representing

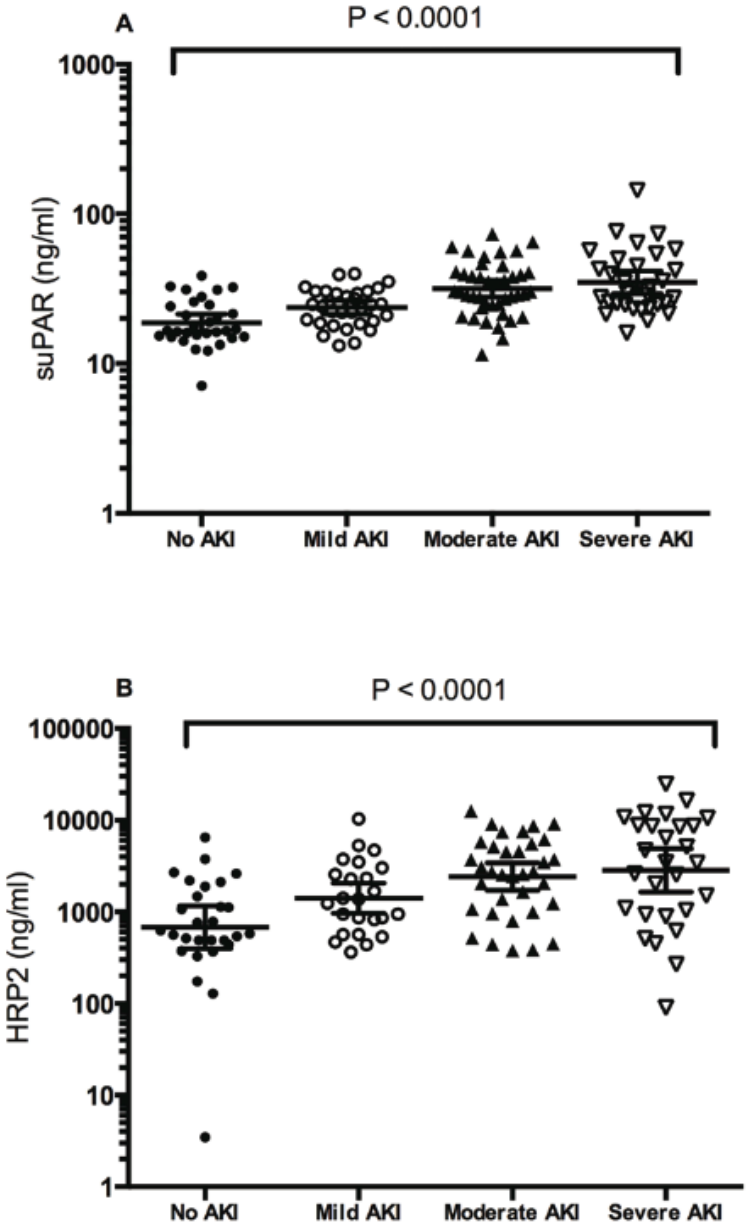


Figure 4.1.2 Scatterplots of cohort stratified by admission acute kidney injury severity. (A) Plasma soluble urokinase–type plasminogen activator receptor (suPAR), and (B) plasma HRP2 concentrations. Concentrations for both suPAR and HRP2 increased with increasing severity of AKI (test-for-trend *P* <0.0001). Geometric mean and 95% confidence intervals displayed. AKI = acute kidney injury; HRP2 = *P. falciparum* histidine rich protein 2.

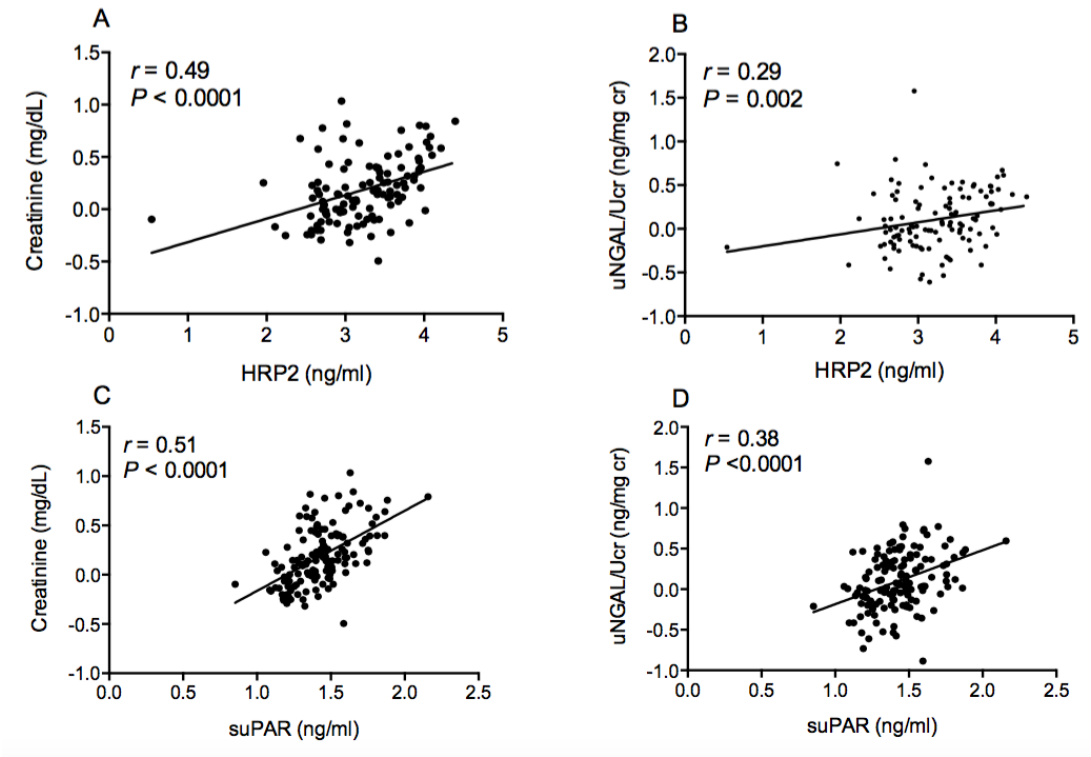


Figure 4.1.3. Correlations of variables involved in acute kidney injury. Correlations of variables proposed to be involved in the pathogenesis of acute tubular necrosis in malaria associated AKI with marker of renal dysfunction (creatinine) and marker of kidney damage (uNGAL/Ucr). Pearson's correlation coefficient between plasma *P. falciparum* histidine rich protein 2 (HRP2) and **(A)** serum creatinine; and **(B)** uNGAL/Ucr. Pearson's correlation coefficient between suPAR and **(C)** serum creatinine; and **(D)** uNGAL/Ucr. All variables were log transformed (log_{10}) for correlation plots and Pearson's analysis. suPAR = soluble urokinase–type plasminogen activator receptor; uNGAL/Ucr = urine neutrophil gelatinase-associated lipocalin corrected for urine creatinine.

acute tubular necrosis. In the univariate analysis suPAR, *Pf*HRP2 and suPAR x *Pf*HRP2 were associated with a higher concentration of uNGAL/Ucr (Table 4.1.3). Multivariate analysis showed that only plasma suPAR and *Pf*HRP2 were independently associated with higher uNGAL, predicting 18% of the variation in uNGAL/Ucr concentrations (R^2 = 0.18; Table 4.1.3).

Plasma suPAR and *Pf*HRP2 concentrations were higher in patients requiring RRT (*P* <0.001, Figure 4.1.4). Logistic regression was performed to further evaluate this relationship with RRT as the binary dependent variable, representing severe tubular damage in SM. In univariate logistic analysis, increased suPAR, *Pf*HRP2 and suPAR x *Pf*HRP2 were associated with increased odds for RRT requirement (Table 4.1.4). In a stepwise, multivariate, logistic regression model, increasing plasma suPAR, *Pf*HRP2 and age increased the odds of RRT requirement (R^2 = 0.27; Table 4.1.4).

Relationship between plasma soluble urokinase-type plasminogen activator receptor, PfHRP2 and mortality

Plasma suPAR and *Pf*HRP2 concentrations were similar in patients who died compared with those who survived: 29.03 (25.38-33.22) versus 25.57 (23.27-28.10) ng/ml for suPAR (*P* = 0.12) and 1,685 (1,162-2,444) versus 1,629 (1,185-2,237) for *Pf*HRP2 (*P* = 0.89). Plasma suPAR and *Pf*HRP2 were elevated in patients who were acidotic (base deficit >3.3 mmol/L) compared to those who were not: 29.63 (27.07-32.44) versus 20.67 (18.02-23.71) ng/ml for suPAR (*P* <0.001) and 2,025 (1,569-2,613) versus 816 (469–1,420) for *Pf*HRP2 (*P* = 0.001). Standard base deficit was elevated in

Table 4.1.3. Linear regression analysis of variables contributing to urine NGAL/Ucr variability in patients with severe malaria

	Univariate analysis		**Multivariate model (R^2 = 0.18)**	
Variable	β **(95% CI)**[a]	***P***	β **(95% CI)**[a]	***P***
suPAR	21.57 (12.20-30.95)	**<0.001**	16.54 (6.36-26.71)	**0.002**
*Pf*HRP2	0.11 (0.06-0.15)	**<0.001**	0.07 (0.02-0.11)	**0.003**
suPAR x *Pf*HRP2	0.002 (0.001-0.003)	**<0.001**	---	**---**
SBP	0.16 (−8.62-8.94)	0.971	---	---
Age	−12.02 (−25.32-1.28)	0.076	---	**---**

[a]Regression coefficient (β) with 95% confidence intervals (CIs) showing the estimated increase in the urine concentration of NGAL (pg/ml) corrected for urine creatinine (uNGAL/Ucr pg/mg of creatinine) predicted by 1 unit increases in the independent (predictor) variables using robust regression analysis. Significant in bold.

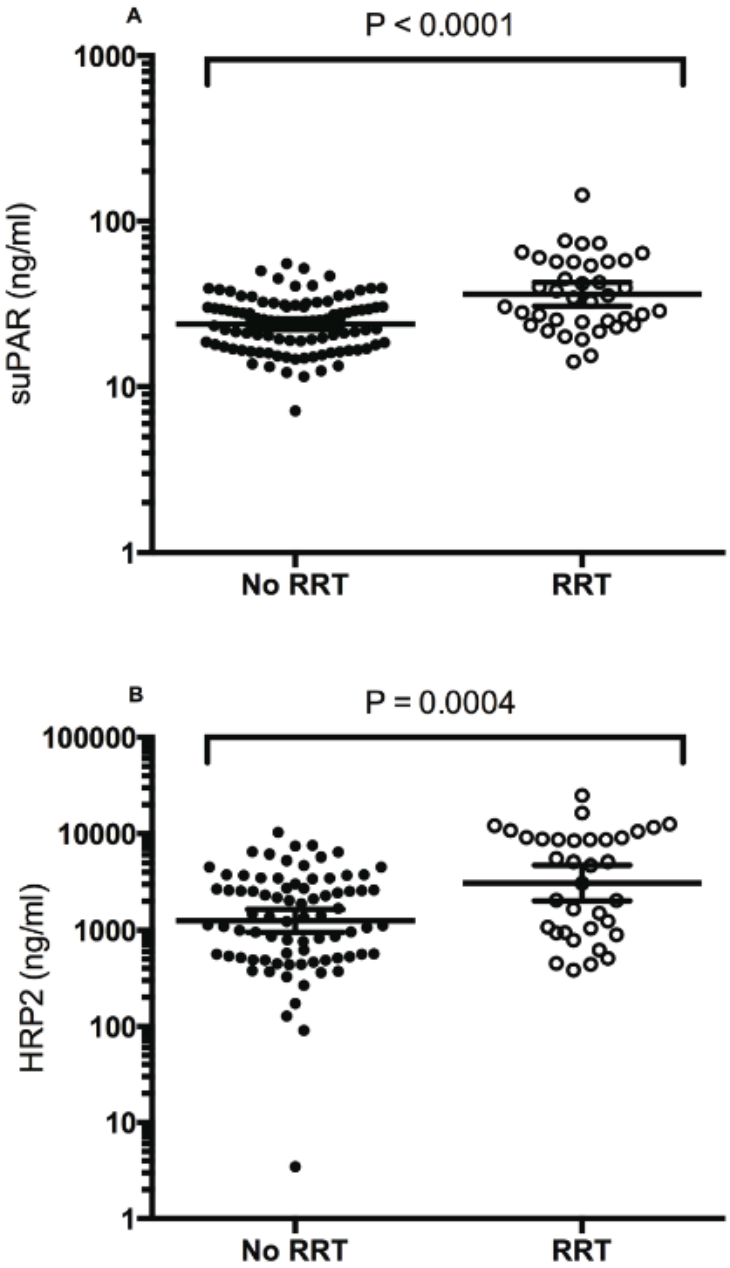


Figure 4.1.4. Scatterplots of cohort stratified by renal replacement therapy requirement. (A) Plasma suPAR, and (B) plasma HRP2. Biomarkers were log transformed (log_{10}) for normality. *T*-tests were performed for comparisons between groups. RRT = renal replacement therapy; suPAR = soluble urokinase–type plasminogen activator receptor; HRP2 = *P. falciparum* histidine rich protein 2.

Table 4.1.4. Logistic regression analysis of variables predicting renal replacement therapy in patients with severe malaria

	Univariate analysis		**Multivariate model (R^2 = 0.27)**	
Variable	**OR (95% CI)[b]**	***P***	**OR (95% CI)[b]**	***P***
suPAR	1.07 (1.04-1.11)	**<0.001**	1.07 (1.03-1.12)	**0.002**
*Pf*HRP2$^{1/2}$	1.03 (1.02-1.05)	**<0.001**	1.02 (1.00-1.04)	**0.026**
suPAR$^{1/2}$ x *Pf*HRP2$^{1/2}$	1.01 (1.00-1.01)	**<0.001**	---	**---**
SBP	1.01 (0.99-1.03)	0.522	---	---
Age	1.02 (0.99-1.05)	0.123	1.05 (1.01-1.10)	**0.010**

[b]Odds ratios (OR) with 95% CIs were determined for renal replacement therapy (RRT) associated with 1-U changes in the independent variables. Significant variables are shown in bold. *Pf*HRP2 was square root transformed to normalize skewed data. Significant in bold. *Abbreviations:* OR = odds ratio; CI = confidence intervals; uNGAL/Ucr = urine neutrophil-associated gelatinase lipocalin corrected for urine creatinine; suPAR = soluble urokinase plasminogen activator receptor; *Pf*HRP2 = *P. falciparum* histidine rich protein 2; SBP = systolic blood pressure.

patients who died compared to survivors; mean (SD), 11.8 (7.5) versus 6.5 (5.1) (P <0.001).

4.1.5 Discussion

In this study of adult patients with severe falciparum malaria, admission plasma suPAR and *Pf*HRP2 were strongly and independently associated with AKI, and a later requirement for RRT. As suPAR is an indirect measure of mononuclear cell activation, and *Pf*HRP2 a measure of sequestered parasite biomass, these results suggest that both mechanisms contribute to the pathogenesis of AKI. These findings are concordant with *post-mortem* renal histopathological findings in patients with falciparum malaria that shows both PRBC sequestration and mononuclear cell infiltration in glomerular and peritubular capillaries [171].

Multivariate regression analysis showed that plasma *Pf*HRP2 independently correlated with uNGAL, and a requirement for RRT. Histopathological ultrastructural studies of falciparum malaria-associated AKI in 67 adult Southeast Asian patients showed an increase in sequestration among patients with a creatinine >3 mg/dL, which defines AKI according to WHO malaria treatment guidelines [171]. Overall, disease severity and the severity of metabolic acidosis have also been shown to correlate strongly with plasma *Pf*HRP2 concentrations [167, 171, 353, 423-425]. *Pf*HRP2 was significantly elevated in patients who were acidotic. Peripheral parasitaemia does not reflect the pathogenic-sequestered parasite burden that causes impaired microvascular flow, and the correlation between parasitaemia and total parasite burden is variable. This might explain why these results

contrast with other studies, where an association between hyperparasitaemia and AKI severity was not found [171, 180, 181]. However, plasma *Pf*HRP2, which is a better estimate the total parasite biomass as it includes the circulating and sequestered parasites, strongly correlated with AKI defined by either the WHO or CrCl classification. The correlation between *Pf*HRP2 and creatinine was also stronger than the correlation between peripheral parasitaemia and creatinine. Sequestered PRBCs occluding the renal microcirculation may explain why increasing the overall renal blood flow in SM does not result in a change in renal oxygen consumption [239]; as only patent vessels free of sequestered PRBCs would continue to be perfused and PRBC occluded vessels would not be recruited.

Plasma suPAR increased with deteriorating renal function defined by CrCl classification and WHO criteria, urine NGAL and independently predicted RRT requirement. Plasma suPAR is positively correlated with creatinine in patients with Hantavirus and critically ill patients, and inversely correlated with estimated GFR in chronic kidney disease [434-437]. Histology of brain tissue from cerebral malaria patients shows an increase in uPAR expression in macrophage and endothelial cells spatially limited to areas with PRBCs sequestered in the microvasculature [438]. This is the first time elevated suPAR has been shown to be associated with kidney injury and dysfunction in malaria. This suggests that the histopathological feature of mononuclear cell accumulation in glomerular and peritubular capillaries could contribute to acute tubular injury in patients with a creatinine above and below 3 mg/dL, despite a difference in observed sequestration of PRBCs. It is possible that

sequestration acts as an endothelial nidus and subsequent positive feedback loop for increased suPAR-mediated mononuclear cell recruitment and immune activation resulting in more severe tubular damage. Cell-to-cell contact between endothelial cells and monocytes increases suPAR release [427] and suPAR subsequently recruits more monocytes via its chemo-attractant effector function [439, 440]. Evidence of endothelial cell damage in malaria-associated AKI is supported by the elevation of urinary nuclear factor kappa B and its correlation with creatinine, although the urinary concentration was not normalised to urine creatinine [441]. Upon schizont rupture, haemozoin (malaria pigment) acts as antigen to activate monocytes resulting in release of pro-inflammatory cytokines and chemokines [442, 443]. This could initiate a positive feedback loop since TNF-α increases expression of uPAR on monocytes resulting in accelerated cleavage of suPAR and consequent monocyte recruitment [439, 443]. Indeed, mononuclear cells loaded with pigment are observed in glomerular and peritubular microvessels on histopathology [171]. As elevated suPAR concentrations have not been shown to be directly pro-inflammatory [444], the accumulation of activated monocytes releasing cytokines are the likely effectors contributing to renal tubular cell damage in SM.

In this study there was no association between suPAR and mortality, which contrasts with a study of African children with malaria where increased suPAR concentrations were associated with poor clinical outcomes [429]. This may be because there was a low mortality rate (2%) in the African study, suggesting less severe disease. The entire cohort presented here

was severely ill with a high mortality rate and higher suPAR concentrations compared to the African study [429]; including UM patients in this analysis may have shown an association between suPAR concentration and mortality.

There are limitations to this study. Both suPAR and HRP2 measured in the plasma are not organ specific markers of monocyte activation and sequestration, respectively. However, kidney specific markers of these phenomena currently do not exist. The justification of the analyses of plasma suPAR and HRP2 is based on the histopathology that has shown sequestration of PRBCs and mononuclear cell infiltration in the kidney. Since suPAR was measured in plasma, the concentrations measured likely represent production by activated host immune cells, in particular mononuclear cells, however endothelial sources could also contribute. As suPAR has renal clearance, elevated plasma levels can be a consequence of reduced GFR [435, 437]. However, in the multiple regression models, interaction terms of CrCl, suPAR and *Pf*HRP2 did not reveal a significant contribution, and the association between suPAR and indication for RRT was also found in patients presenting without AKI on admission. It has been shown that plasma HRP2 half-life is not correlated with biochemical evidence of kidney dysfunction [353]. Urine NGAL as a biomarker for renal tubular damage has limitations, as its excretion depends on the timing in relation to the initial renal injury [445]. However, NGAL is an ‘induced’ biomarker, thus excretion is continued with ongoing renal stress [445]. Using renal biopsy as a reference standard for ATN was not feasible or ethically

indicated in this study, so RRT requirement was used as an alternative endpoint. Finally, the sample size in this study was relatively small and patient data were reviewed retrospectively. However, this assessment was done according to pre-defined criteria and three independent specialist physicians were in full agreement.

4.1.6 Conclusion

This study suggests that both parasite sequestration and immune activation contribute to the pathogenesis of AKI in severe falciparum malaria. Early diagnosis of falciparum malaria and treatment with artemisinin-based anti-malarials will rapidly eliminate ring-staged parasites, preventing their maturation and microvascular sequestration in vital organs, including the kidney. Deeper understanding of the immunopathological mechanisms underlying malaria-associated AKI might reveal novel, targeted, treatment options to further prevent this common and life-threatening complication.

4.2 Cell-free haemoglobin and oxidative stress

4.2.1 Background

As discussed in Chapter 1, severe falciparum malaria is characterized by intravascular haemolysis, where CFH increases with disease severity [50]. Sources of CFH include rupture of parasitized RBCs at schizogony, and destruction of uninfected erythrocytes, most prominently in patients with BWF (haemoglobinuria). This condition of fulminant haemolysis has been associated with kidney dysfunction in up to 64% of patients [33], but the underlying mechanisms have not been fully characterised.

CFH is independently associated with AKI in patients post-cardiopulmonary bypass, and with mortality in bacterial sepsis [337, 376, 446]. Haemoproteins, haemoglobin and myoglobin, can cause oxidative damage via haem redox cycling between ferric and ferryl states, which results in lipid peroxidation [58]. *In vivo* studies on oxidative injury have been hampered by the paucity of stable and specific markers of oxidative stress.

CFH-mediated lipid peroxidation of arachidonic acid generates F2-isoprostanes and isofurans, which are considered the most reliable amongst *in vivo* measures of oxidative stress [61, 62]. Arachidonic acids in membrane phospholipids are particularly vulnerable to free radical-mediated lipid peroxidation, which can disrupt cell membranes by altering membrane organization and integrity. In malaria, the high arachidonic acid content of infected RBC membranes reduces with parasite maturation, suggesting

membrane peroxidation may occur during parasite development [190]. Oxidative stress-induced reduction of RCD is proposed to play a role in renal insufficiency [185]. F2-isoprostanes are potent vasoconstrictors and have been associated with AKI in patients with rhabdomyolysis and haemolysis post-cardiopulmonary bypass [65, 69, 191]. Other plasma and urinary markers of oxidative stress have been shown to be elevated in SM compared to UM [70, 447-449].

4.2.2 Study aim

The role of plasma CFH-mediated lipid peroxidation in the pathophysiology of SM has not been described. This study examined the generation of CFH-mediated lipid peroxidation and its role in AKI and malaria severity by analyzing the associations between CFH, oxidative stress, RCD, and creatinine clearance in falciparum malaria patients.

4.2.3 Methods

This prospective observational study was conducted at CMCH, Bangladesh from 2011 to 2013. Patients ≥ 16 years admitted with slide confirmed severe *P. falciparum* malaria were recruited consecutively. Criteria for SM are defined in chapter 2.3.4.2. Enrollment venous blood biochemistry was analysed using a bedside analyzer (iSTAT, Abbott). Serum LDH and creatinine were measured using an Olympus analyser (Beckman coulter Inc.). Blood and urine were collected daily for 72 hours.

Plasma and urine F2-IsoPs and IsoF, plasma CFH and *Pf*HRP2 concentrations were determined as described in chapter 2.10. For

comparison, CFH was measured in an additional forty UM patients. RCD was measured using a laser-assisted optical rotational cell analyzer (LORCA, Mechatronic) immediately after blood collection [29, 90]. The deformability was measured by ellipticity of RBCs and described by the elongation index; (long minus short axes lengths) divided by (long plus short axes lengths). RCD was assessed at shear stresses ranging from 0.3 to 30 Pa. In capillaries, shear stresses of 1.7 Pa and above are encountered [450].

AKI was defined by CrCl using the Cockcroft-Gault formula as described in section 2.9.1 [344]. KDIGO criteria were not used because of missing follow-up data. Patients were classified according to AKI status based on enrolment CrCl with a cut-off of 60 ml/min. Longitudinal data were analysed according to the clinical course of renal function: (i) No admission AKI and CrCl remained ≥ 60 ml/min; (ii) No admission AKI but developed AKI; (iii) AKI on admission and CrCl remained < 60 ml/min, and (iv) AKI on admission but recovered without dialysis. The percent change from baseline was calculated for longitudinal data since patients presented at different stages of illness.

Statistical analysis

Continuous and categorical data were analysed as described in Chapter 2. Percentage change from baseline calculated at each time point was compared using Wilcoxon matched-pairs signed-rank or Kruskal-Wallis tests. Correlations between variables were assessed using Pearson and Spearman's correlation coefficient depending on distributions. Backward

stepwise robust regression was used to assess contribution of oxidative stress markers, CFH, and RCD to CrCl. The initial model included known risk factors for AKI (SBP, mean arterial pressure, age, *Pf*HRP2), variables were removed on the basis of $P \geq 0.05$. Robust regression was used because linear regression model diagnostics showed non-normality of residuals and influential points. Interaction terms between CFH and oxidative stress measures did not improve model fit. Risk factors for haemodialysis were assessed using logistic regression with each patient fitted as a random effect. LDH was analysed as a proxy measure for haemolysis.

4.2.4 Results

Sixty-nine adults with SM were included in this analysis (Tables 4.2.1 and 4.2.2). In the AKI group, 43% (20/47) met WHO criteria for AKI and 66% (31/47) met KDIGO criteria for AKI. Of those with AKI, 17% (8/47) recovered kidney function without haemodialysis. Of the patients without AKI, 36% (8/22) subsequently developed AKI. Overall mortality was 35%. (24/69) where patients with AKI died more quickly than those without (P =0.008). The mortality rate in AKI patients who did not receive dialysis was approximately 3.5-fold higher (15/28; 54%) compared to those who received dialysis (3/19; 16%).

CFH and oxidative stress

CFH was increased in patients with SM, compared to UM (P = 0.026), and correlated with other measures of haemolysis, including LDH, AST, total

Table 4.2.1 Baseline demographics and clinical characteristics by AKI status

Variable	Total (n= 69)	No AKI (CrCl >= 60) (n = 22)	AKI (CrCl<60) (n = 47)	*P*
Demographics				
Age (years)[a]	30 (24-40)	26 (22-30)	35 (25-45)	**0.021**
Males (%) [c]	43 (62)	14 (64)	29 (62)	0.475
Admission clinical parameters				
Glasgow Coma Score (max = 15)[a]	9 (8-12)	9 (8-10)	9 (7-14)	0.790
Systolic blood pressure (mmHg)	111 (14)	110 (14)	112 (15)	0.447
Mean arterial pressure (mmHg)[a]	81 (72-90)	75 (71-85)	81 (72-95)	0.170
Pulse rate (beats/min)[a]	113 (95-132)	111 (93-136	113 (95-124)	0.694
Respiratory rate (breaths/min)[a]	32 (28-40)	30 (28-39)	34 (26-42)	0.718

All values are mean (SD) unless otherwise specified: [a]median (interquartile range (IQR)), [b]geometric mean (95% CI), [c]number (%). *P* <0.05 using student t-test or Mann U Whitney; significant in bold. *Abbreviations:* AKI= acute kidney injury.

Table 4.2.2. Baseline admission laboratory parameters and outcome by AKI status

Variable	Total (n= 69)	No AKI (CrCl >= 60) (n = 22)	AKI (CrCl<60) (n = 47)	*P*
Haemoglobin (x10^3/)[a]	8.7 (7.0-11.1)	9.8 (6.7-11.9)	8.6 (7.2-10.6)	0.401
Cell-free haemoglobin (ng/ml)[b]	112,247 (82,896-151,991)	72,648 (47,213-111,786)	138,852 (93,585-206,016)	**0.044**
Peak cell-free haemoglobin (ng/ml)[b]	164,319 (124,084-217,601)	107,339 (67,043-171,855)	202,348 (143,360-285,607)	**0.033**
Mean corpuscular volume (mg/dL)[b]	87 (5)	86 (6)	87 (5)	0.74
Total serum bilirubin (mg/dL)[b]	2.7 (1.8-4.2)	1.4 (0.7-2.7)	3.7 (2.2-6.3)	**0.033**
Indirect bilirubin (mg/dL)[b]	1.1 (0.7-1.6)	0.6 (0.32-1.1)	1.4 (0.9-2.3)	**0.033**
Lactate dehydrogenase (U/L)[b]	700 (620-790)	549 (446-677)	783 (679-903)	**0.006**
Serum creatinine (umol/L)[b]	156 (132-185)	82 (73-92)	211 (176-254)	**<0.001**
Blood urea nitrogen (mg/dL)[b]	41 (35-49)	21 (18-23)	57 (48-67)	**<0.001**
Glucose (mmol/L)[b]	112 (85 -152)	113 (85 - 147)	107 (83 -155)	0.81
WHO AKI (creatinine > 265 umol/L, %)[c]	20 (29)	0 (0)	20 (43)	**<0.001**
KDIGO AKI (%)[c]	32 (46)	1 (5)	31 (66)	**<0.001**
Serum potassium (mmol/L)[a]	4.0 (3.6-4.6)	3.9 (3.4-4.0)	4.3 (3.8-4.7)	**0.027**
Serum base excess (mmol/L)	-7 (7)	-3 (6)	-9 (7)	**<0.001**
Venous bicarbonate (mmol/L)	17 (6)	21 (5)	16 (5)	**<0.001**
Parasitaemia (parasites/µL)[b]	33,265 (18,376-60,217)	68,774 (30,442-155,374)	23,677 10,823-51,797)	0.10
*Pf*HRP2 (ng/ml)[a]	2,243 (1,637-3,074)	1,652 (1,186-2,302)	2,588 (1,671-4,010)	0.19
Oxidative stress markers				
Plasma F2-isoprostanes (pg/ml)[b]	42.7 (35.2-51.8)	26.4 (21.5-32.4)	52.7 (41.7-66.6)	**<0.001**
Plasma Isofurans (pg/ml)[b]	66.2 (50.0-87.7)	32.3 (21.0-49.6)	90.8 (66.4-124.1)	**<0.001**
Clinical outcomes				
Received RRT (%)[c]	20 (29)	1 (5)	19 (40)	**0.002**
Death (%)[c]	24 (35)	6 (27)	18 (38)	0.37
Study hours to death (%)[c]	33 (19-93)	106 (51-121)	24 (16-38)	**0.008**

All values are mean (SD) unless otherwise specified: [a]median (IQR), [b]geometric mean (95% CI), [c]number (%). *P* <0.05 using student t-test or Mann U Whitney; significant in bold. *Abbreviations:* AKI= acute kidney injury; *Pf*HRP2= *P. falciparum* histidine rich protein 2; RRT= renal replacement therapy.

bilirubin and indirect bilirubin (r_s= 0.43, P < 0.001; r_s= 0.40, P < 0.001; r_s= 0.28, P = 0.023; and r_s= 0.40, P = 0.003, respectively). Parasite biomass as estimated from *Pf*HRP2, but not peripheral blood parasitaemia, positively correlated with CFH (r_p= 0.51, P <0.001; r_p= 0.04, P = 0.73) (Figure 4.2.1A) and enrolment creatinine r_p= 0.31, P = 0.010; r_p= -0.07, P = 0.53). CFH correlated with plasma F2-isoprostanes (r_p = 0.34, P = 0.018), as a measure of oxidative stress (Figure 4.2.1B), the correlation with isofurans was not significant (r_p = 0.20, P = 0.180).

Both plasma oxidative stress measures were inversely associated with pH (r_p = -0.47, P <0.001; r_p = -0.39; P <0.005) (Figure 4.2.1C, and 4.2.1D). When stratified by AKI, the F2-isoprostane correlation with pH remained significant in those with AKI (r_p = -0.63, P = 0.012) and without AKI (r_p = -0.37, P = 0.030). The 24-hour urinary excretion of urine F2-isoprostanes and isofurans were available for a minority of cases, but correlated inversely with admission CFH in those without AKI (r_s = - 0.80, P = 0.010 (n=9); r_s = - 0.56, P = 0.090 (n=10), respectively), but not in those with AKI (r_s = - 0.26, P = 0.366 (n=14); r_s = 0.21, P = 0.464 (n=14), respectively).

CFH, oxidative stress, and renal function

CFH was higher in the AKI group compared to the group without AKI (P = 0.044) (Figure 4.2.2, Table 4.2.1). Increased admission CFH was associated with deterioration in CrCl at 48 hours compared to baseline (r_p= -0.44, P =0.016) (Figure 4.2.3D). F2-isoprostanes and isofurans were higher in the AKI group (P < 0.001; P < 0.001) (Figure 4.2.2). F2-isoprostanes and

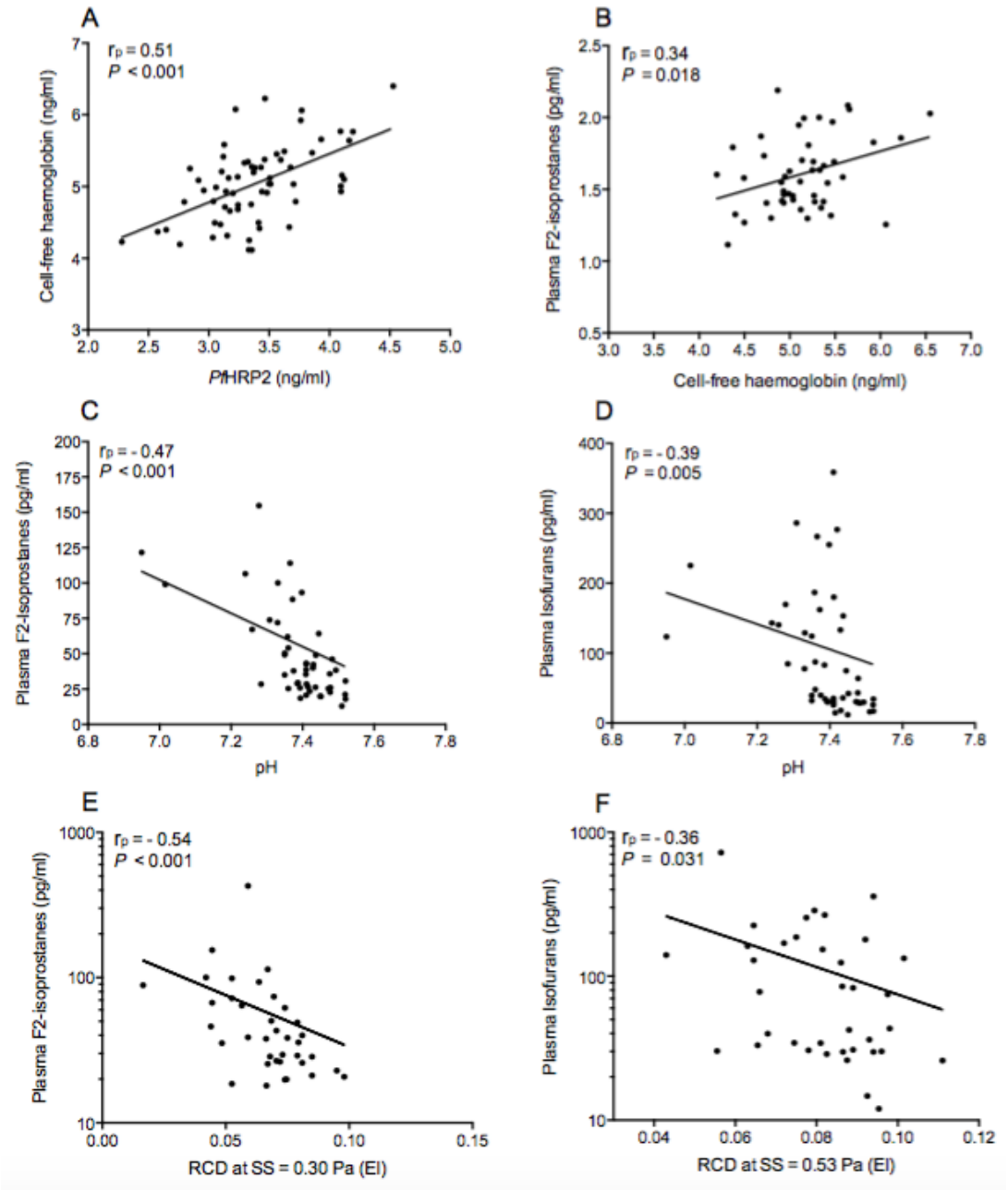


Figure 4.2.1 Correlation of oxidative stress markers in severe malaria. (A) Parasite biomass, as measured by *Pf*HRP2 concentration, was positively correlated with plasma cell-free hemoglobin, (B) cell-free hemoglobin was positively correlated with plasma F2-isoprostanes. Acidaemia (venous pH) was inversely correlated with (C) plasma F2-isoprostanes, and (D) plasma isofurans. Red cell deformability was inversely correlated with (E) plasma F2-isoprostanes, and (F) plasma isofurans. All correlations were performed with enrolment values. *Abbreviations*: *Pf*HRP2, *Plasmodium falciparum* histidine rich protein 2; RCD, red cell deformability; SS, shear stress; EI, elongation index; Pa, pascals; r_p, Pearson's correlation coefficient.

isofurans inversely associated with CrCl (r_p= -0.63, P <0.001; r_p= -0.67, P <0.001) (Figure 4.2.3A and 4.2.3B). The 24-hour urine F2-isoprostane and urine isofuran excretion concentrations were lower in the AKI group compared to those without (median: 1.0 (0.6-2.5) versus 2.7 (1.4-4.0), P = 0.034; and 8.1 (4.0-19.3) versus 9.2 (5.5-56.1), P = 0.318, respectively).

In evaluating the association of oxidative stress with development of AKI not present on admission, patients who developed AKI were found to have higher admission total plasma oxidative stress concentrations compared to those who maintained normal renal function (86 pg/ml (29-252) (n=8) versus 51 pg/ml (39-64) (n=13), P = 0.119), and the highest 24-hour urinary F2-isoprostane and isofuran excretion (Supplementary Table 4.2.1). LDH, F2-isoprostanes and isofurans concentrations increased during the first three days in patients who developed AKI (Figure 4.2.4A, Supplementary Table 4.2.1). Patients without AKI had low baseline F2-isoprostane concentrations that increased over time, however the magnitude of change was small compared to those who developed AKI (Supplementary Table 4.2.1). The 8 patients whose renal function recovered without dialysis showed a decreasing trend of LDH, F2-isoprostanes and isofurans (Figure 4.2.4B).

The fractional change at 24 hours in CrCl, LDH, and F2-isoprostanes differed in patients that developed AKI compared to patients who recovered (P = 0.006, P = 0.014, and P = 0.007, respectively).

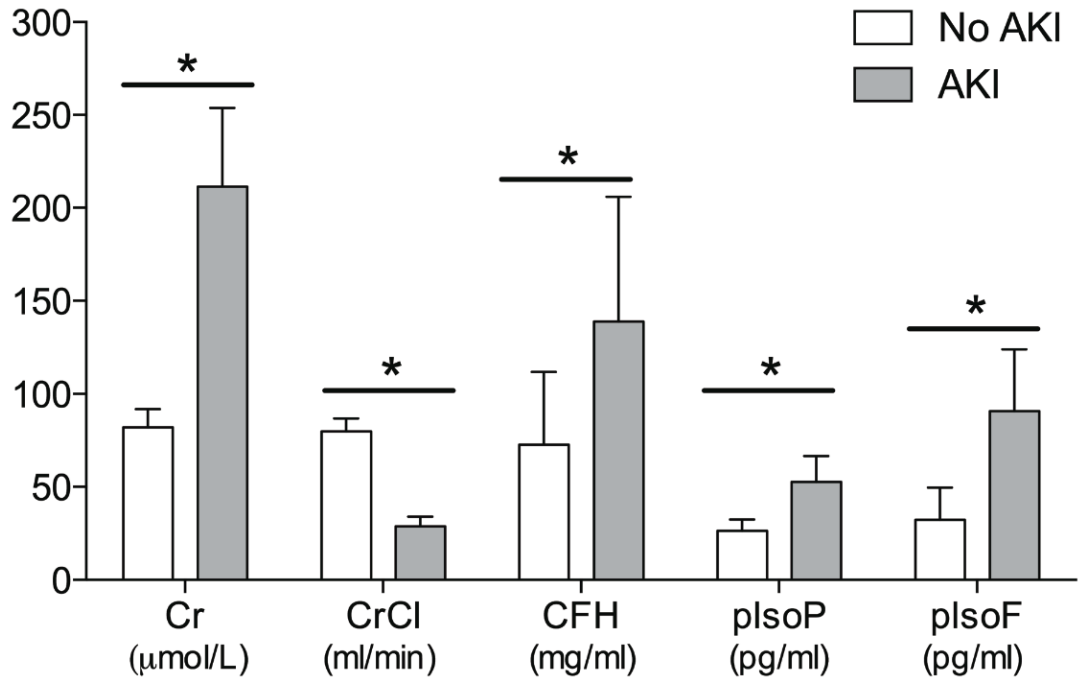


Figure 4.2.2 Kidney function, cell-free hemoglobin, and oxidative stress measures on admission by AKI status. Geometric mean plasma creatinine, cell-free hemoglobin, F2-isoprostanes and isofurans were significantly more elevated on admission in the acute kidney group (*= *t*-test: *P* <0.001; *P* = 0.044; *P* = 0.001; *P* < 0.001, respectively). Creatinine clearance was significantly reduced in the AKI group on admission (*=*t*-test: *P* <0.001). Error bars represent 95% confidence intervals. *Abbreviations:* AKI, acute kidney injury; Cr, creatinine; CrCl, creatinine clearance; CFH, cell-free hemoglobin; pIsoP, plasma F2-isoprostanes; pIsoF, plasma isofurans.

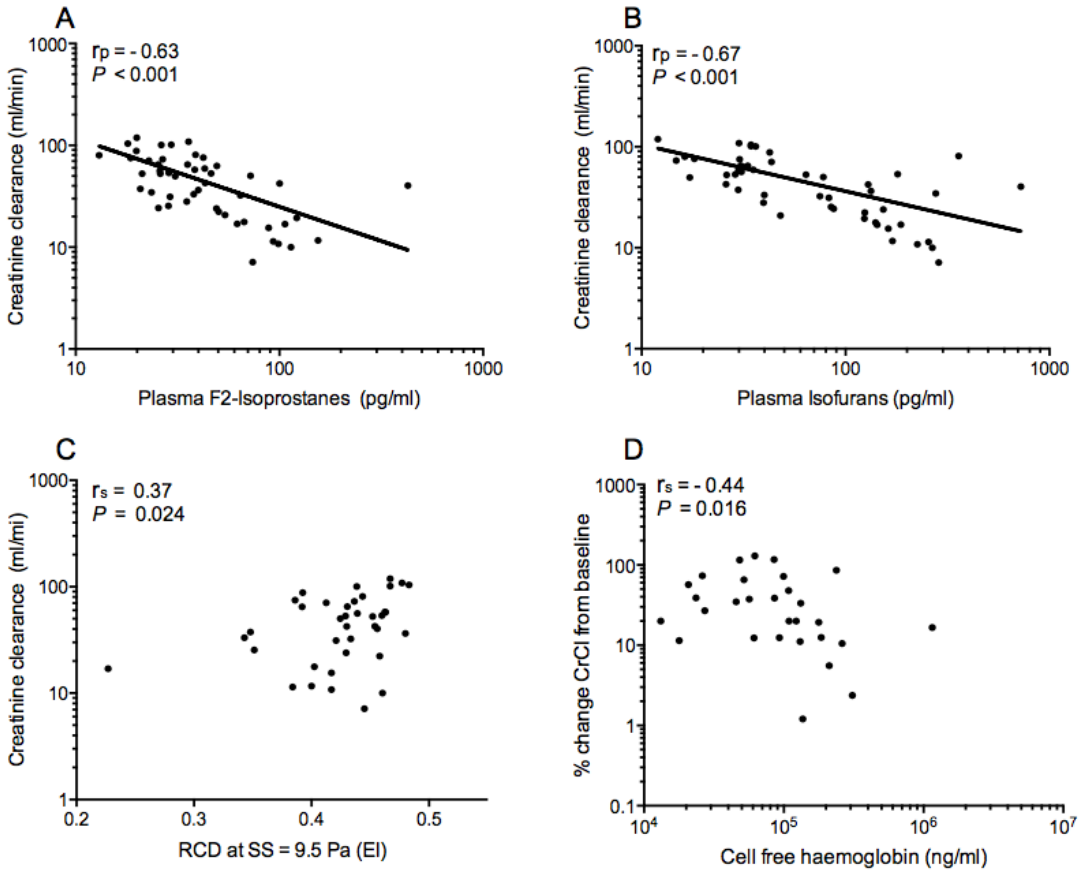


Figure 4.2.3 Correlations with creatinine clearance. Creatinine clearance on enrolment correlated inversely with (A) plasma F2-Isoprostanes, (B) plasma isofurans, and (C) red cell deformability at shear stress 9.5 Pa. (D) Plasma cell-free hemoglobin correlated with percent change in creatinine clearance at 48 hours from baseline. Regression lines not appropriate for Spearman's scatter plots (C, D) as this is a rank correlation. *Abbreviations:* CrCl, creatinine clearance; RCD, red cell deformability; SS, shear stress; EI, elongation index; Pa, pascals; r_p, Pearson's correlation coefficient; r_s, Spearman's correlation coefficient.

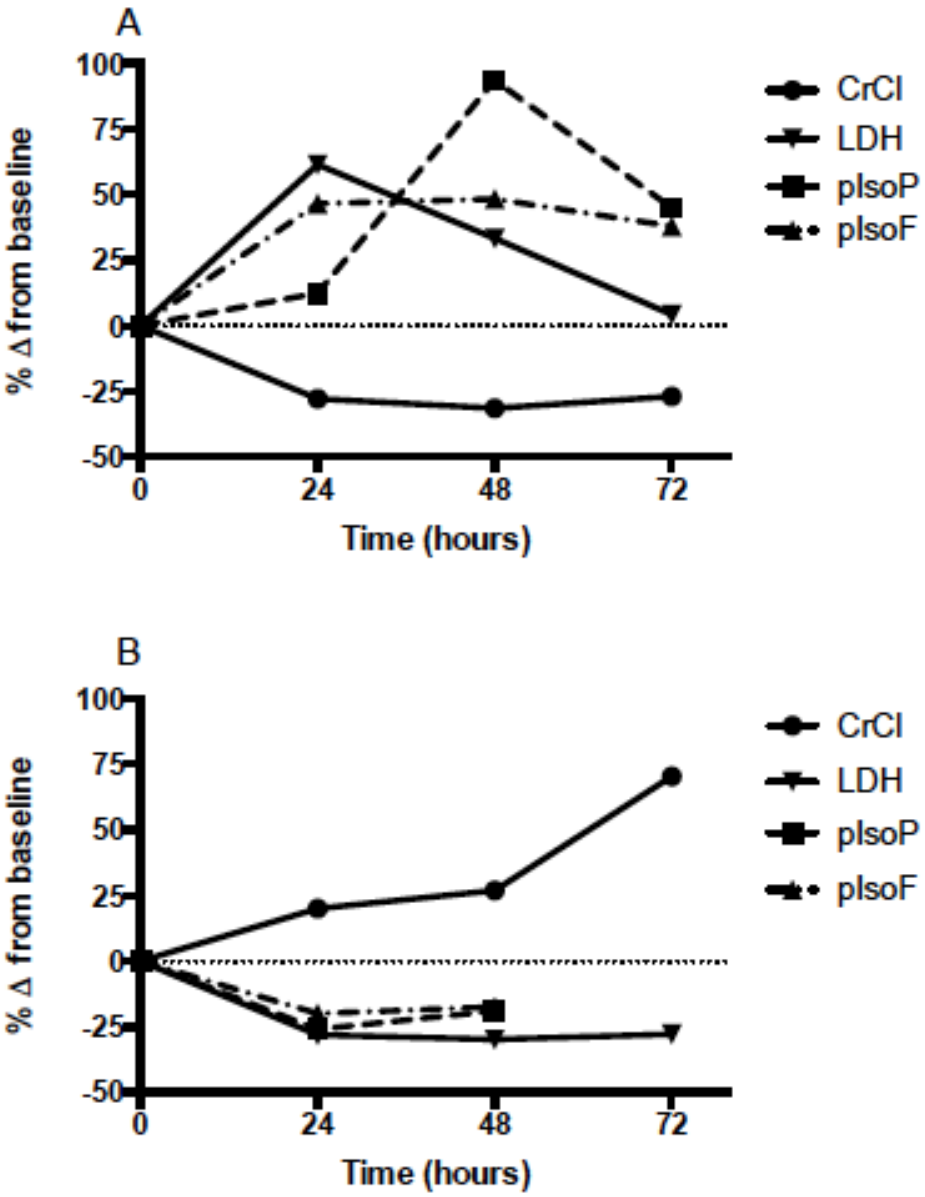


Figure 4.2.4 Trends in creatinine clearance, oxidative stress and hemolysis markers stratified by clinical course of renal function during hospital admission**.** (A) Patients classified as no AKI on enrolment who subsequently developed AKI during admission (N=8). (B) Patients classified as AKI on enrolment whose renal function recovered during admission in the absence of renal replacement therapy (N=8). All variables presented as fractional change from baseline value. *Abbreviations*: AKI, acute kidney injury; CrCl, creatinine clearance; LDH, lactate dehydrogenase; pIsoP, plasma F2-isoprostanes; pIsoF, plasma isofurans.

Haemodialysis was performed in 40% (19/47) of AKI patients. Plasma F2-isoprostanes and isofurans on admission were higher in those who later received haemodialysis compared to those who did not (geometric mean: 61 (43-86) versus 38 (30-48), *P*=0.036; 139 (97-199) versus 52 (38-72), *P* = 0.002) (Figure 4.2.5A, 4.2.5C).

Red cell deformability, renal function and oxidative stress

RCD at low shear stresses inversely associated with both plasma F2-isoprostanes and isofurans (r_p = - 0.54, *P* <0.001; r_p = - 0.36, *P* = 0.031) (Figure 4.2.1E, 4.2.1F). Patients with AKI on admission and those who developed AKI had a similar reduction in RCD at enrolment, which was lower than patients without AKI or recovered from AKI without dialysis (*P* = 0.007). Decreased RCD correlated with lower CrCl at all shear stresses and most strongly at 9.5 Pa (r_s= 0.37; *P* =0.024) (Figure 4.2.3C).

Predictors of renal function

In a multivariable linear regression model, increased plasma F2-isoprostanes, isofurans and decreased RCD were independently associated with a decrease in CrCl, predicting 64% of the variation in CrCl (R^2 = 0.64; Table 4.2.3). In the longitudinal logistic model, adjusted for known predictors of haemodialysis and patients fitted as a random effect, elevated isofurans were independently associated with subsequent need for RRT (*r*= 0.60).

CFH, oxidative stress and survival

Those who died had higher plasma F2-isoprostanes and isofurans compared to survivors (geometric mean: 59 pg/ml (39-88) versus 35 pg/ml

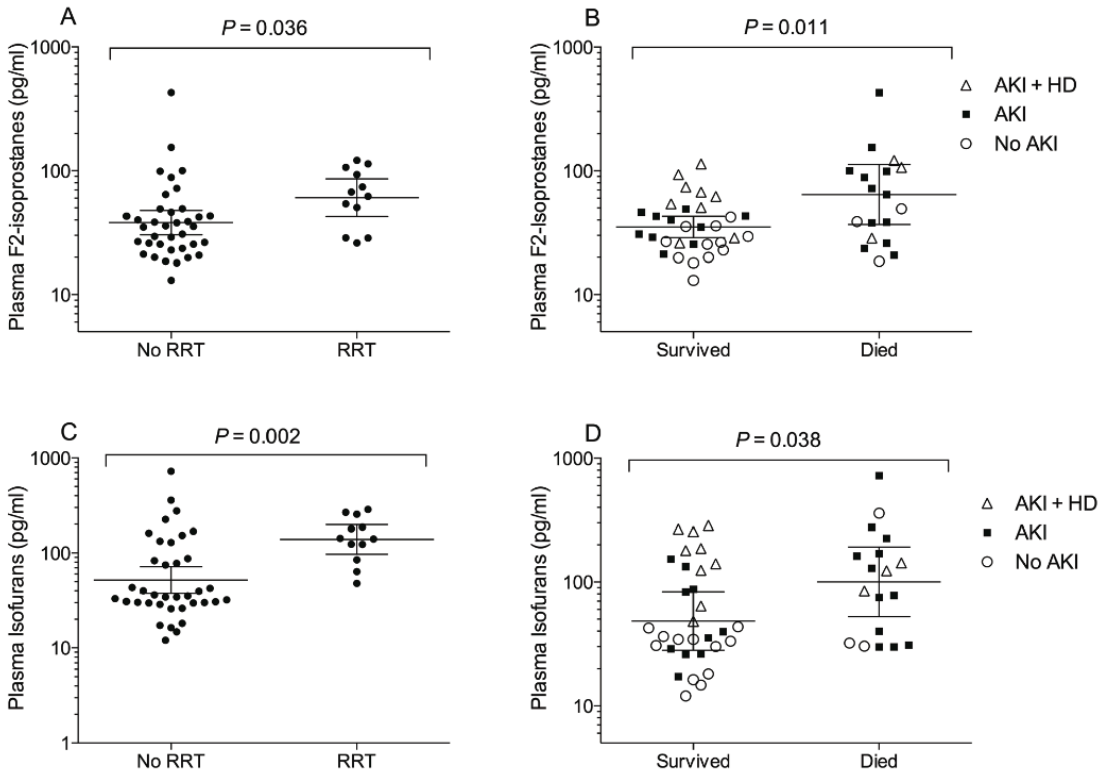


Figure 4.2.5 Comparison of plasma oxidative stress measures by clinical outcomes. Plasma F2-isoprostanes at enrolment by (A) renal replacement therapy, and (B) outcome. Plasma isofurans at enrolment by (C) renal replacement therapy, and (D) outcome. *Abbreviations*: RRT, renal replacement therapy; HD, hemodialysis; AKI, acute kidney injury.

Table 4.2.3. Association of variables with creatinine clearance on admission in patients with severe malaria

	Univariate analysis		Multivariate model (R^2=0.64)	
Variables	β (95% CI)[a]	*P*	β (95% CI)[a]	*P*
Log CFH[§]	- 0.08 (-0.22 to 0.06)	0.274	---	**---**
Log F2-IsoP	- 0.67 (-0.91 to -0.43)	**<0.001**	- 0.56 (-0.89 to -0.22)	**0.002**
Log IsoF	- 0.49 (-0.65 to -0.33)	**<0.001**	- 0.42 (-0.63 to -0.21)	**<0.001**
Log CFH*F2-IsoP	- 0.05 (-0.06 to -0.03)	**<0.001**	---	**---**
Log CFH*IsoF	- 0.04 (-0.05 to -0.03)	**<0.001**	---	**---**
Log*Pf*HRP2[§]	- 0.29 (-0.44 to -0.15)	**<0.001**	---	**---**
RCD at SS1.69Pa[§]	19.2 (-3.3 to 41.6)	0.092	---	**---**
RCD at SS9.49Pa[§]	7.3 (0.6 to 14.0)	**0.033**	3.7 (0.01 to 7.5)	**0.049**
Log Age[§]	- 0.45 (-0.91 to -0.001)	0.050	---	---
MAP <65mmHg	- 0.13 (-0.63 to 0.37)	0.607	---	---

[a]Regression coefficient (β) with 95% confidence intervals (CIs) showing the estimated decrease in creatinine clearance predicted by 1-U changes in the independent (predictor) variables. [§]Univariate robust regression applied for variables with non-normally distributed residuals, containing data with high leverages and residuals. A backward stepwise robust regression multivariate model included all variables in univariate analyses, which were removed on the basis of $P \geq 0.05$. Interaction terms did not improve the model. *Abbreviations*: CFH, cell-free hemoglobin; F2-IsoP, F2-isoprostanes; IsoF, isofurans; CFH*F2-IsoP, cell-free hemoglobin and F2-isoprostane interaction term; CFH*IsoF, cell-free hemoglobin and isofuran interaction term; *Pf*HRP2, *P. falciparum* histidine rich protein 2; RCD, red cell deformability at shear stress 1.69 and 9.49 pascals; MAP, mean arterial pressure less than 65mmHg.

(30-43), *P* = 0.011; 96 pg/ml (60-157) versus 53 pg/ml (38-75), *P* = 0.038) (Figure 4.2.5B, 4.2.5D). There was no difference in CFH between those who died and survived (*P* = 0.146).

4.2.5 Discussion

In this study CFH-mediated oxidative stress, as measured by plasma F2-isoprostanes and isofurans, was strongly and independently associated cross-sectionally with AKI on admission, and prospectively with haemodialysis requirement during admission in adult patients with falciparum malaria.

Intravascular haemolysis involves both parasitized and unparasitised RBCs [91]. In the current study, while the source of CFH was not assessed its plasma concentration was associated with increased estimated parasite biomass, consistent with previous studies [50]. This suggests that haemoglobin released at schizont rupture, the end of the 48-hour intra-erythrocytic lifecycle, is an important source of CFH. In agreement with another recent study, *Pf*HRP2, a more accurate estimate of parasite biomass accounting for both sequestered and peripheral parasites, correlated with kidney dysfunction whereas peripheral parasitaemia did not [179].

Redox cycling of haemoglobin forms a radical ferryl state that can initiate lipid peroxidation of arachidonic acid to generate F2-isoprostanes and isofurans [61, 62, 189]. Isofurans are formed preferentially at higher oxygen

tension compared to F2-isoprostanes, thus measuring both lipid peroxidation products provides a more reliable measure of oxidative stress [62]. This is the first study to assess both plasma F2-isoprostanes and isofurans in SM and show that they are elevated in patients with AKI. Elevated lipid peroxidation, as measured by maldialdehyde, has been observed to be associated with disease severity in falciparum malaria but did not correlate with kidney dysfunction [447]. This may be due to maldialdehyde and the quantification method used not being as specific for lipid peroxidation compared the markers (F2-isoprostanes and isofurans) assessed in this study [451, 452]. CFH correlated with increased levels of plasma F2-isoprostanes, a marker of oxidative injury. Several factors influence the generation of lipid peroxidation markers. The oxidative capacity of plasma CFH is largely dependent on the fate of the haem moiety: haem is water insoluble and binds to haemopexin, albumin, lipoproteins, and cell membranes [453], and its oxidative capacity differs considerably between these fractions. In addition, other sources of free radicals may initiate lipid peroxidation to generate plasma F2-isoprostanes and isofurans. Reactive oxygen intermediates produced during phagocytic oxidative burst may contribute radicals involved in generating plasma F2-isoprostanes and isofurans. Indeed, renal histopathology of malaria-associated AKI shows accumulation of host monocytes in addition to parasitized RBCs [171]. Haem-containing myoglobin concentration is also increased in SM, although to a much lesser extent than CFH [50]. The oxidative capacity of myoglobin increases at low pH because of increased pseudoperoxidase activity [58]. This could also apply to CFH. In our study both plasma F2-isoprostanes and

isofurans were associated with reduced venous blood pH. This correlation remained strong when stratified by AKI, which supports the association being independent of renal dysfunction.

CFH-mediated oxidative stress has been shown to contribute to AKI in diseases and medical procedures characterized by haemolysis [65, 66, 69, 191]. F2-isoprostanes are potent renal vasoconstrictors, which reduce renal blood flow and GFR [61]. In this study, concentrations of plasma CFH, F2-isoprostanes, and isofurans were higher in the AKI group, and were inversely associated with creatinine clearance.

The impact of CFH-mediated oxidative stress on renal function may be delayed given the observation that the CFH on enrolment inversely associated with the percent change creatinine clearance at 48 hours. Moreover, the subgroup of non-AKI patients at enrolment who developed AKI had the highest increase of plasma and 24-hour urine F2-isoprostanes and isofurans. This finding also suggests that intrarenal oxidative stress generated from filtered CFH dimers may be an early contributing mechanism to renal injury. The lower 24-hour urine F2-isoprostane excretion concentrations in patients with established AKI suggest that haemoglobin dimers are not filtered well at low creatinine clearances. A common problem of AKI biomarkers is that their concentrations can be increased as a result of renal dysfunction, rather than be a cause of it. However, the group without AKI on enrolment that subsequently developed AKI showed concomitant increases in haemolysis parameter LDH, plasma and 24-hour urine F2-

isoprostanes and isofurans. This further suggests that plasma F2-isoprostane and isofuran concentrations contribute to the pathogenesis of AKI rather than their elevated concentrations resulting from a decreased GFR.

Plasma F2-isoprostanes and isofurans were also associated with reduced RBC deformability. Red cell rigidity has been well described in SM. It is thought to be caused by oxidative damage to RBC membranes, and contribute to microvascular flow obstruction [29, 90]. RBC membranes are rich in arachidonic acid. Haem-mediated lipid peroxidation of RBC membranes could contribute to the development of reduced RCD, and thus be a source of plasma F2-isoprostanes and isofurans in SM. RCD at low shear stresses is mainly determined by membrane properties, whereas at high shear stresses surface-to-volume relationships become more important [454, 455]. In this study, the correlation between plasma oxidative stress measures and decreased RCD was strongest at low shear stresses, suggesting RBC membrane damage. Reduced RCD through non-enzymatic glycation and oxidation of membrane glycoproteins has also been proposed to play a pathogenic role in diabetic nephropathy, since advanced glycation end products independently associated with reduced RCD [185]. In the current study, red cell rigidity inversely correlated with creatinine clearance. Furthermore, reduced RCD and high plasma F2-isoprostanes were present in patients with AKI, but also in those who developed AKI after admission, suggesting that RBC rigidity mediated by oxidative injury was not due to reduced filtration. Potentially, oxidative stress induces medullary

vasoconstriction that results in intrarenal regional hypoxia, which could be further exacerbated by limited flow of RBCs with reduced deformability in renal vessels containing sequestered parasitized RBCs.

This study has limitations. The sample size of this detailed study meant patient numbers became relatively small when considering subgroups. Also, LDH analysed as a proxy for CFH in the longitudinal analysis is not a specific marker of haemolysis. However, LDH did positively correlate with plasma CFH and F2-Isoprostanes, and negatively with haemoglobin. As a consequence, most analyses focused on admission concentrations of the variables proposed to contribute to this mechanism of kidney injury. Myoglobin was not assessed in the current study, although myoglobin can cause oxidative damage to the kidney. However, the elevation in plasma myoglobin concentration was shown to be 100-fold less than CFH in SM [50]. Plasma CFH and oxidative stress markers were not assessed for comparison to other forms of AKI or disease processes, such as septic patients, due to financial constraints. However, CFH has been shown to be significantly higher in patients with severe malaria compared to moderately severe malaria and healthy controls [50]. The KDIGO criteria to classify AKI were not used in this analysis for two reasons. Firstly, 11% of creatinine values during hospital admission were missing in surviving patients and in the 14 patients who died before a second creatinine could be measured. Secondly, while the application KDIGO definitions are accepted for epidemiological comparisons of AKI incidence the definitions have not been well validated for pathophysiologic studies of AKI. The KDIGO diagnosis of

AKI has low specificity thus patients without AKI may be falsely identified as AKI. Further, the KDIGO staging implies both that there is homogeneity within each stage and heterogeneity between stages [456]. Further, there is a lack of correlation between creatinine and GFR in the setting of AKI where a rising creatinine may be observed when the GFR is actually improving. Although there are limitations with all formulae for estimated glomerular filtration in the setting AKI, they may be a more accurate reflection of GFR compared to creatinine in isolation [103].

4.2.6 Conclusions

In summary, the study findings suggest that CFH-mediated oxidative stress generating systemic F2-isoprostanes and isofurans contributes to the pathogenesis of malaria-associated AKI and is associated with need for haemodialysis and in-hospital mortality. Lipid peroxidation may have a dual effect in the mechanism of AKI in malaria. Firstly, the lipid peroxidation metabolites, plasma F2-isoprostanes, may contribute to renal vasoconstriction. Secondly, lipid peroxidation of RBC membranes may result in reduced RCD, which in turn may exacerbate ischaemia in the renal microvasculature.

5.0 Paracetamol as adjunctive treatment

5.1 Pharmacokinetics and pharmacodynamics of paracetamol in falciparum malaria

5.1 Background

"Fever" was recognized as a disease in itself in the early 6th century BC [457], which called for treatment ranging from food restriction in the era of Hippocrates (460-370 BC) to phlebotomy in the era of Galen (130-200 AD). Today, the debate of the benefits and risks of fever in response to bacterial and viral infections continues [458-469], and thus the use of antipyretics remains controversial in these clinical scenarios. Paracetamol is the antipyretic recommended by the World Health Organization for the treatment of malaria for temperatures above 38.5°C [21]. While ibuprofen reduces temperature in UM [326, 341, 342], it is no longer recommended due to potential adverse effects such as renal impairment, and gastrointestinal bleeding [21]. However, the therapeutic benefit of paracetamol in patients with malaria has also been put into question and possible interactions with artemisinin antimalarials have not been evaluated *in vivo*.

While fever is a salient feature of malaria, the exact mechanism and its effects on both the host and parasite are not completely understood [470]. *In vitro* studies show conflicting results, where some studies have found that elevated temperatures negatively affect parasite growth, whereas others have shown accelerated parasite maturation and increased sequestration in response to febrile temperatures [331, 471-473]. Two randomised controlled

studies in UM have shown that antipyretics prolonged parasite clearance time [324, 341] with no accompanying clinical deterioration. Clinical studies assessing the effect of a single oral dose of paracetamol on fever in UM have shown an effective reduction in temperature [326, 327]. However, RCTs of multiple dose paracetamol in children with UM have not shown significant reductions in fever clearance time compared to controls [324, 325]. One of these studies [324] administered paracetamol suppositories at a dose that has been proposed to result in sub-therapeutic concentrations [293, 294, 474, 475], and neither of these studies measured paracetamol plasma concentrations to determine if the patients had antipyretic concentrations between 10 and 20 μg/ml to support the conclusions of a lack of antipyretic effect.

In Thai adults with acute UM, the clearance, elimination half-life and apparent volume of distribution of single dose oral paracetamol administered as a tablet are similar when compared to the convalescent phase of illness, suggesting that Phase II conjugation of paracetamol is not affected in uncomplicated falciparum malaria [284]. While the plasma glucuronide metabolite concentration was lower in the acute illness phase, the formation clearance was not significantly different. The pharmacokinetics of dihydroartemisinin, which is cleared by glucuronidation [476], is affected in pregnant women with falciparum malaria infection [477], but an *in vitro* study suggests that paracetamol is a poor inhibitor of dihydroartemisinin glucuronidation [476]. In practice it is frequently not possible to administer

regular oral paracetamol in tablet form since malaria patients may be nauseated or unconscious, thus patients receive either rectal suppositories or intramuscular injections. Pharmacokinetic studies of rectal paracetamol in non-malaria patients show that higher dosing is required to achieve therapeutic serum concentrations [290, 293, 294]. Due to the practical obstacles of physically delivering adequate doses to adults by suppositories, the majority of patients are under-dosed by this route [475]. A study of intramuscular paracetamol in children undergoing minor surgery showed that intramuscular paracetamol achieved higher drug levels compared to suppositories [478]. However, in sick patients with poor muscle perfusion the relative bioavailability of intramuscular paracetamol could be reduced. The relative bioavailability and antipyretic efficacy of intramuscular paracetamol in patients with malaria have never been investigated.

The effect of disease severity on the pharmacokinetics of paracetamol has been reported. In critically ill non-malaria patients, a pharmacokinetic study of paracetamol suggested that 1,000 mg of *intravenous* paracetamol was not sufficient to achieve a therapeutic effect [479]. In a rat malaria model, glucuronidation of paracetamol was found to be impaired, potentially explained by the increased severity of disease in this model [287]. Currently, there is no pharmacokinetic data available for paracetamol in severe falciparum malaria patients. Given the widespread administration of paracetamol in severe falciparum malaria patients treated with artesunate, the potential for drug interactions and effects on clinical outcomes, studies of paracetamol are warranted in this population.

Therefore, trials of paracetamol in both uncomplicated and severe falciparum malaria administered by different routes were conducted to address the pharmacokinetics of paracetamol and define the optimal dosing strategies to ensure therapeutic concentrations in these populations.

5.1.1 Intramuscular versus oral paracetamol in uncomplicated malaria

5.1.1.1 Study aim

To investigate the preferred route of paracetamol administration by determining the pharmacokinetics of paracetamol given by intramuscular route versus oral route in patients with uncomplicated *P. falciparum* malaria and high body temperature to compare the relative bioavailability and antipyretic effects.

5.1.1.2 Study design

Study design and patients

The study was a randomised, open-label, two treatment crossover study of oral syrup and intramuscular paracetamol conducted at Mae Sot Hospital, Tak, Thailand from May to June 2001, as described in Chapter 2. Briefly, consecutive non-pregnant adult (≥15 years) patients admitted with slide confirmed uncomplicated *P. falciparum* malaria were enrolled into the study if they had an aural temperature > 38°C, and required oral or intramuscular paracetamol. Patients were excluded if paracetamol had been taken within

the previous 12 hours, or if there was any contraindication to paracetamol or requirement for interacting drugs.

Study participants were randomly assigned to receive on the first day of enrolment, either a single dose of 600 mg paracetamol syrup suspension (oral or via NG tube) (Tylenol ®; Janssen), followed by 100 ml of water (Group 1), or a single dose of 600 mg intramuscular paracetamol (Partamol ®; Atlantic) divided in 2 doses into the upper legs (Group 2). After a period of 24 hours, the patients received paracetamol by the alternate route. Study participants whose aural temperature exceeded 40°C despite the study dose of paracetamol, received additional tepid sponging and a bedside fan. If the aural temperature exceeded 40°C after 6 hours from the first dose of paracetamol, further doses were administered to a daily maximum of 4 g.

Lithium heparin plasma samples for paracetamol concentration measurement were collected on enrolment (pre-dose), and then at 0.5, 1.0, 1.5, 2, 3, 4, 6, 8, 10, 12 hours post-dose. Paracetamol plasma concentrations and plasma protein binding were quantified in Bangkok, Thailand, using high-performance liquid chromatography, as previously described [284, 295].

This study was designed and performed in 2001, at this time there was a paucity of information available describing the expected differences between groups and inter-individual variability to estimate sample size. Population

pharmacokinetic, pharmacodynamic analysis and parasite clearance endpoints are described in Chapter 2.10. Descriptive statistics analyses and software used are described in chapter 2.10.14. Secondary pharmacokinetic parameters in both groups (i.e. IM and oral administration) were compared using the Wilcoxon matched-pairs signed rank test.

5.1.1.3 Results

Baseline characteristics

Twenty-one adults with acute falciparum malaria were included in this analysis (Figure 5.1.1.1). Admission temperature ranged from 38.1°C to 41.2°C. Baseline characteristics are shown in Table 5.1.1.1. Of the 21 patients, seven received extra doses of paracetamol during the 2-day study period. One patient in Group 1 withdrew from the study 12 hours after the initial oral dose and did not receive the intramuscular dose on the second day. One patient in Group 2 was given extra oral doses starting 6 hours after the initial intramuscular study dose for high fever (>39°C) and did not receive the scheduled oral study dose the second day. Participants in group 2 were older, weighed less and had increased ALT and ALP concentrations compared to Group 1 (Table 5.1.1.1). One patient in group 2 was diagnosed with SM post-enrolment based on a total bilirubin >3 mg/dl with a parasitaemia >100,000 /μl. Concomitant drugs taken by the patients included dimenhydrinate (n=2), metoclopramide (n=4) and domperidone (n=1). No patient reported excessive alcohol intake.

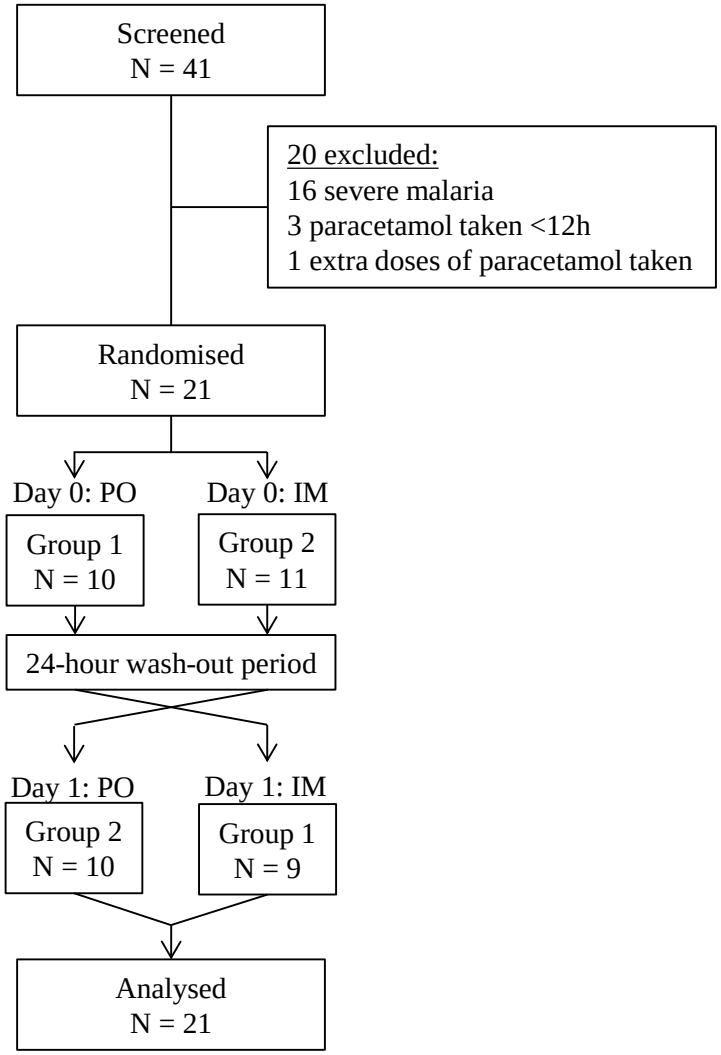


Figure 5.1.1.1. Consort diagram of paracetamol crossover trial. After enrolment to the studies, patients admitted to Mae Sot Hospital had blood collected prior to paracetamol administration and then timed plasma collections. Group 1 received oral syrup paracetamol (PO) on Day 0 then intramuscular paracetamol (IM) on Day 1; Group 2 received intramuscular paracetamol on Day 0 then oral syrup paracetamol on Day 1. One patient in group 1 did not receive the intramuscular dose of paracetamol on Day 1 due to self-discharge from the hospital; one patient from group 2 did not receive oral paracetamol on Day 1 as the patient received multiple doses of paracetamol during the study period. All patients were included in the pharmacokinetic analysis.

Table 5.1.1.1. Baseline characteristics stratified by treatment group

Variable	Total (n=21)	Group1 (n=10)	Group2 (n=11)
Age (years)	25 (22-37; 15,54)	23 (20-30; 15,37)	33 (22-52; 20-54)
Males (%)[a]	19 (90)	9 (90)	10 (91)
Weight (kg)	58 (55-63; 47,70)	60 (58-65; 55,70)	55 (50-60; 47,70)
Temperature (°C)	39.6 (39.0-40.5; 38.1,41.2)	40.1 (38.2-40.5; 38.1,41.2)	39.4 (39.0-40.5, 38.6,40.8)
Heart rate (beats/min)	100 (92-115; 80,122)	109 (95-120; 92,122)	94 (90-104; 80,120)
Parasitaemia (parasites/µl)[b]	47,573 (19,769-114,477)	46,793 (13,117-166,929)	48,293 (11,436-203,926)
Haematocrit (%)	44 (38-46; 22,54)	44 (36-45; 34,51)	45 (41-48; 22,54)
Creatinine (µmol/L)	101 (87-112; 70,136)	99 (83-113; 70,131)	105 (92-111; 81,136)
BUN (mg/dL)	16 (15-22; 12,34)	15 (14-21; 13,34)	16 (15-22; 12,29)
AST (IU/L)	40 (27-49; 14,132)	32 (26-42; 14,67)	45 (30-52; 25,132)
ALT (IU/L)	16 (10-28; 9,40)	11 (10-15; 9,28)	26 (16-36; 9,40)
ALP (IU/L)	88 (69-105; 54,311)	72 (63-96; 54,218)	98 (87-116; 70,311)
Total bilirubin (mg/dL)	1.6 (1.2-2.2; 0.6,6.9)	1.9 (1.5-2.4, 1.0,2.7)	1.3 (0.9-1.6; 0.6,6.9)
Indirect bilirubin (mg/dL)	0.4 (0.3-0.5; 0.2,2.4)	0.5 (0.4-0.6; 0.2,0.7)	0.4 (0.3-0.4, 0.2,2.4)

All values are compared using the Mann U Whitney test and reported as median (IQR; range), unless otherwise specified. [a] Reported as number (%) and compared using the Fisher's exact test.[b] Reported as geometric mean (95% CI) and compared using the Student's *t*- test. Group 1= Oral syrup paracetamol on Day 0 then intramuscular paracetamol on Day 1; Group 2= Intramuscular paracetamol on Day 0 then oral syrup paracetamol on Day 1. *Abbreviations:* BUN= blood urea nitrogen; AST= aspartate transaminase; ALT= alanine transaminase ALP= alkaline phosphatase.

Pharmacokinetics

Of the 21 patients, 363 plasma paracetamol concentrations were included in the pharmacokinetic analysis. Paracetamol concentration-time data were well described by a two-compartment disposition model (ΔOFV = −63.3). Adding an additional disposition compartment improved the model fit (ΔOFV = −26.2), but not goodness-of-fit plots and visual predictive checks. However, the main driver of this significant drop in OFV was due to one patient contributing an individual ΔOFV of −22.0, resulting in a non-significant ($p > 0.05$) improvement in model fit when this patient was removed. Furthermore, the terminal half-life estimated from the three-compartment disposition model was inappropriately long (median terminal half-life of 600 h) compared to the literature (1.5 - 2.5 h)[285]. Thus, the two-compartment disposition model was carried forward. Zero-order absorption for intramuscular administration and first-order absorption for oral syrup administration best described the absorption phase of paracetamol compared to all other absorption models (ΔOFV > -62.1).

Allometric scaling of pharmacokinetic parameters did not improve model fit (ΔOFV = 6.67). Thus, body weight was not incorporated into the final model. The stepwise covariate search showed no significant covariate relationships in this population. Inter-individual variability in the duration of zero-order absorption and apparent of volume of distribution were less than 1% and fixed to zero, without affecting the OFV. Parameter estimates from the final

Table 5.1.1.2. Parameter estimates of the final population pharmacokinetic model of paracetamol in patients with falciparum malaria

Parameter	Population estimate[a] (%RSE)[b]	95% CI[b]	IIV (%CV)[a] (%RSE)[b]	95% CI[b]
F_{IM}	1 Fixed	-	-	-
F_{PO}	0.844 (8.4)	0.682-0.951	287 (49.1)	76.5-1,038
DUR_{IM} (h)	0.689 (6.2)	0.621-0.784	-	-
$k_{a,PO}$ (h^{-1})	4.15 (44.5)	1.95-9.73	232 (49.7)	39.9-574
CL (L/h)	10.7 (16.9)	7.35-14.7	81.8 (69.7)	24.4-164
V_C (L)	45.5 (8.5)	36.7-51.5	-	-
Q (L/h)	10.3 (36.8)	4.80-20.1	77.4 (44.0)	21.1-105
V_P (L)	11.3 (42.7)	5.01-29.0	428 (46.5)	112-2,351
σ	0.376 (7.8)	0.316-0.436	-	-

[a] Population mean values and inter-individual variability (IIV) estimated by NONMEM. The coefficient of variation (%CV) for IIV was calculated as $100 \times \sqrt{\exp(\text{estimate}) - 1}$

[b] The relative standard error (%RSE) was calculated as $100 \times (\frac{\text{SD}}{\text{Mean value}})$ from the non-parametric bootstrap results (n=1,000). The 95% confidence interval (95% CI) is presented as the 2.5 to 97.5 percentiles of the bootstrap estimates.

Abbreviations: F_{IM}, bioavailability after intramuscular administration; F_{PO}, relative bioavailability after oral syrup administration; DUR_{IM}, duration of zero-order absorption after intramuscular administration; $k_{a,PO}$, absorption rate constant after oral syrup administration; CL, apparent elimination clearance; V_C, apparent volume of distribution of the central compartment; Q, inter-compartment clearance; V_P, apparent volume of distribution of the peripheral compartment; σ, variance of the residual variability.

Table 5.1.1.3. Secondary parameters of paracetamol after intramuscular and oral syrup administration in patients with falciparum malaria

Secondary parameters	Intramuscular administration	Oral administration	*P* value
C_{MAX} (mg/L)	11.4 (10.8-11.8)	8.52 (7.42-9.55)	< 0.0001
T_{MAX} (h)	0.689	0.705 (0.577-1.00)	0.442
$t_{1/2}$ (h)	3.18 (2.67-4.30)	3.03 (2.07-3.53)	0.196
AUC_{0-12} (h×mg/L)	37.9 (27.5-44.9)	31.6 (27.0-39.3)	0.229

Secondary parameters were calculated as the median and range of the empirical Bayes estimates. The *P* values were calculated with the Wilcoxon matched-pairs signed rank test. *Abbreviations:* C_{MAX}, maximum concentration; T_{MAX}, time to reach maximum concentration; $t_{1/2}$, terminal half-life; AUC_{0-12}, area under the concentration-time curve from time zero to 12 hours.

paracetamol pharmacokinetic model are shown in Tables 5.1.1.2 and 5.1.1.3.

The final paracetamol pharmacokinetic model showed satisfactory goodness-of-fit diagnostics (Figure 5.1.1.2) and visual predictive performance (Figure 5.1.1.3). Eta-shrinkage was less than 30% except for inter-individual variability of inter-compartmental clearance and absorption rate constant (54.9% and 35.8%, respectively). The numerical predictive check after intramuscular administration resulted in 0.556% (95% CI: 0.556 to 12.8%) and 2.22% (95% CI: 1.11 to 10.6%) of observations below and above the simulated 90% prediction interval, respectively. The numerical predictive check after oral administration resulted in 1.09% (95% CI: 0 to 13.1%) and 6.56% (95% CI: 1.09 to 10.9%) of observations below and above the simulated 90% prediction interval, respectively.

Two simulated dosing regimens of 1,000 mg intramuscular and oral syrup every 6 hours (Figure 5.1.1.4) achieved mean steady state therapeutic levels of paracetamol (>10mg/L). However, lower peak concentrations were seen after the first dose, which might suggest the need of a 1,500 mg loading dose in order to achieve a rapid onset of maximum therapeutic efficacy (Figure 5.1.1.4). A fixed maintenance dose of 600 mg intramuscular or oral syrup every 4 hours also showed adequate steady state concentrations but this regimen failed to reach the therapeutic levels during the first 6 hours after administration (Figure 5.1.1.4).

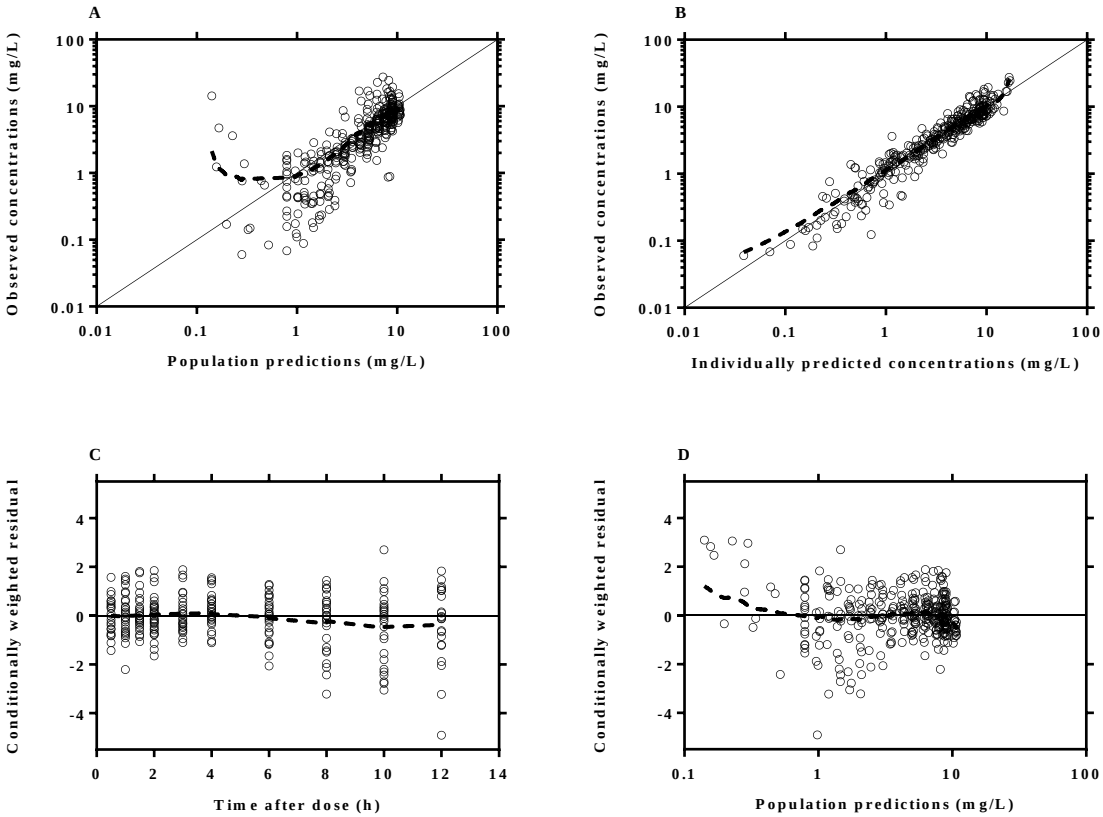


Figure 5.1.1.2. Goodness-of-fit diagnostics of the final population pharmacokinetic model for paracetamol in patients with falciparum malaria. Observations are represented by the black circles, the solid black lines represents the line of identity or zero line, and the local polynomial regression fitting for all observations is represented by the dashed black line. The observed paracetamol concentrations, population predictions, and individual predictions were transformed into their logarithms (base 10).

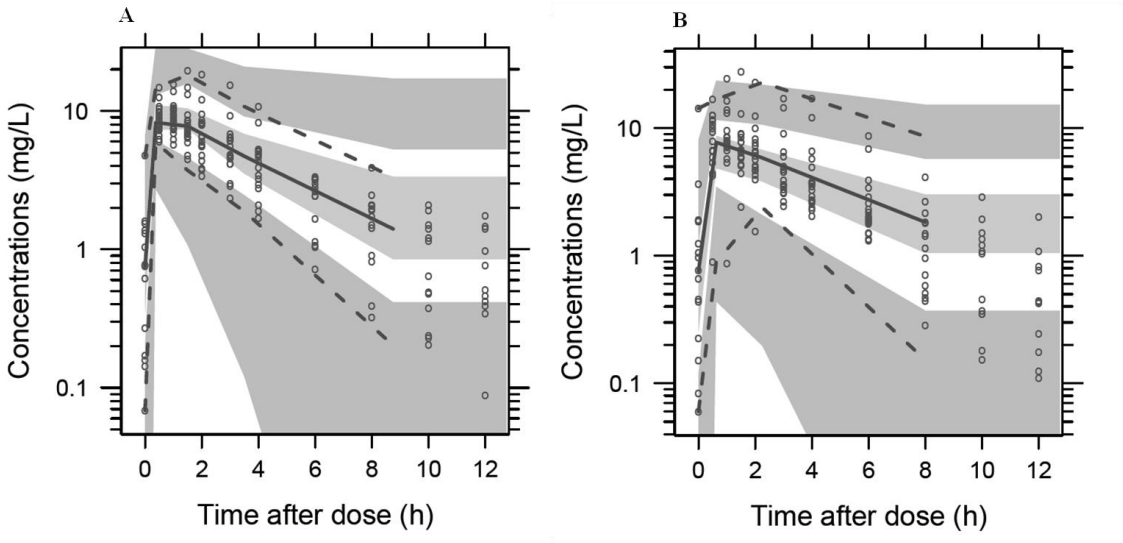


Figure 5.1.1.3. Visual predictive check of the final population pharmacokinetic model for paracetamol in patients with falciparum malaria stratified by route of drug administration, (A) intramuscular administration and (B) oral syrup administration. Open circles, observed data points; solid lines, 5th, 50th, and 95th percentiles of the observed data; shaded area, 95% confidence intervals of the simulated 5th, 50th, and 95th percentiles (n=2,000).

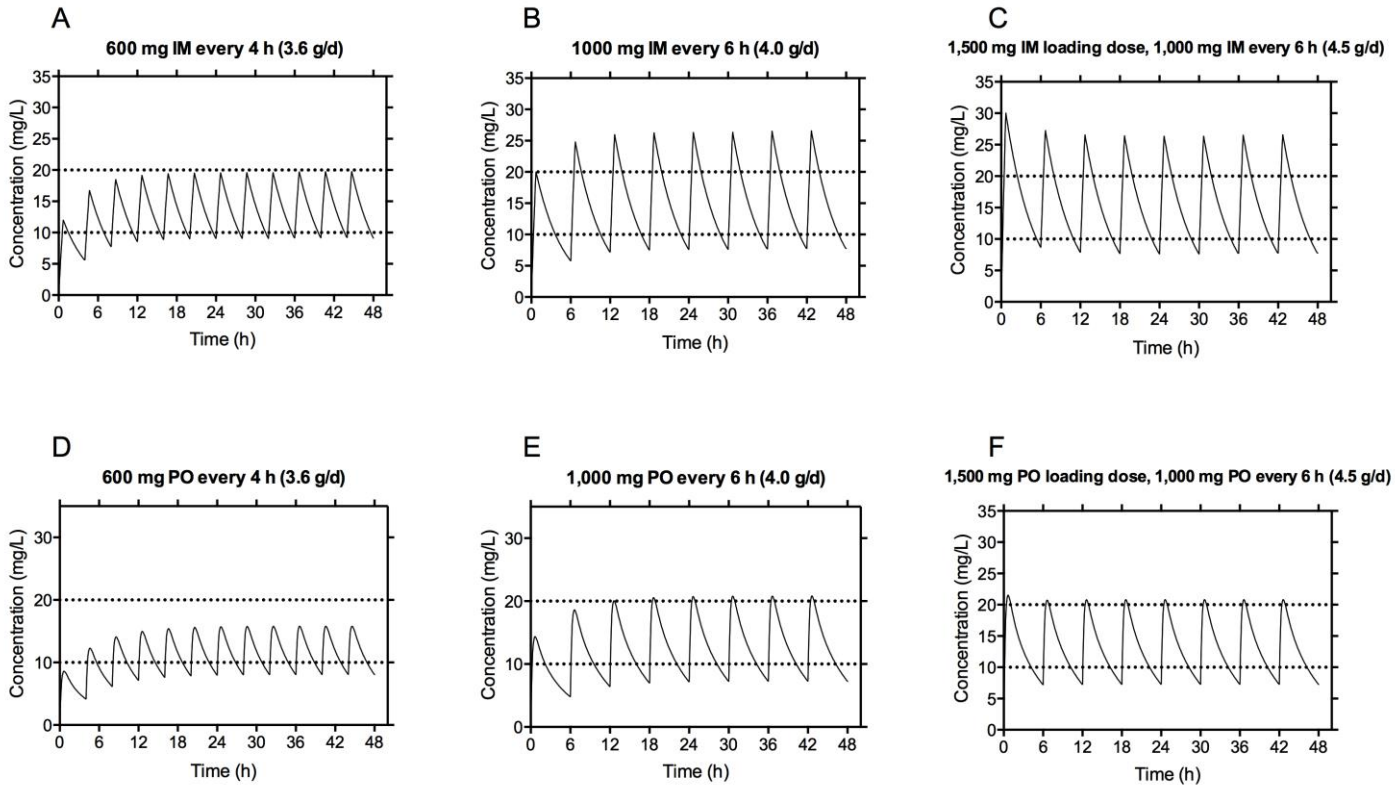


Figure 5.1.1.4. Simulations from the final population pharmacokinetic model for paracetamol. The lower and upper dashed lines represent the therapeutic plasma concentration range of paracetamol for fever control (10 – 20 mg/L).
Upper panel: population mean plasma concentration-time profiles after intramuscular (IM) administration of (A) study dosing regimen; 600 mg every 4 hours, (B) normal dosing regimen; 1,000 mg every 6 hours and (C) modified dosing regimen; 1,500 mg loading dose followed by 1,000 mg every 6 hours.
Lower panel: population mean plasma concentration-time profiles after oral (PO) syrup administration of (D) study dosing regimen; 600 mg every 4 hours, (E) normal dosing regimen; 1,000 mg every 6 hours and (F) modified dosing regimen; 1,500 loading followed by 1,000 mg every 6 hours.

Pharmacodynamics

The linear regression analysis showed no statistically significant relationship between C_{MAX} and temperature reduction 2 hours post-dose (P = 0.65). There was a trend of a longer parasite half-life with higher C_{MAX} but this was not statistically significant (P =0.20). When stratified by administration route, the relationship between the intramuscular C_{MAX} and parasite clearance half-life was more evident (P = 0.030). However, the pharmacodynamic model assessing the relationship of paracetamol concentrations and the relative change in parasite clearance half-life, showed no statistical improvement of adding paracetamol concentrations as a direct-effect on to the estimated parasite clearance rate (ΔOFV = −2.85). Adding a hypothetical effect-compartment to account for a delayed effect of paracetamol on parasite clearance showed no improvement of the model (ΔOFV = -0.06).

5.1.1.4 Discussion

Although paracetamol has been used clinically for over 100 years, there is a paucity of literature describing its pharmacokinetic properties after intramuscular administration. Since patients with malaria are often vomiting and in cerebral malaria are unconscious, the intramuscular route provides an alternative administration option in the absence of severe thrombocytopenia. The disposition of paracetamol was best described by a two-compartment disposition model, which is consistent with previous pharmacokinetic reports [480]. Since one-compartment structural models of oral paracetamol have been reported [481], separate analyses of structural models for intramuscular and oral administration were performed. The results showed

that the two-compartment disposition of the final pharmacokinetic model was driven by the intramuscular data. A one-compartment disposition model was adequate for oral syrup administration demonstrating that the rapid distribution phase after intramuscular administration was fully suppressed by the oral absorption phase. As expected, the absorption of paracetamol administered intramuscularly and orally was best described by zero-order and first-order absorption, respectively. A more flexible transit absorption model did not result in a statistical improvement when fitting the absorption data after oral administration. This may be a consequence of a small amount of data in the absorption phase. This resulted in C_{MAX} at approximately 40 minutes after both intramuscular and oral syrup administration, which is similar to previous reports [285, 482]. However, few data points in the absorption phase might bias these estimates and they should be interpreted with caution. The bioavailability of intramuscular paracetamol was fixed at 100% in order to estimate the relative bioavailability of oral administration, resulting in a relative oral syrup bioavailability of 84.4% compared to intramuscular administration. This is in agreement with a previously reported absolute oral bioavailability of paracetamol syrup of 87% [483]. This bioavailability differs compared to oral tablets of paracetamol (i.e. 63%-90%), which may be explained by the increased absorption of the syrup formulation [484, 485]. The C_{MAX} of paracetamol after intramuscular and oral administration (600 mg) was 11.4 and 8.52 mg/L, respectively. The lower C_{MAX} of oral paracetamol can be explained by incomplete absorption and the first-pass metabolism that occurs during absorption before paracetamol enters the systemic circulation [284].

The population pharmacokinetic model of paracetamol showed large inter-individual variability in most pharmacokinetic parameters. This was likely due to the small sample size and limited data for each route of administration. However, the visual predictive check of the final pharmacokinetic model resulted in adequate predictive performance.

Based on the simulated mean plasma concentration of paracetamol, intramuscular and oral syrup administration of 1000 mg every 6 hours resulted in therapeutic mean concentration-time profiles. However, dosing simulations of a 1,500 mg loading dose followed by a maintenance dose of 1,000 mg every 6 hours resulted in more favorable paracetamol plasma concentration-time profiles, reaching maximum therapeutic concentrations rapidly after the first dose. This suggests that a loading dose might be needed for a rapid onset of maximum therapeutic effect. Dosing simulations of intramuscular and oral syrup paracetamol administered at a dose of 600 mg every 4 hours showed that this regimen reached therapeutic steady state concentrations but with a delayed onset of action. The simulated total daily dose of paracetamol administered when using the loading dose regimens was 4.5 g per day, which is above the recommended maximum daily dose of 4 g, thus there is some concern for hepatotoxicity with this dosing regimen.

Administration of tablets and suppositories may not reach therapeutic concentrations due to an even lower bioavailability compared to orally administered paracetamol syrup [285, 286, 474, 475, 486]. Thus, these recommended dosing regimens may not be adequate for tablets and suppositories to reach similar steady state concentrations compared to paracetamol syrup. Therefore, the minimal pharmacodynamic effect of paracetamol on fever clearance reported in UM studies that administer suppositories or tablets, or do not directly observe therapy may be due to sub-therapeutic paracetamol levels [324, 325].

High doses of paracetamol have been studied both as single loading doses and as multiple dosing regimens that exceed 4 g per day. A single 2 g dose of intravenous (IV) paracetamol for post-operative pain has been shown to be more efficacious and safe in patients undergoing dental surgery compared to a 1 g IV dose [487]. A multiple dose regimen of 2 g IV paracetamol followed by 1 g IV every 6 hours (total, 5 g in 24 hours) in healthy subjects showed no drug accumulation during the regimen and no hepatotoxicity at 72 hours after the first dose [488]. The mean C_{MAX} measured 15 minutes after the 2 g IV infusion was 67.9 $\pm$ 21.8 μg/ml, which is below the toxic range. Multiple-dose regimens of 6 mg per day for three days (1g PO 4-hourly) studied in stroke patients showed a temperature lowering effect and no hepatotoxicity compared to placebo [489, 490]. In the current study, a simulated 1,500 mg loading dose followed by 1,000 mg every 6 hours rapidly achieved therapeutic concentration-time profiles of

paracetamol administered by either route. Although the total daily dose of this regimen (4.5 g per day) exceeds the recommended maximum adult daily dose, the simulated maximum plasma concentrations were well below the potential hepatotoxic threshold concentration of 150 mg/L measured 4 hours post-dosage [491]. Also, the bioavailability of the loading dose paracetamol regimen administered by intramuscular and oral syrup routes is expected to be lower than the intravenous (5 g per day) regimen used in other studies [488]. Further pharmacokinetic studies including clinical safety and efficacy outcomes of this regimen would be required to confirm the applicability of loading doses in this population.

It is important to stress that this study reflects the pharmacokinetics of paracetamol in Thai adults. Pharmacogenetic studies suggest that polymorphisms may be contribute to the altered paracetamol metabolism in children (lower CL and higher AUC) and different ethnicities [492]. Upregulation [493] and polymorphisms [494, 495] of the UDP-glucuronosyl transferases may contribute to increased glucuronidation in older children and adults. The decreased expression of CYP2E1, responsible for oxidative metabolism producing the hepatotoxic N-acetyl-*p*-benzoquinoneimine (NAPQ1) metabolite, in infants [496] and African-Americans [497] may result is a decreased risk of hepatotoxicity in these populations. Similarly, the CYP2E1*2 polymorphism suggested to decrease protein expression and activity may be protective in Chinese populations [498]. A study of Thai adults with β-thalassemia/HbE found that paracetamol elimination half-life

was shorter, and its CL faster with higher AUC of glucuronide and sulphate conjugates compared to healthy controls [499]. The authors suggest that UDP-glucuronidation transferase expression may be upregulated in thalassemic patients. However, the thalassaemic patients in this study were significantly older than the healthy controls.

Febrile temperatures have been shown to accelerate and increase cytoadherence of parasitized erythrocytes *in vitro* [331]. In a study of African children receiving regularly dosed rectal paracetamol, it was suggested that those receiving paracetamol had a prolonged parasite clearance time compared to patients treated with mechanical antipyresis [324]. One hypothesis is that paracetamol reduces fever, which may then result in less cytoadherence, sequestration and thus increase circulating peripheral blood parasitaemia. This effect could be interpreted as a prolonged parasite clearance time. The pharmacodynamic model in this study showed that higher paracetamol concentrations resulted in an insignificant temperature reduction, and there was a trend to prolongation of parasite clearance rate. The lack of a pharmacodynamic effect observed in this study is likely due to the single daily low dose of paracetamol administered and the small sample size.

The small sample size and limited number of pharmacokinetic samples are limitations of the study. Related to the small sample size, the model showed large inter-individual variability, which should thus be interpreted with

caution. Haemoglobinopathies and paracetamol metabolites and were not assessed in this study. Therefore, we are unable to comment on the contribution of host genetic factors that may have contributed to the final model.

5.1.1.5 Conclusion

In conclusion, the population pharmacokinetic properties of paracetamol was characterised after both intramuscular and oral syrup administration, and resulted in an oral bioavailability of 84.4% relative to intramuscular administration in patients with uncomplicated falciparum malaria. Modeling and simulation showed that paracetamol administered by intramuscular or oral syrup routes at the conventional repeated interval dosing regimens predicted therapeutic steady state concentration-time profiles. Maximum therapeutic steady state concentrations were reached more rapidly by adding a loading dose to the standard regimen. However, the safety and efficacy of these regimens need to be verified in this population.

5.1.2 Pharmacokinetics of oral paracetamol in severe and moderately severe malaria

5.1.2.2 Study aim

The aim of this study was to assess the population pharmacokinetic properties of multiple oral doses of paracetamol in adult Bangladeshi patients with severe and moderately severe *P. falciparum* malaria.

5.1.2.3 Study design

Pharmacokinetic data from adult Bangladeshi patients with severe and moderately severe malaria collected as part of a multicentre, open-label, randomised comparison of the effect of paracetamol and on paracetamol on kidney function (Chapter 6) were used for the analysis. A detailed description of the trial is outlined in Chapter 2. Briefly, febrile (>38°C on admission or fever during preceding 24 hours) patients over 12 years with slide confirmed severe or moderately severe malaria were included provided written informed consent was obtained. Criteria for SM are listed in section 2.3.2.1. Moderately severe malaria was defined as a requirement for inpatient parenteral therapy due to inability to reliably tolerate oral antimalarials, but without severity criteria.

Consecutive eligible patients were randomly assigned to supervised treatment with paracetamol or no paracetamol. Paracetamol tablets (500mg tablets, Bristol Laboratories Ltd., Hertfordshire, UK) were given to adults > 50kg at a dose of 1,000 mg on enrolment then 6-hourly for 72 hours if oral medication could be taken reliably. Patients < 50kg were given paracetamol

12.5-15 mg/kg/dose 6-hourly for 72 hours. Paracetamol syrup (250 mg/5 ml, Rosemont Pharmaceuticals Ltd., Leeds, UK) was given via NG tube to patients who were unconscious or unable to take oral medication. If patients vomited within one hour of paracetamol administration, the dose was repeated. Patients in the control arm who developed a fever > 40°C were given ibuprofen in the absence of renal impairment, dehydration and dengue infection. If ibuprofen was contraindicated, then febrile control patients were given paracetamol as per local practice (500 mg tablet or suppository).

Patients were treated with parenteral artesunate (Guilin Pharmaceutical Factory, China) at a dose of 2.4 mg/kg on admission, 12 hours, 24 hours and daily thereafter, followed by a standard course of artemether/lumefantrine (Coartem, Novartis, Switzerland) as soon as oral medication was tolerated. Supportive treatment was according to WHO malaria treatment guidelines [101].

Plasma samples for paracetamol concentration analysis were collected prior to each dose plus dense sampling after the first (0 hours) and last (72 hours) dose with the following sampling schedule: 0, 0.5, 1.5, 2.5, 4, 6, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66, 72, 72.5, 73.5, 74.5, 76, 78, and 84 hours in paracetamol arm patients. Control arm patients had samples for paracetamol analysis collected every 6 hours for 72 hours to assess unplanned paracetamol intake. Paracetamol concentrations were measured from frozen EDTA plasma samples using a liquid chromatography-tandem

mass spectrometry method as described in Chapter 2.10.1. Population pharmacokinetic analysis was performed as described in Chapter 2.10.2.

5.1.2.4 Results

The analysis was conducted using data from adults with severe and moderately severe malaria (n=28) where dense venous sampling was incorporated into a randomised control trial of paracetamol (Table 5.1.2.1). Of the 31 patients randomised to the paracetamol arm, three patients did not receive paracetamol after randomisation due to haemodynamic instability precluding NG tube insertion and death within 30 hours of enrolment. 21/28 (43%) patients received oral paracetamol tablets, 3/28 (11%) patients initially received paracetamol syrup by NG tube then switched to oral tablets to complete the 72 hours of paracetamol, and 4/28 (14%) patients received all paracetamol doses as syrup via NG tube. 26/28 (93%) patients completed the 6-hourly dosing for 72 hours as one patient died after the first dose and a second patient withdrew from the study after nine doses of oral tablets. Additional doses of paracetamol were administered to 7/62 patients (median, range: 2, 1-3 tablets) within the 72 hour period; 3 patients in the paracetamol arm required a repeat dose due to vomiting within one hour, and 4 patients in the control arm required paracetamol for fever > 40°C. Two patients in the control arm received a diclofenac suppository for fever > 40°C without any elevation in creatinine. Concomitant medications administered during hospital admission included domperidone (n=4), proton pump inhibitors (n=18), ceftriaxone (n=26) or a fluoroquinolone (n=3).

Table 5.1.2.1 Demographic and laboratory characteristics of study population

	All data	PO	NG
Study size	28	21 (75)	7 (25)
Number of PK samples	571	490 (86)	81 (14)
Number of PK samples per patient	22 (5-26)*	22 (9-26)*	11 (6-24)*
Age (years)	27 (20-28)	26 (20-35)	30 (20-65)
Gender, male[c]	18 (64)	12 (57)	6 (86)
Body weight (kg)	50 (47-54)	50 (46-53)	50 (50-54)
Temperature (°C)	39 (37-40)	39 (37-40)	39 (38-39)
Severe malaria[c]	21 (75)	14 (67)	7 (100)
Moderately severe malaria[c]	7 (25)	7 (33)	0 (0)
Heart rate (beats/min)	105 (95-116)	106 (94-116)	103 (96-109)
Parasitaemia (parasites/µl)[b]	49,598 (21,148-116,319)	74,474 (27,178-204,071)	14,650 (2,886-74,358)
*Pf*HRP2 (mg/ml)	1,052 (128-4,486)	467 (93-2,404)	3,835 (1,356-12,478)
Haematocrit (%)	34 (24-41)	38 (25-42)	23 (16-35)
Cell-free haemoglobin (ng/ml)	40,537 (17,077-98,528)	37,241 (16,839-71,708)	94,086 (19,490-176,201)
Creatinine (µmol/L)	84 (72-132)	82 (71-127)	96 (79-268)
Blood urea nitrogen (mg/dL)	22 (12-32)	18 (12-29)	23 (20-122)
Alanine aminotransferase (IU/L)	20 (13-27)	20 (14-26)	15 (7-28)
Aspartate aminotransferase (IU/L)	42 (27-79)	40 (25-77)	44 (42-84)
Total bilirubin (mg/dL)	1.1 (0.9-1.7)	1.1 (0.9-1.6)	1.6 (0.7-2.4)
Indirect bilirubin (mg/dL)	0.6 (0.4-1.2)	0.6 (0.5-1.0)	0.6 (0.3-1.6)
Received haemodialysis[c]	2 (7)	1 (5)	1 (14)
Study time to haemodialysis (hours)	7 (6-8)	6 (6-6)	8 (8-8)

Values are presented as median (interquartile range) unless otherwise specified. [b]geometric mean (95% confidence intervals), [c]number (%). *(range). *Abbreviations*: *Pf*HRP2, *Plasmodium falciparum* histidine rich protein 2.

Pharmacokinetics

The structural model was best described by a one-compartment disposition model. The addition of inter-individual variability on bioavailability improved the model fit (ΔOFV = -10.7). A transit absorption model with fixed number of transit compartments (n = 1) optimally described the absorption phase of paracetamol compared to other absorption models (ΔOFV = -11.6). The absorption rate constant (k_a) was set to equal the transit-compartment rate constant (k_{tr}) without alteration of model fit compared to estimating k_a and k_{tr} separately (ΔOFV = 1.35).

Body weight implemented as an allometric covariate on all clearance and distribution parameters did not improve the model fit (ΔOFV = -0.085) and was excluded from the model. The stepwise covariate evaluation included age, gender, ALT, AST, INR, bilirubin (total, indirect and direct), creatinine, creatinine clearance, haemoglobin, parasitaemia, *Pf*HRP2, severity, haemodialysis and route of administration. The route of administration significantly affected the bioavailability of the drug in a linear relationship and was also retained in the more parsimonious backward elimination analysis. Paracetamol administered via NG tube resulted in a 67.6% higher relative bioavailability compared to oral paracetamol administration (P value < 0.001). Analysis stratified by severity and by route of administration was performed, due to a high correlation between severity and route of administration, to assess this result further. Subgroup analysis by severity showed that route of administration was a significant covariate on bioavailability (relative increase of 58.5% by NG tube) in the severe group

(n=8 oral, n=7 NG). Subgroup analysis of the moderately severe group was not possible since only one patient received paracetamol by NG route. Subgroup analysis by route of administration was only possible in the oral administration group since only one patient in the moderately severe group received paracetamol by NG route. Analysis of the patients administered oral paracetamol (n=8 severe, n=17 moderately severe) did not show disease severity as a significant covariate. These subgroup analyses support that route of administration had an impact on bioavailability as opposed to disease severity.

Goodness-of-fit diagnostics of the final pharmacokinetic model showed accurate and precise predictions of paracetamol concentration (Figure 5.1.2.1). A total of 27 (4.5%) concentrations were below the level of detection and omitted from the pharmacokinetic analysis without affecting the model. The visual predictive check (n=2000) showed acceptable predictive power of the model (Figure 5.1.2.2). The numerical predictive check resulted in 5.25% (95% CI: 2.28-8.76%) and 1.75% (95% CI: 2.28-8.58%) of paracetamol concentrations below and above the simulated 90% prediction interval, respectively. Eta shrinkage was less than 25% except for inter-individual variability of clearance and volume of distribution (45.6% and 31.9%, respectively) and epsilon shrinkage was 6.9%. Parameter estimates from the final paracetamol pharmacokinetic model are shown in Tables 5.1.2.2 and 5.1.2.3.

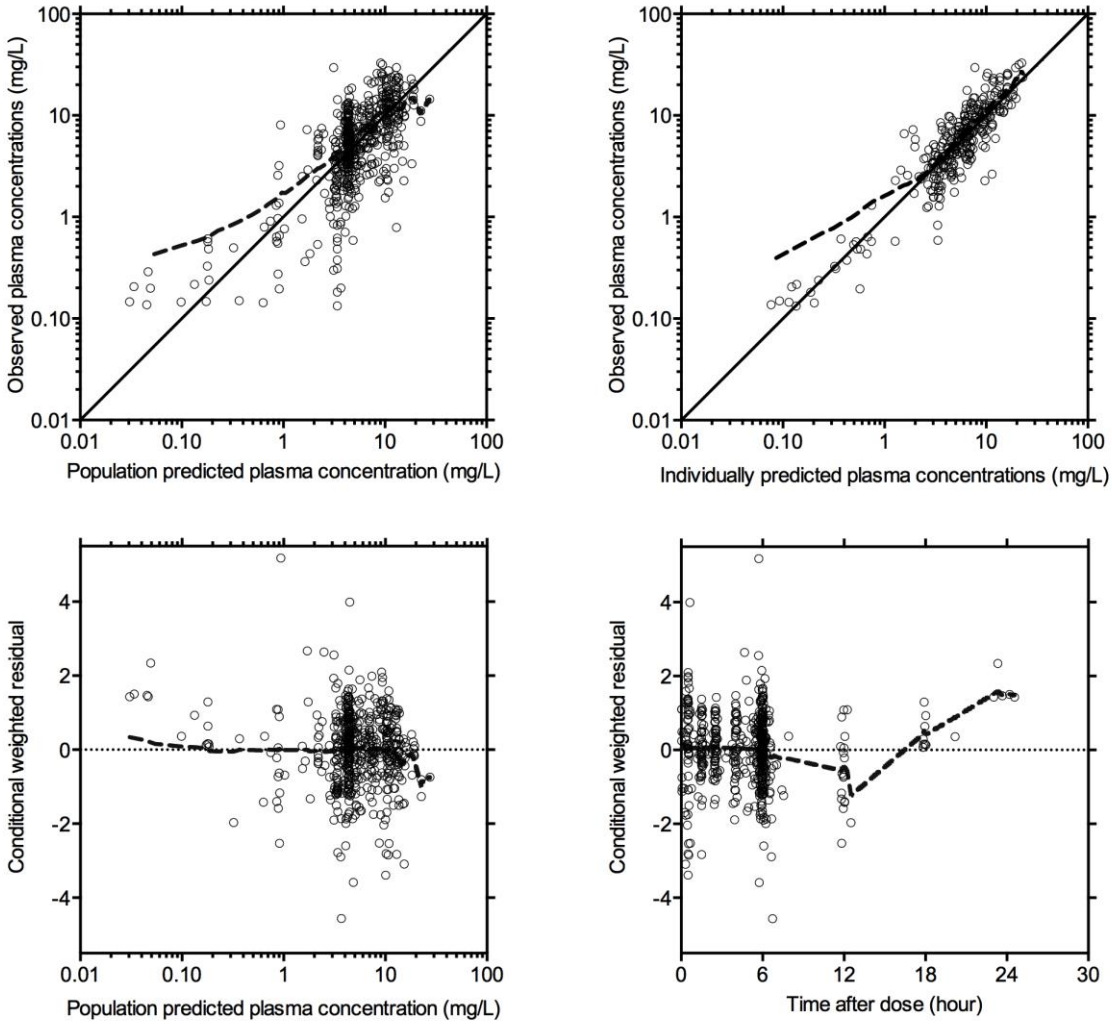


Figure 5.1.2.1. Goodness-of-fit diagnostics of final paracetamol population pharmacokinetic model. Observations are represented by the black circles; solid black line represents the line of identity (zero line), dashed black line represents the local polynomial regression. Observed plasma paracetamol concentrations, population predictions, and individual predictions were log transformed (base 10).

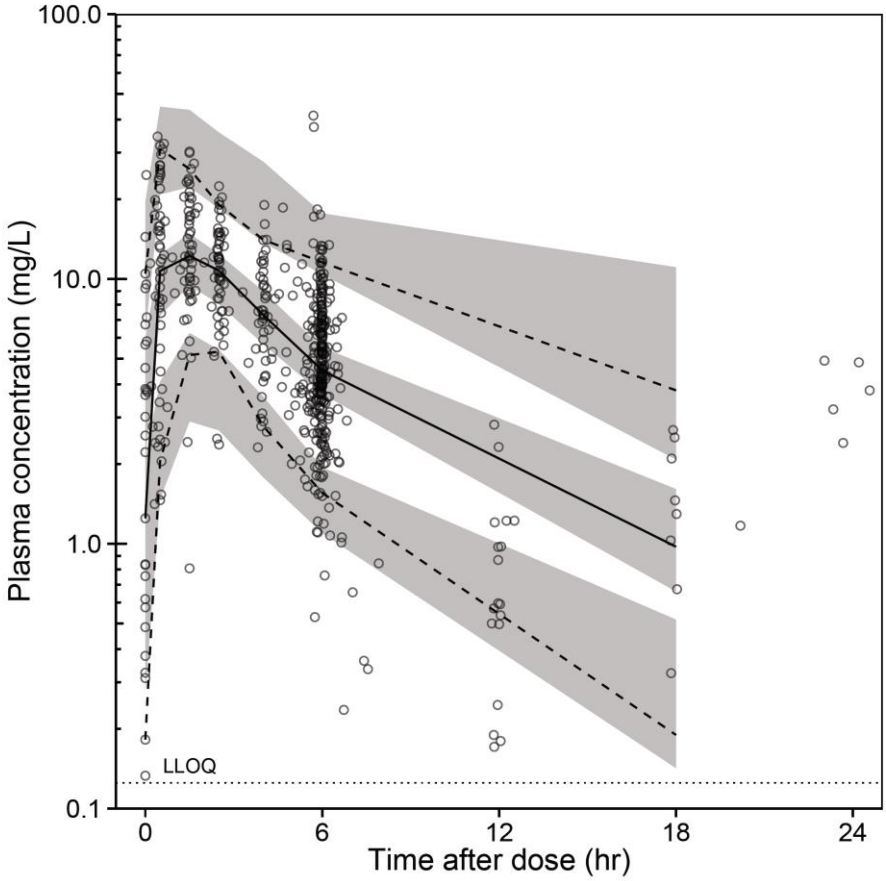


Figure 5.1.2.2 Prediction-corrected visual predictive check of the final paracetamol population pharmacokinetic model in adult patients with severe and moderately severe falciparum malaria. Open circles represent observed data points; solid and dashed lines represent the 5th, 50th, and 95th percentiles of the observed data; shaded areas represent the 95% confidence intervals of the simulated 5th, 50th and 95th percentiles (n=2,000).

Table 5.1.2.2 Parameter estimates of the final population pharmacokinetic model of paracetamol in patients with severe and moderately severe falciparum malaria

Parameter	Population estimates[a] (%RSE[b])	95%CI[b]	IIV ;%CV)[a] (%RSE)[b]	95%CI[b]
F	1 (*fixed*)	-	33.1 (31.8%)	20.0-40.2
CL/F (L/h)	18.5 (7.8%)	15.5-21.3	12.5 (64.2%)	4.1-22.7
V/F (L)	69.5 (9.7%)	56.6-83.6	18.8 (57.8%)	3.7-25.9
MTT (h)	0.617 (18.2%)	0.444-0.883	104.2 (52.9%)	27.9-195.9
Effect of admin route on F	0.676 (21.9%)	0.317-0.874	-	-
σ	0.509 (6.8%)	0.446-0.577	-	-

[a] Population mean values and inter-individual variability (IIV) estimated by NONMEM. The coefficient of variation (%CV) for IIV was calculated as $100 \times \exp(\text{estimate}) - 1$

[b] The relative standard error (%RSE) was calculated as $100 \times (\frac{\text{SD}}{\text{Mean value}})$ from the non-parametric bootstrap results (n=1,000). The 95% confidence interval (95% CI) is presented as the 2.5 to 97.5 percentiles of the bootstrap estimates.

F: relative bioavailability, MTT: mean transit absorption time, CL/F: apparent oral elimination clearance, V/F: apparent volume of distribution of the central compartment, σ: variance of the residual variability.

Table 5.1.2.3. Secondary parameters of paracetamol

Secondary parameters	Median (Range)
C_{MAX} (mg/L)	13.5 (4.67-24.5)
T_{MAX} (h)	0.50 (0.028-1.94)
$t_{1/2}$ (h)	2.61 (1.79-3.54)
AUC_{0-24} (mg*h/L)	209 (80.2-548)

C_{MAX}, maximum concentration; T_{MAX}, time to reach maximum concentration; $t_{1/2}$, terminal half-life; AUC_{0-24}, area under the concentration-time curve from time zero to 24 hours.

Dosing simulations showed that SM patients receiving paracetamol syrup via NG tube (1,000 mg every 6 hours) achieved a steady state concentration (15.1 mg/L) within the therapeutic range (10-20 mg/L) (Figure 5.1.2.3). Simulations of oral paracetamol in severe and moderately severe malaria patients showed that paracetamol tablets administered at the maximum recommended dose (1,000 mg every 6 hours) resulted in steady state level (9.01 mg/L) below the therapeutic range (Figure 5.1.2.4). A loading dose of oral 1,500 mg followed by 1,000 mg *per os* every 6 hours did not result in a therapeutic steady state concentration, however regular dosing of 1,500 mg *per os* every 6 hours and 1,000 mg every 4 hours did achieve steady state concentration of 13.5 mg/L (Figure 5.1.2.4).

5.1.2.5 Discussion

Paracetamol is extensively used as an antipyretic for the management of fever in severe malaria, however the pharmacokinetics have not been described in this population. In uncomplicated falciparum malaria, the pharmacokinetics of oral paracetamol are not altered [284], however it was shown in Chapter 5.1.1 that paracetamol suspension had a bioavailability of 84.4% relative to intramuscular administration. In critically ill non-malaria patients [479] and experimental SM [287], the pharmacokinetics of paracetamol show increased clearance and reduced metabolism, respectively.

In this study the pharmacokinetics of paracetamol administered to severe malaria patients as oral tablets or syrup via nasogastric tube was assessed

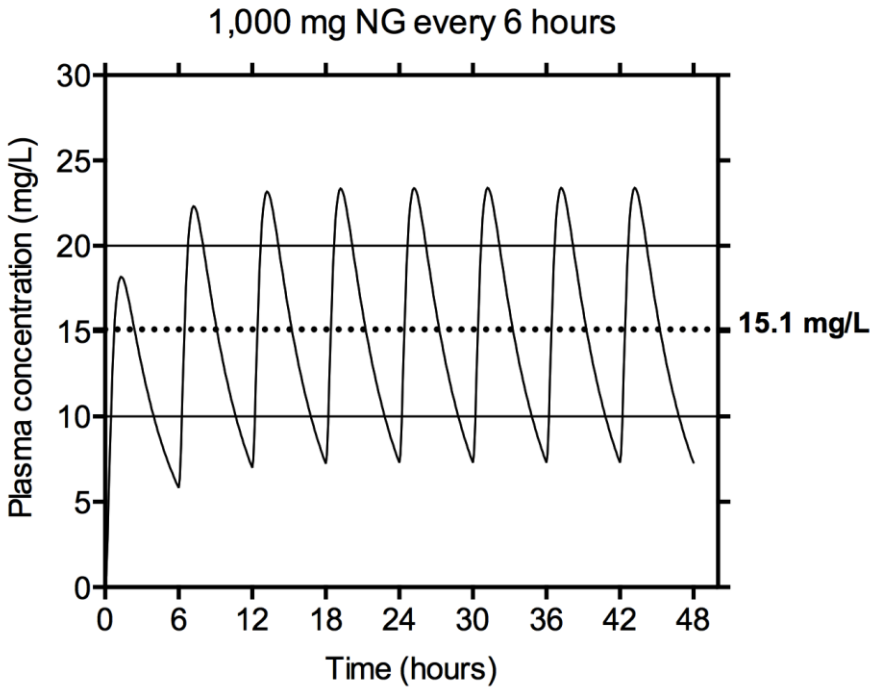


Figure 5.1.2.3. Simulation from the final population pharmacokinetic model for paracetamol administered via nasogastric tube. Solid lines represent the upper and lower therapeutic plasma concentration range of paracetamol for fever control (10 – 20 mg/L). Dashed line represents the steady state plasma paracetamol concentration with corresponding numeric value of steady state achieved. *Abbreviation*: NG, nasogastric.

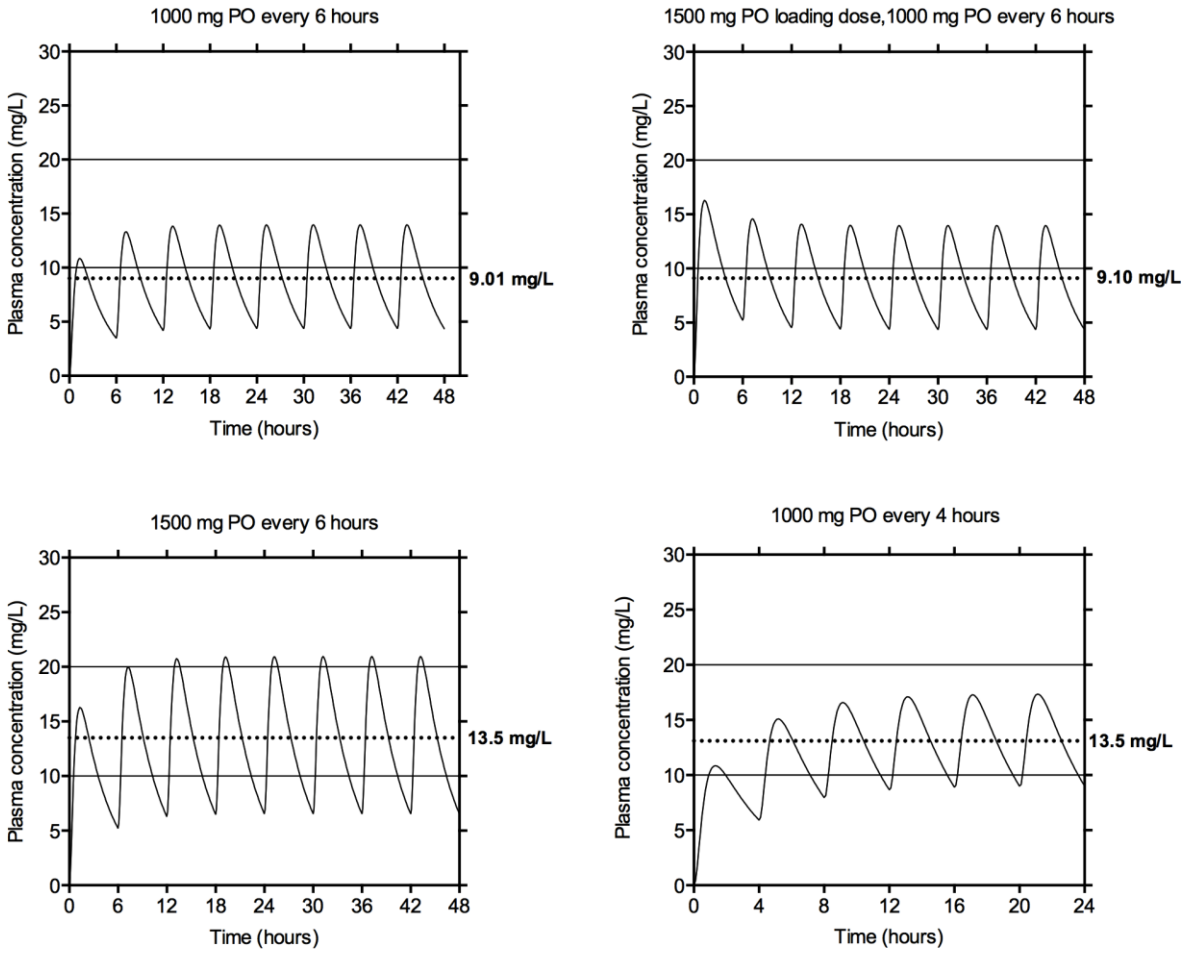


Figure 5.1.2.4. Simulations from the final population pharmacokinetic model for oral paracetamol tablets. Solid lines represent the upper and lower therapeutic plasma concentration range of paracetamol for fever control (10 – 20 mg/L). Dashed line represents the steady state plasma paracetamol concentration with corresponding numeric value of steady state achieved. *Abbreviation*: PO, *per os*.

since rectal paracetamol is incompletely and erratically absorbed [474, 475, 500], thrombocytopenia often precludes intramuscular injection, and intravenous formulations are not widely available. The pharmacokinetic analysis showed that oral paracetamol was best described by a one-compartment disposition model. This result differs from the two-compartment disposition model proposed by other studies [480, 501] likely because of the larger number of samples analysed. The one-compartment model is in agreement with previous reports [480, 502, 503] and as reported for oral suspension in uncomplicated falciparum malaria (Chapter 5.1.1). The absorption phase was best described by a more complex one-compartment transit first-order absorption model, which has previously been described in patients without malaria [503]. The high number of paracetamol concentrations analysed during the absorption phase provided more information to describe the absorption with this more complex model. However, the median T_{MAX} was 30 minutes, which is comparable to previous studies [285, 482].

The relative bioavailability of paracetamol was 67.6% higher when given by nasogastric tube compared to oral administration. Since patients required administration by nasogastric tube had more severe disease, additional subgroup analysis stratified by severity was performed. The results suggested that the higher relative bioavailability of nasogastric administration is likely due to the syrup formulation of paracetamol as opposed to disease severity. Sorbitol included in the formulation used in the current study may have contributed, as this excipient has been shown to

increase absorption [504]. The pre-pyloric nasogastric tube route itself is unlikely to have contributed [505]. While the rate of paracetamol absorption is dependent on the rate of gastric emptying [285], domperidone could potentially increase absorption and thus bioavailability. However, since only one patient receiving nasogastric administration had this drug co-prescribed, this interaction is an unlikely explanation of the increased bioavailability. The effect of proton pump inhibitors on pyloric motility is unclear [506] but difficult to ascertain an impact in this study, as although many patients were prescribed this drug, accurate administration records of this particular drug were not available. The C_{MAX} reached was 13.5 (4.67-24.5) mg/L, which is similar to a study in UM [284]. This is higher than the C_{MAX} achieved after administering 600 mg as intramuscular or suspension formulation shown in Chapter 5.1.1. The larger range seen in this study is likely due to the different drug formulations but all concentrations were far below the toxic threshold.

The volume of distribution, clearance and half-life were similar compared to studies in UM in Chapter 5.1.1 [284] (Chapter 5.1.1), suggesting that the metabolism and distribution of paracetamol is not altered by disease severity. Further, these parameters were similar compared to a population pharmacokinetic study of paracetamol in critically ill non-malaria patients [507]. This suggests that the metabolism of paracetamol is not altered in the presence of artemisinin antimalarials despite the shared metabolic pathway of both drugs via glucuronidation involving a common UDP-glucuronosyltranferase isoform [476, 508]. However, the current study did

not assess the metabolic products of paracetamol to definitely support this conclusion.

The antipyretic efficacy of paracetamol in UM has been questioned, however these studies do not include drug concentrations to show whether therapeutic levels were attained [324, 325]. Dosing simulations revealed that the maximum recommended daily dose (1,000 mg 6 hourly) did not reach therapeutic plasma concentrations at steady state when administered as oral tablets. Thus, sub-therapeutic concentrations may explain the borderline antipyretic effect of paracetamol observed in previous studies. However, simulations of oral paracetamol dosed at 1,000 mg every 4 hours or 1,500mg every 6 hours did result in therapeutic antipyretic concentrations (10-20 µl/ml) [328, 509]. These doses are above the maximum dose of 4,000 mg recommended in the label. In addition, although liver failure is very rare in SM, liver involvement with increased plasma transaminases is very common, thus extra caution is warranted. However, dosing of paracetamol in this high range, including a loading dose of intravenous 2 g [487, 488], have been reported and used in stroke patients without inducing liver toxicity [489, 490, 510]. So although the simulations predicts paracetamol concentrations within the safe range, actual use of these higher doses in patients with SM will have to be assessed carefully before further recommendations can be made. Notably, the simulation of 1 g 6-hourly by nasogastric paracetamol suspension in this study is highly comparable to the same dosing simulation shown in Chapter 5.1.1 (Figure 5.1.1.4), both of which achieved therapeutic steady state concentration of paracetamol and did not exceed the

recommended maximum dose in adults. This suggests that the best dosing regimen for non-pregnant adult patients with severe and uncomplicated malaria, which reaches steady state without exceeding the dosage maximum, is 1 g of paracetamol suspension administered every 6 hours. Severe and moderately severe patients receiving this regimen had mild elevation in aminotransferases at 72 hours but no significant hepatotoxicity was observed (detailed description in Chapter 6).

Limitations to this study include the small sample size, which limits the power to detect covariates influencing the pharmacokinetics and may underestimate variability. However, the dense sampling protocol assists to determine a robust and precise model. An uncomplicated comparator group was not included in this study to firmly conclude that the pharmacokinetic properties of paracetamol are unaltered in severe malaria. However, the pharmacokinetic parameters were similar to previous studies in UM [284] (Chapter 5.1.1) and the subgroup analysis of moderate compared severe malaria patients showed similar values.

5.1.2.6 Conclusions

The population pharmacokinetic properties of paracetamol in severe and moderately severe malaria patients were best described by first-order, one-compartment transit absorption with one distribution compartment and first-order elimination. Nasogastric tube administration resulted in a 67.6% higher relative bioavailability, which is likely due to drug formulation as opposed to disease severity. Drug simulations showed that nasogastric administration of

the maximum recommended daily dose resulted in therapeutic steady state concentrations, whereas oral tablets given at the same dose resulted in sub-therapeutic concentrations. These findings may explain the conflicting reports of the antipyretic efficacy of oral paracetamol in malaria patients. Further clinical studies are required to determine the optimal regular dosing schedule of paracetamol tablets for sustained temperature reduction.

5.2 An *in vitro* study of the potential interaction between paracetamol and artesunate on cultured *P. falciparum* parasites

5.2.1 Background

Adjunctive and preventative therapies targeted to the pathophysiological mechanisms of AKI may reduce the morbidity and mortality associated with this complication of SM. Haemoglobin released from RBCs can cause severe oxidative damage and kidney injury [65, 66, 511] and the evidence presented in Chapter 4.2 suggested this contributes to malaria-associated AKI. Recent studies in a rat model of rhabdomyolysis showed that paracetamol improved renal function, reduced oxidized haemoprotein-associated ferryl (Fe^{4+}) iron and prevented lipid peroxidation [66]. The latter has also been shown in children and adults after surgery involving extra-corporal circulation [66, 335, 336] and in an observational study of septic adults with high haemoprotein concentrations, paracetamol was associated with reduced mortality [337] . A randomised controlled trial was therefore done as part of this thesis to test the hypothesis that paracetamol may decrease kidney dysfunction in patients with severe and moderately severe falciparum malaria by inhibiting haemoglobin-induced oxidative damage.

However, a theoretical concern with this approach was that the reducing properties of paracetamol could inhibit the parasitocidal activities of the artemisinins. Paracetamol is widely used concomitantly with artemisinins for the treatment of fever in severe and uncomplicated malaria [21].

Artemisinins are the most effective anti-malarials available, and are unique structurally by virtue of an endoperoxide bridge that is essential for their activity [318]. The precise mechanism of artemisinin activation and its cellular targets is still debated. Activation by opening the endoperoxide bridge is proposed to be mediated by ferrous iron [512] or ferrous haem [306, 513] inducing reductive scission yielding a reactive carbon centered radical and subsequent activation via a ferryl-oxo intermediate [303-305, 514]. An alternative hypothesis is that ferrous iron facilitates ionic endoperoxide bridge opening to activate artemisinins [515], which may then form an unsaturated hydroperoxide [516]. This could in theory also produce ferryl iron as a by-product, which could contribute to the parasitocidal activity of the drug. The precise details of how the resulting free radicals complex with haem to cause cell damage and parasite death are still being defined and there may be an alternative artemisinin target pathway [517, 518].

In both these scenarios, paracetamol could potentially inhibit (i) artesunate activation, and/or (ii) artesunate action by reducing the ferryl intermediate, thereby preventing oxidative damage. Trolox (vitamin E analog), which similarly reduces haemoprotein-oxidation [66], has been shown to reduce the antimalarial effect of artemisinins *in vitro* [519, 520] and increase the risk of parasitaemia in HIV co-infected women when administered with another ferryl-reductase (ascorbic acid) [521].

The source of ferrous iron and/or ferrous haem responsible for artemisinin activation is not completely defined. Within the intraerythrocytic parasite,

host haemoglobin digestion could begin as early as mid-ring stage (14 to 20 hours post-invasion) when the digestive vacuole is formed and is required for artemisinin activation, which supports an intraparasitic source of ferrous and/or ferrous haem [317, 522]. However, early ring stages are also killed by artemisinins suggesting that another source of ferrous iron/ferrous haem is necessary for earlier drug activation [523]. It has been suggested that artesunate may be activated intravascularly [520], which could result in decreased efficacy of the drug as it is not activated in the target compartment. Alternatively, since paracetamol may inhibit the activation of artesunate it could potentially act synergistically with artesunate by reducing its activation in the intravascular space until it reaches its intracellular target. No studies of the potential interaction between paracetamol and artesunate have been conducted, so as part of this thesis the potential interaction between the two drugs was assessed using an *in vitro* study, on cultured *P. falciparum*–infected erythrocytes. In brief, both laboratory and field parasite isolates from Bangladeshi adult patients with malaria were studied, using standardised trophozoites and schizont stage maturation assays as a way of examining the potential modulation of the effectiveness of artesunate by paracetamol.

5.2.2 Study aim

To investigate the potential modulating effect of paracetamol on the anti-malarial activity of artesunate using *in vitro* drug sensitivity assays, as measured by the 50% inhibition concentration (IC_{50}) from schizont and

trophozoite maturation inhibition, in *P. falciparum* laboratory and clinical isolates.

5.2.3 Study design

Plasmodium falciparum laboratory stain (TM267, Thailand) and seven *P. falciparum* parasite isolates from Bangladeshi patients were cultured as outlined in chapter 2.10.14.1. The drug susceptibility assessment from patient isolates was nested into a larger RCT of paracetamol in severe and moderately severe malaria (Chapter 6). Out of a total of 28 patients enrolled at this site in 2014, only 14 field isolates were assessed in Bangladesh due to logistical reasons and 20 patient samples were cryopreserved as packed RBCs in liquid nitrogen for analysis at the Mahidol Oxford Research Unit Malaria Laboratory in Bangkok, Thailand. Harvesting and drug susceptibility testing in Bangladesh was performed on 7 fresh isolates as the other 7 clinical samples failed to grow, likely due to pre-treatment with anti-malaria prior to enrolment. Drug susceptibility testing was successful in only 4 cryopreserved samples as the remaining 16 either had low parasitaemia, did not grow or haemolysed after thawing. Schizont maturation inhibition and trophozoite maturation inhibition (TMI) assays are described in Chapter 2.10.14.2 to 2.10.14.4.

Statistical analysis

In the trophozoite and schizont maturation assays, the effects were defined as % maturation inhibition, as described in Chapter 2.10.13. The anti-malarial effect of artesunate and paracetamol and the combination of the two drugs, was assessed by calculating the 50% inhibition concentration (IC_{50}).

The IC_{50} was defined as the drug concentration that resulted in 50% inhibition of parasite growth from ring to trophozoite (TMI, 24 hour assay) or ring to schizont stage (WHO, 48 hour assay). To calculate the IC_{50}, the sigmoidal dose response curve was fitted using WinNonlin computer software version 4.1. The median IC_{50} was presented as with interquartile ranges. Differences in anti-malarial activity between IC50s in the different drug combinations were assessed by Wilcoxon matched-pairs signed-rank test.

5.2.4 Results

Quantitation of drug activity

Anti-malarial activity of artesunate and paracetamol were determined *in vitro* in one laboratory strain and 11 out of 28 clinical isolates. 17 clinical field isolates failed to grow in culture, thus drug susceptibility testing was not possible. Prior to drug exposure of clinical isolates, ring-stage parasites (0-16 hours) were predominant in 8/11 isolates. However, 5/11 patients presented with a mixture of large ring-stage parasites and 3/11 patients presented with a mixture of ring and early trophozoite-stage parasites. All isolates developed to trophozoites in the control wells, and 11/11 IC_{50} values were calculated from the TMI tests. One isolate failed to grow in the control and WHO test resulting in 10/11 IC_{50} values calculated from the WHO tests. 3 isolates showed delayed growth with trophozoites appearing after 48 hours.

Inhibition of schizont maturation to 50% (IC_{50}) by artesunate was reached at a median (IQR) concentration of 0.2 (0.1-0.3) ng/ml in the reference strain (TM267). In the clinical strains, complete inhibition of trophozoite and schizont maturation was achieved with 200 ng/ml of artesunate. The median (IQR) schizont maturation inhibition IC_{50} of artesunate was 1.1 (0.4 - 1.9) and median (IQR) trophozoite maturation inhibition IC_{50} of artesunate was 1.2 (0.6 - 4.4). (Figure 5.2.1). Paracetamol alone had minimal effect on parasite growth of the reference strain and minimal schizont and trophozoite inhibition at toxic concentrations of over 10,000 µg/ml in the clinical isolates.

Effects of paracetamol on the anti-malarial activity of artesunate

The potential modifying effect of paracetamol on schizont and trophozoite maturation inhibition was assessed in wells containing artesunate and a fixed paracetamol concentration of 160 µg/ml, in parallel to control wells with artesunate and paracetamol alone. In the reference strain, median (IQR) schizont maturation inhibition IC_{50} of artesunate in combination with paracetamol was 0.2 (0.1 - 0.2) compared to artesunate alone, 0.2 (0.1 - 0.3) ng/ml, P = 0.29. In the clinical isolates, inhibition of schizont maturation IC_{50} of artesunate in combination with paracetamol was 1.2 (0.9 - 2.1) compared to artesunate alone, 1.12 (0.39-1.92), P =0.17. Inhibition of trophozoite maturation IC_{50} of artesunate in combination with paracetamol was 0.6 (0.3 - 3.7) compared to artesunate alone, 1.2 (0.6 - 4.4), P =0.93.

Clinical correlation

No patients in this cohort had a parasite clearance time > 72 hours or a parasite clearance half-life of > 5 hours, indicative of artesunate resistance.

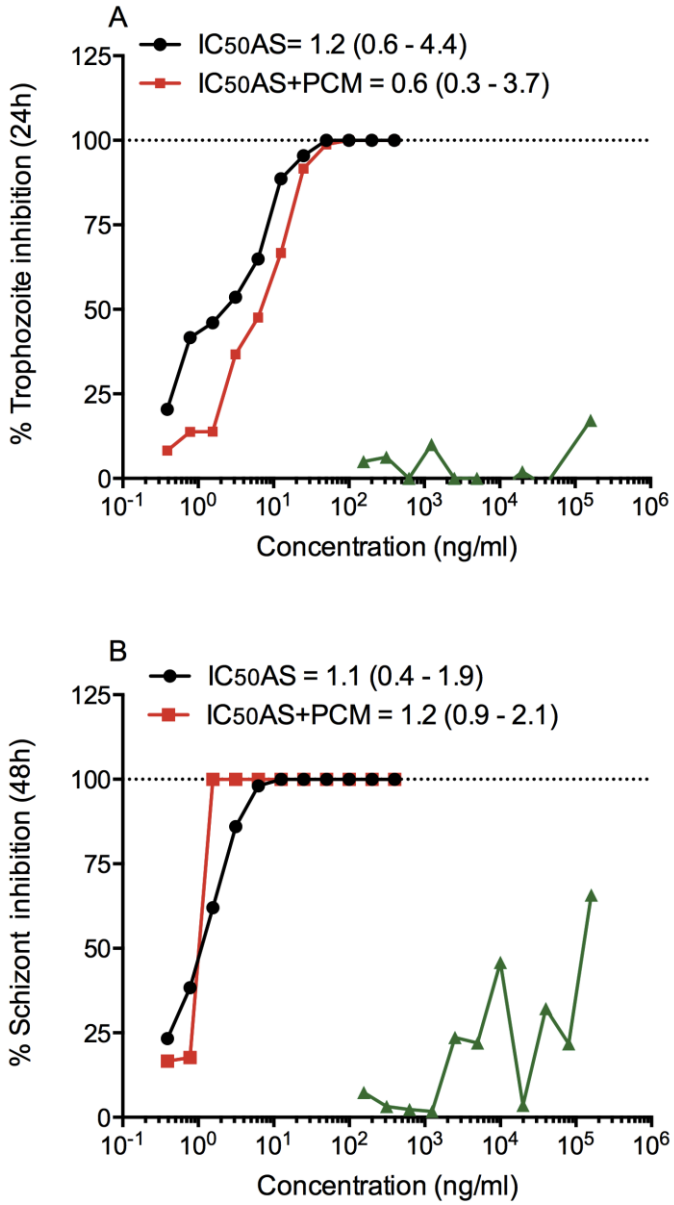


Figure 5.2.1. *In vitro* anti-malarial activity of artesunate, paracetamol and artesunate plus paracetamol in combination. (A) Trophozoite maturation inhibition, and (B) Schizont maturation inhibition, both expressed as a percentage from control, by artesunate (●), paracetamol (▲), and artesunate plus paracetamol (■) were assessed in 11 clinical isolates of *P. falciparum.*

All patients were wildtype at the *Kelch* 13 locus, as previously reported [524]. The parasite clearance time of patients with an artesunate plus paracetamol TMI $IC_{50} \leq 0.6$ were similar to those with TMI $IC_{50} > 0.6$ (39 (36 – 51) versus 48 (48 – 60), $P = 0.26$). The parasite clearance half-life of patients with an artesunate plus paracetamol TMI $IC_{50} \leq 0.6$ were similar to those with TMI $IC_{50} > 0.6$ (2.1 (1.6 – 3.0) versus 2.4 (2.2 – 3.5), $P = 0.31$). There was no correlation between artesunate plus paracetamol TMI IC_{50} and parasite clearance time ($r_s = 0.43$; $P = 0.19$) or parasite clearance half-life ($r_s = 0.54$, $P = 0.11$).

5.2.5 Discussion

This study showed that paracetamol did not inhibit the anti-parasitic effect of artesunate up to concentrations well above those reached in therapeutic use.

Limitations to this study include the small samples size. However if there is an interaction between paracetamol and artesunate, the high concentration of paracetamol used in the assays should have revealed any effect even in this small sample.

The TMI assay was performed specifically to assess whether there might be an interaction during the ring stage of parasite development, when artesunate is potentially activated by an alternative non-host haemoglobin digestion-derived haem/iron source [318]. One hypothesis is that artesunate is activated intravascularly [520], which could deactivate the drug due to its

activation in the wrong compartment [318]. In this situation paracetamol could act synergistically by inhibiting artesunate activation. However, there was no difference in the trophozoite maturation inhibition IC_{50} when paracetamol was tested in combination with artesunate compared to artesunate alone. The absence of any clinical correlation between the TMI IC_{50} also argues against a synergistic effect of paracetamol on artesunate.

The results suggest that paracetamol either: (1) does not possess ferryl-reductase activity in the absence of bound haemoglobin, or (2) is a weak inhibitor of potential artesunate-mediated lipid peroxidation. Alternatively, artesunate either is not activated via a ferryl intermediate, or does not exert its mechanism of action via a ferryl intermediate. However, the lack of obvious paracetamol inhibition on artesunate activity is reassuring, suggesting that the use of paracetamol as a supportive antipyretic treatment in combination with artesunate, or potentially as a renoprotective agent, would not diminish the antimalarial activity of artesunate.

5.2.6 Conclusion

There was no interaction observed between artesunate and paracetamol when assessed by trophozoite and schizont maturation *in vitro* assays. These findings provide reassurance for further testing of paracetamol to assess the effect on kidney function in patients with severe and moderately severe malaria.

6.0 A randomised controlled trial of oral paracetamol to prevent AKI in severe and moderately severe malaria

6.1 Background

A major advance in the treatment of SM has been the introduction of artesunate, which has significantly reduced the risk of death in both adults and children [19, 20, 525]. However, mortality in severe adult malaria remains ~15% despite this best available parasiticidal antimalarial therapy [19]. Kidney dysfunction in SM is an independent predictor of mortality in all ages [12, 19]. RRT is the only supportive treatment modality shown to reduce mortality in SM complicated by AKI from 75% to 26% [22]. Prevention of AKI would be an important step in reducing mortality, particularly since RRT is frequently unavailable in endemic areas.

Evidence suggests that CFH contributes to AKI in several conditions characterised by haemolysis including post-cardiopulmonary bypass [65, 376, 446, 526], paroxysmal nocturnal haemoglobinuria [527, 528], sickle-cell disease [529], and massive transfusion [45]. CFH can cause oxidative kidney damage via haem redox cycling between ferric ($Fe^{3+)}$ and ferryl ($Fe^{4+)}$ states, which results in lipid peroxidation [58]. This mechanism is supported by the observation that patients who developed AKI post-cardiopulmonary bypass were found to have higher peak CFH, and lipid peroxidation markers (F2-isoprostanes and isofurans) compared to controls [65]. In adults with SM, CFH-mediated oxidative stress was associated with kidney dysfunction,

need for haemodialysis and mortality (Chapter 4.2). Research focused on reducing CFH-mediated damage has shown that infusions of haptoglobin, a CFH scavenger, prevented AKI in patients with haemolytic conditions [44, 72-74, 76, 78, 80, 81, 530]. However, limited research has targeted inhibition of CFH-mediated lipid peroxidation.

Paracetamol was recently shown to have ferryl reductase activity where it inhibits lipid peroxidation and reduces the ferryl haemoproteins, haemoglobin and myoglobin [66]. In an experimental rat model of rhabdomyolysis, paracetamol decreased markers of oxidative stress and reduced kidney dysfunction [66]. Paracetamol also had a protective association against mortality and reduced oxidative stress markers in patients with sepsis and high CFH [337]. In two RCTs, perioperative paracetamol decreased lipid peroxidation in adults and children undergoing cardiopulmonary bypass associated with high CFH and lipid peroxidation [335, 336]. These two studies were not powered to assess the effect of paracetamol on kidney function.

6.2 Hypothesis

The potential renoprotective role of paracetamol in severe malaria has not been investigated. This study examined the hypothesis that paracetamol reduces kidney dysfunction by inhibiting cell-free haemoglobin mediated oxidative damage in severe and moderately severe malaria.

6.3 Study design

This was a multicentre, open-label, randomised comparison of paracetamol, versus no paracetamol in adult patients admitted to hospital with severe and moderately severe malaria treated with antimalarials. It was conducted between June 2012 and September 2014, as described in Chapter 2. Briefly, patients over 12 years with slide confirmed severe or moderately severe malaria (defined in Chapter 2.3), and fever (temperature >38° on admission or fever during preceding 24 hours) were included, provided written informed consent was obtained. Criteria for SM are listed in Panel 6.1. Moderately severe malaria was defined as a requirement for inpatient parenteral therapy due to inability to tolerate oral medication, but no SM criteria (Panel 6.1). Patients were not included if they had a history of chronic liver disease or alcohol use (more than three drinks per day), contraindication to paracetamol or NG tube insertion, or were pregnant.

Panel 6.1 Pre-specified modified criteria for severe falciparum malaria

Upon hospital admission, at least one of the following:

- Glasgow coma score < 11/15
- Generalised convulsions (>2 in 24 hours)
- Acute kidney injury (creatinine >150 µmol/L)
- Jaundice (total bilirubin >45 µmol/L + parasitaemia >100,000/µl
- Severe anaemia (haematocrit <20% + parasitaemia >100,000/µl)
- Hyperparasitaemia (>5% or > 200,000 parasites/µl)
- Hypoglycaemia (glucose <2.2 mmol/L)
- Venous bicarbonate <15 mmol/L
- Venous lactate > 4 mmol/L
- Shock (systolic blood pressure < 80 mmHg with cool extremities)
- Pulmonary oedema
- Spontaneous bleeding
- Haemoglobinuria
- Severe prostration (inability to walk or sit without assistance)
- Impaired consciousness (behavioral change, confusion, or drowsiness)

Consecutive eligible patients were randomly assigned to treatment with paracetamol or no paracetamol. Paracetamol tablets (500mg tablets, Bristol Laboratories Ltd., Hertfordshire, UK) were given to adults > 50kg at a dose of 1,000 mg on enrolment then 6-hourly for 72 hours if oral medication could be taken reliably. Patients < 50kg were given paracetamol 12.5-15 mg/kg/dose 6-hourly for 72 hours. Paracetamol syrup (250mg/5ml, Rosemont Pharmaceuticals Ltd., Leeds, UK) was given via NG tube to patients who were unconscious or unable to take oral medication. Patients in the control arm who developed a fever > 40°C were given ibuprofen in the absence of renal impairment, dehydration and dengue infection. If ibuprofen was contraindicated, then febrile control patients were given paracetamol as per local practice (500 mg tablet or suppository).

Patients were treated with parenteral artesunate (Guilin Pharmaceutical Factory, Guangxi, China), followed by a standard course of oral artemether/lumefantrine (Coartem, Novartis, Basel, Switzerland) once oral medication was tolerated. Patients were supportively managed according to WHO treatment guidelines [101]. On enrolment, if patients did not have shock, anuria nor severe dehydration, intravenous normal saline was administered at a rate of 250 ml/h for 6 hours followed by maintenance at 100ml/h until tolerating oral fluids. If patients were severely dehydrated or were oliguric, intravenous normal saline was administered at a rate of 1000 ml/h for 2 hours followed by 500 ml/h for 2 hours if oliguria persisted and there were no signs of fluid overload. Patients remaining oliguric after initial fluid resuscitation were referred to nephrology. In Chittagong, haemodialysis

was initiated according to the local nephrologist. Patients enrolled in Ramu potentially requiring haemolysis were transferred to the Chittagong nephrology ward and study procedures continued uninterrupted by the study team located at this site.

A venous blood sample was analysed for baseline biochemistry using a bedside analyzer (iSTAT, Abbott Laboratories, IL, USA). Assessment of temperature and parasitaemia were 6-hourly, kidney function 12-hourly, and CFH 24-hourly. Samples for paracetamol pharmacokinetic assessment were collected as described in Chapter 2. Plasma transaminases to monitor paracetamol toxicity were assessed on admission and after 72 hours. Urine color was assessed using a standardised urine color chart (Figure 6.1). Haemoglobinuria was defined as dark urine with a colorimetric level of 6 or greater with a urine dipstick positive for haemoglobin. Technical aspects of the assays are described in Chapter 2.10.

Patients were discharged from hospital at the discretion of local physicians and survivors were seen in follow-up at weekly intervals for six weeks for haematological and renal function assessment.

Study outcomes

The primary outcome measure was change of creatinine at 72 hours compared to baseline stratified by the level of intravascular haemolysis, as measured by CFH, compared between groups on an intention-to-treat basis.

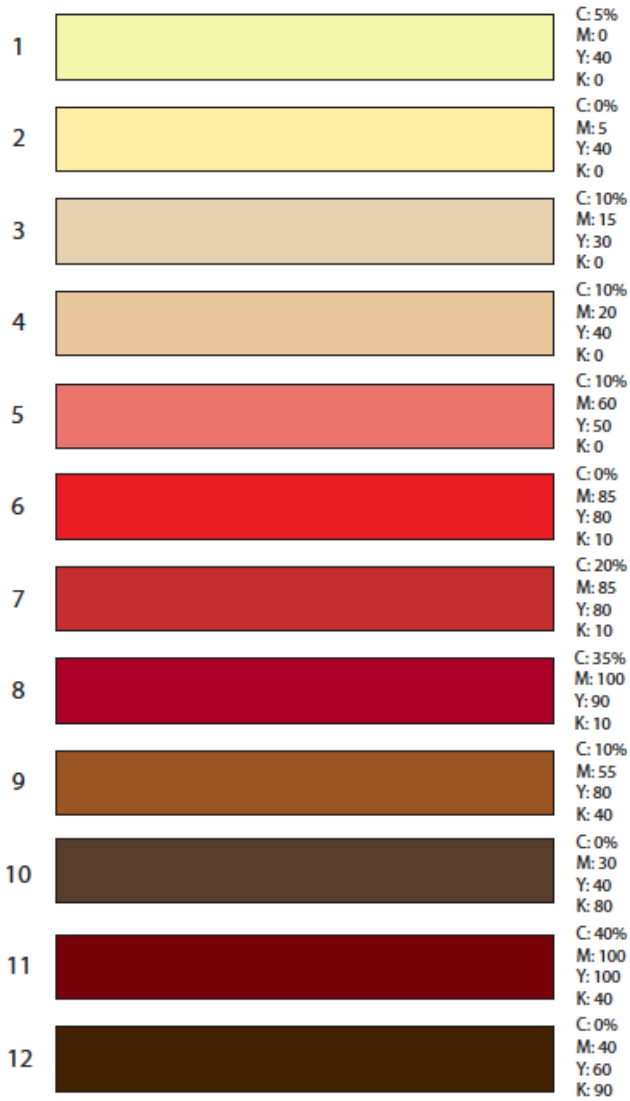


Figure 6.1. Urine color scale to detect haemoglobinuria. Color scale was adapted from Hillmen *et al.* [340] to monitor for incidence of haemoglobinuria in falciparum malaria patients. Color scale was created in Photoshop using cyan, magenta, yellow and key (black) (CMYK) color model as listed for reproducibility of the chart. Haemoglobinuria was prospectively defined as a urine color of 6 or greater. Urine was aliquoted into clear 10 ml collection tubes with a 0.5 cm diameter in order to standardise color assessments between and within patients. Tubes filled with urine were held against a white background in a well-lit room in order to assess the color with reference to the color scale.

Secondary outcome measures were: (1) the proportion of patients developing AKI as defined by the KDIGO criteria; Stage 1: increase in serum creatinine by 0.3 mg/dL from enrolment creatinine within 48 hours; (2) the effect on plasma oxidative stress markers, as measured by plasma F2-isoprostanes and isofurans, and the relationship to CFH and change in creatinine at 72 hours; (3) fever clearance time, defined as i) time at which aural temperature first dropped below 37.5°C, (ii) time at which aural temperature remained below 37.5°C for 24 hours, and (iii) area under the temperature-time curve from admission to 72 hours, above the 37.5°C; (4) parasite clearance, defined as (i) the time interval between start of treatment and the time of the first of 2 sequential negative thick films, and (ii) slope half life; (5) coma recovery time, defined as time for Glasgow Coma Score (GCS) to recover to a score 15; (6) safety of regularly dosed paracetamol in severe and moderately severe malaria, and (7) the pharmacokinetics of paracetamol in severe and moderately severe malaria. Secondary outcome measures were also assessed per-protocol, excluding those that died before receiving paracetamol, or died or withdrew within 72 hours. A subgroup analysis was performed for patients with high or low admission CFH, using a cut-off concentration of 48,000 ng/ml.

Statistical analysis

Descriptive statistics analyses and software used are described in chapter 2.10.14. Inclusion of more than 50 adults with severe and moderately severe malaria was required to show, with 80% power and 95% confidence, a 50% reduction in creatinine from admission baseline. This calculation was based

on an estimated mean admission creatinine value of 1.7 mg/dL. Parasite clearance times, fever clearance times, and coma recovery times were assessed by means of survival analysis with the use of the Kaplan-Meier method and the log rank test or the Wilcoxon-Breslow-Gehan test if lines crossed. Receiver operating characteristic (ROC) analysis was performed to quantify the threshold enrolment CFH to discriminate between those with and without AKI on enrolment, defined by KDIGO criteria. KDIGO diagnosis and staging on enrolment was assessed using the estimated baseline creatinine obtained from the MDRD formula assuming a GFR of 75 ml/min [531]. Variables with repeated measures were assessed as percent change from baseline since patients had variable creatinine and CFH concentrations on enrolment. Repeated creatinine measurements across time points were missing 8% of the total creatinine time points excluding those who died.

When assessing the changes in creatinine from baseline over time, a multiple imputation procedure using chained equations with five rounds was used to deal with missing data. The number of imputed creatinine observations at each time point is detailed in Table 6.1. Outcomes at all time points in survivors were included in the imputation model. These analyses were followed by complete case analysis as a form of sensitivity of the assumptions used in the imputation models. Apart from those that had missing values, there was one patient that had a single invalid value and this value was coded as missing and a plausible value was imputed. To account for haemodialysis confounding the decline in creatinine concentrations,

Table 6.1. Number of imputed creatinine values for longitudinal creatinine analysis

		Time point (hours)						
		0	**12**	**24**	**36**	**48**	**60**	**72**
Overall	Control	0	2	1	7	2	3	1
	Paracetamol	0	4	0	8	1	4	1
CFH > 48,000 ng/ml	Control	0	2	1	2	0	0	0
	Paracetamol	0	2	0	3	0	1	0
CFH < 48,000 ng/ml	Control	0	0	0	5	2	3	1
	Paracetamol	0	2	0	5	1	3	1

creatinine values were adjusted at each time point following dialysis until time of death by using a creatinine rise of 1.5 mg/dL per day as proposed for anephric states [532]. Mixed effects models were used in analysis because of the longitudinal nature of data, with maximum likelihood as the method of estimation. The parameters of interest were the fixed effect interaction terms between treatment group and times, and treatment group and CFH at enrolment to describe whether change in creatinine differed between the two groups across the observation period, and whether the treatment effect depended on enrolment CFH, respectively. Time-to-event outcome sub-hazard ratio (HRs) was estimated by competing-risks regression to adequately account for death as the competing event that impeded the occurrence of the event of interest, AKI, and to better describe covariates in the model. Competing-risks regression was adjusted for study site.

6.4 Results

Cohort characteristics

Figure 6.2 shows the trial consort diagram. 62 patients were randomised (31 assigned paracetamol, 31 assigned control) of 346 patients assessed for eligibility. Patients who died before 72 hours (4 in paracetamol and 4 in control groups) or withdrew from the study before 72 hours (1 in paracetamol and 1 in control groups) were included in the main intention-to-treat analysis, but were excluded from the per-protocol analysis. Table 6.2 shows the baseline characteristics of all individuals randomised. In the paracetamol group, 5/31 (16%) individuals had AKI as defined by the WHO guidelines

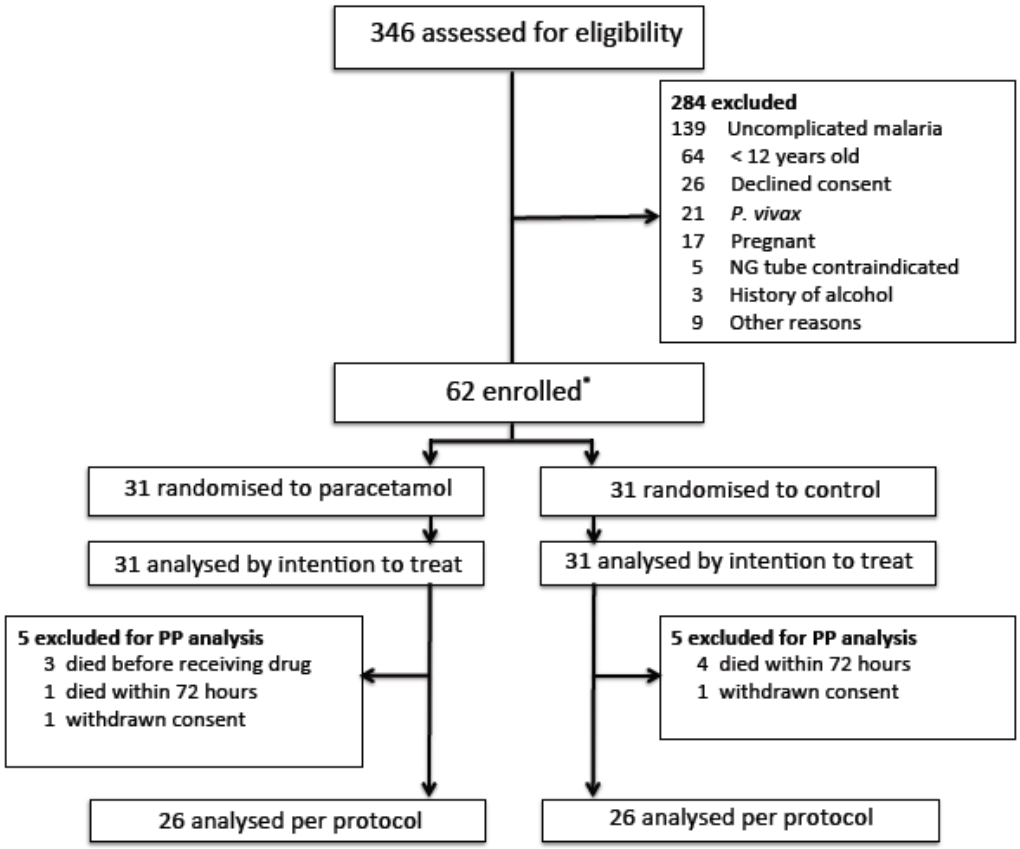


Figure 6.2. Consort diagram of paracetamol randomised controlled trial. * 30 patients recruited in Chittagong, Bangladesh; 32 patients recruited in Ramu, Bangladesh.

Table 6.2. Baseline characteristics in the two treatment groups

	Paracetamol (n=31)	Control (n=31)
Demographics		
Sex (male)	19 (61%)	21 (68%)
Age (years)	28 (20-43, 15-70)	32 (25-40, 13-55)
Fever before enrolment (days)	7 (4-10, 2-30)	7 (5-8, 1-15)
Altered LOC before enrolment (days)	2.5 (1.0-3.0, 1,5)	1.0 (1.0-2.5, 0.3,4)
Red or black urine before enrolment	0 (0%)	4 (13%)
Paracetamol before enrolment*	1 (3%)	0 (0%)
Complications on enrolment		
Coma$^{\delta}$	10 (32%)	13 (42%)
Jaundice	3 (10%)	5 (16)
Severe anaemia	2 (6%)	2 (6%)
Hyperlactataemia (lactate >4 mmol/L)	7 (23%)	9 (29%)
Acute kidney injury (SCr >265 µmol/L)	5 (16%)	6 (19%)
Hyperparasitaemia (>10%)	1 (3%)	4 (13%)
Haemoglobinuria	2 (7%)	9 (29%)
Pulmonary oedema	0 (0%)	1 (3%)
Severe prostration$^{\sharp}$	18 (58%)	19 (61%)
Unable to tolerate oral medications	21 (71%)	24 (77%)
Severity		
Severe malaria	24 (77%)	24 (77%)
Moderately severe malaria	7 (23%)	7 (23%)
Number of severity criteria on enrolment	3 (1-5, 1-10)	4 (1-6, 1-10)
KDIGO stage on enrolment		
Stage 0	20 (65%)	19 (61%)
Stage 1 (≥1.5x baseline)	4 (13%)	5 (16%)
Stage 2 (≥2.0 - 2.9x baseline)	3 (10%)	2 (6%)
Stage 3 (≥3x baseline or (≥ 353.6µmol/L)	4 (13%)	5 (16%)

Data are number (%) or median (IQR, range) unless otherwise indicated. *Paracetamol before enrolment was based on a paracetamol concentration > 10µg/ml on enrolment pharmacokinetic samples. δDepth of coma was assessed by Glasgow coma scale < 11; $^{\sharp}$Severe prostration defined as inability to walk or sit up without assistance. Abbreviations: LOC, level of consciousness; KDIGO, Kidney Disease: Improving Global Outcomes.

(creatinine > 265 μmol) compared with 6/31 (19%) in the control group (*P* = 0.74). The KDIGO criteria classified an additional 12 patients with AKI on admission; 11/31 (35%) in the paracetamol group and 12/31 (39%) in the control group (*P* =1.00). Baseline characteristics and predictors of severity did not differ between groups with the exception of more patients in the control arm presenting with haemoglobinuria (*P* = 0.043) (Tables 6.2 and 6.3). This corresponded to a higher CFH concentration at enrolment in the control group compared to the paracetamol group however this was not significant (*P* = 0.13) (Table 6.3). Comorbidities were more frequent in the paracetamol arm 5/31 (16%) (3 with hypertension and 2 with type 2 diabetes) compared to none in the control arm (*P* = 0.053). Overall the treatment and control groups were well matched.

Paracetamol effect on kidney function

The percent rise in creatinine at 72 hours compared to baseline was lower in the paracetamol group compared to the control group (median, IQR: -23, -37 to -18 versus -14, -29 to 0; *P* = 0.043) (Figure 6.3). When adjusted for baseline CFH and paracetamol area under the concentration time curve (0 to 72 hours), the creatinine change at 72 hours remained lower in the paracetamol group compared to the control group (*P* = 0.016). The ROC analysis of AKI on CFH, showed that AUC was 0.70, 95% CI (0.56-0.84) with a CFH cut-off of 48,000 ng/ml giving a sensitivity of 65% and a specificity of 67% (Figure 6.4). When stratified by intravascular haemolysis, defined as admission CFH greater than 48,000 ng/ml, the paracetamol group had a more pronounced reduction in creatinine at 72 hours compared

Table 6.3. Baseline clinical and laboratory investigations in the two treatment groups

	Paracetamol (n=31)	Control (n=31)
Clinical examination		
Temperature (°C)	39.0 (37.3-40.0; 36.5,41.0)	38.9 (37.5-39.5; 35.5,41.0)
Blood pressure (mmHg)		
Systolic	108 (100-116; 80,170)	110 (95-119; 78,153)
Diastolic	61 (50-70; 43,92)	69 (59-75; 30,97)
Glasgow coma score	15 (9-15; 3,15)	12 (9-15; 4,15)
Respiratory rate (per minute)	30 (24-38; 18,56)	30 (28-40; 14,54)
Heart rate (per minute)	107 (96-118; 61,134)	108 (92-130; 74,148)
Laboratory investigations		
Parasite count per µl[a]	53426 (24596-116048; 160,737272)	17258 (5751-51785; 128,968376)
*Pf*HRP2 (mg/ml)	1356 (146-4486; 1,36675)	1308 (154-4961; 1,49265)
Sodium (mmol/L)	134 (130-138; 126,154)	135 (130-138; 118,146)
Potassium (mmol/L)	3.5 (3.3-4.0; 2.4,5.4)	3.4 (3.0-3.9; 2.6,5.0)
Chloride (mmol/L)	103 (107-99; 96,124)	104 (110-107; 94,120)
Glucose (mmol/L)	5.8 (4.7-8.1; 2.4,10.5)	6.8 (5.5-9.0; 4.6,38.9)
Blood urea nitrogen (mg/dL)	23 (13-36; 4,138)	25 (16-43; 4,140)
Creatinine (mg/dL)	1.2 (1.1-1.9; 0.9,5.1)	1.3 (1.1-1.9; 1.0,6.3)
Haemoglobin (g/dL)	10.1 (8.0-12.7; 2.3,15.4)	11.6 (10.0-13.4; 5.3,15)
Cell-free haemoglobin (ng/ml)	42767 (16839-94086) (819,2041417)	52495 (23388-188802) (8883, 3428982)
Total bilirubin (mg/dL)[a]	1.5 (1.0-2.2; 0.4,28.5)	1.6 (1.2-2.4; 0.5,20.5)
Indirect bilirubin (mg/dL)[a]	0.7 (0.5-0.9; 0.2,3.6)	0.8 (0.6-1.2; 0.1,7.9)
Lactate (mmol/L)	2.89 (1.94-3.70; 1.00,14.14)	2.11 (1.62-4.84; 0.71,9.95)
Bicarbonate (mmol/L)	19.2 (17.3-21.8; 7.9,28.8)	18.5 (15.8-19.9; 13.4,25.9)
Base excess (mmol/L)	-5 (-7 to -3; -21, 5)	-7 (-10 to -3; -13, 3)

Data are number (%), or median (IQR, range), unless otherwise indicated. [a] geometric mean (95% CI, range).
Abbreviations: CI, confidence intervals; *Pf*HRP2, *Plasmodium falciparum* histidine rich protein 2.

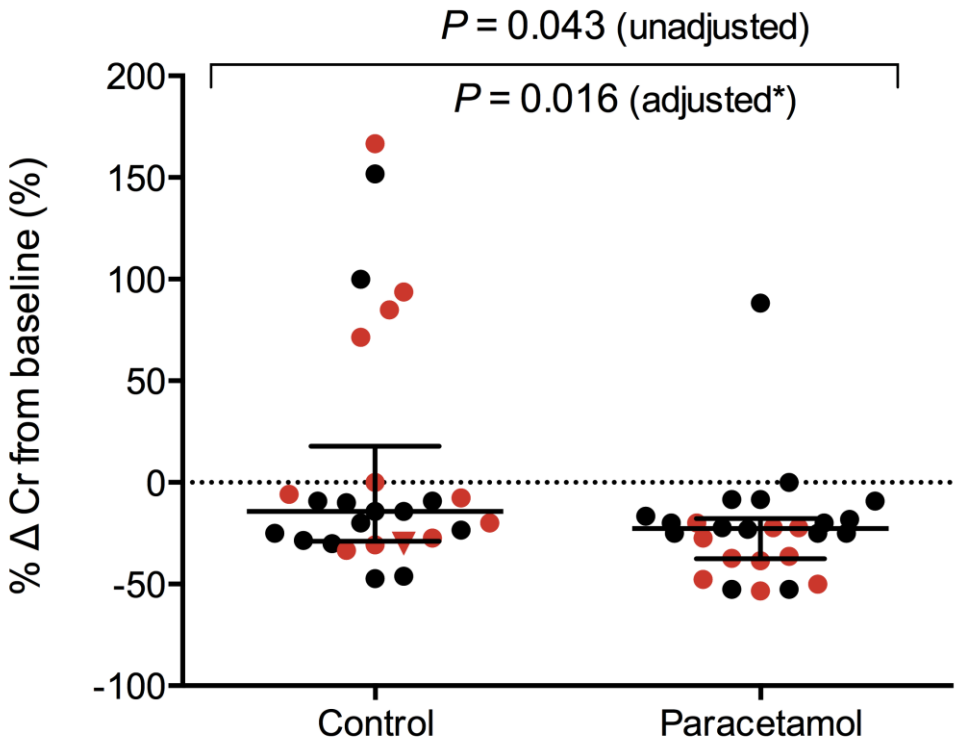


Figure 6.3 Paracetamol effect on 72-hour creatinine. Creatinine percent change from baseline at 72-hours was the primary endpoint. Red and black solid dots represent patients with admission plasma CFH ≥ 48,000 ng/ml, and plasma CFH < 48,000 ng/ml, respectively. Red triangle represents patient in the control group with elevated paracetamol area under the curve. *Adjusting for baseline CFH and paracetamol area under the concentration time curve (0-72 hours), 72-hour percent change of creatinine from baseline remained significant (*P* = 0.016, slope= -42, 95% CI (-77, -8)). *Abbreviations*: Cr, creatinine.

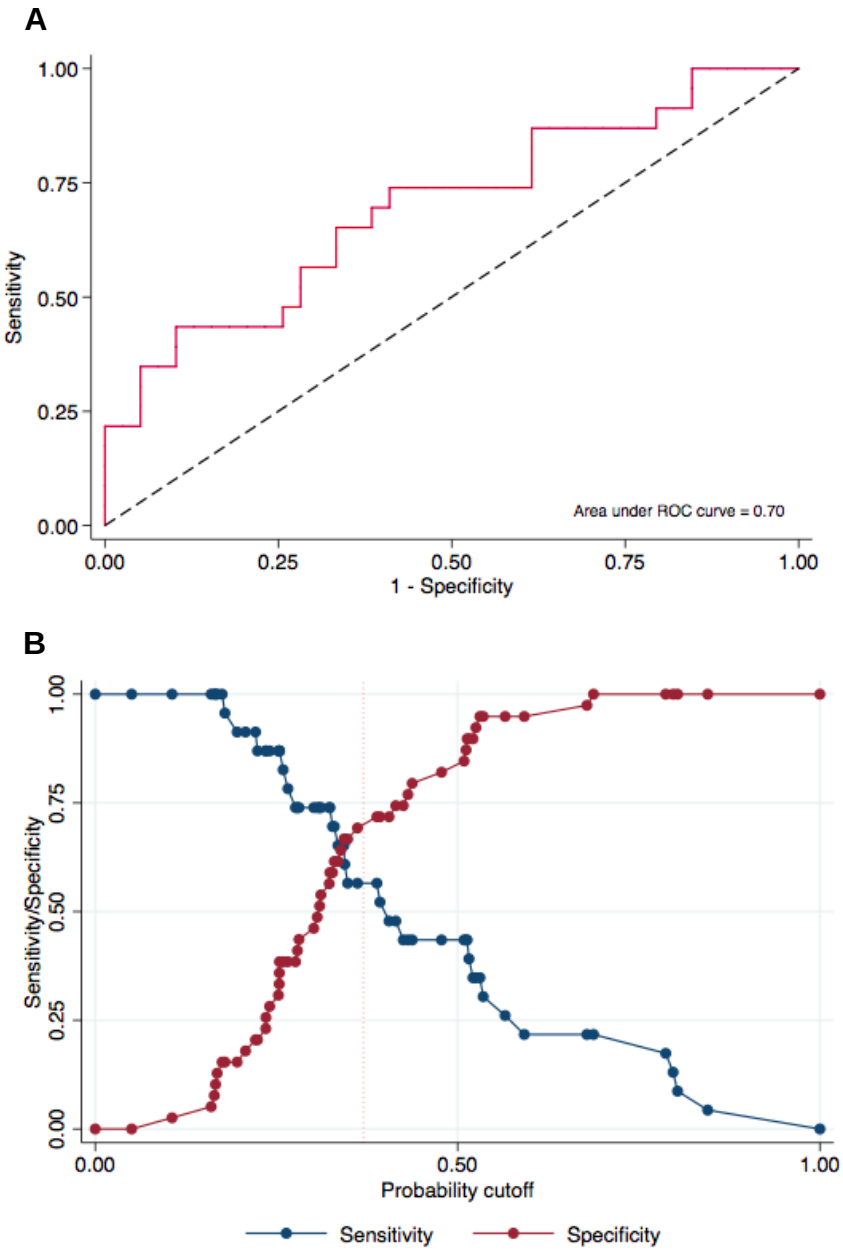


Figure 6.4. Determination of cell-free haemoglobin stratification threshold. (A) Receiver operating curve of AKI on cell-free haemoglobin. AKI defined by KDIGO diagnosis on admission. (B) Sensitivity and specificity versus probability cutoff representing the probability cutoff of 0.339, corresponding to a cell-free haemoglobin concentration of approximately 48,000 ng/ml, predicts AKI with 65% sensitivity and 67% specificity.

to the control group (median (IQR): -37 (-48 to -22) versus -7 (-29 to 78), *P* = 0.004). The subgroup of patients with admission CFH less than 48,000 ng/ml did not show a difference in 72-hour creatinine between groups (*P* = 0.98). The outlier in the paracetamol arm with the elevated creatinine at 72 hours was a patient transferred to CMCH from a primary health care centre with SM and anuric AKI that developed during treatment for SM with quinine. The subject began IHD within 8 hours of enrolment however concomitant sepsis due to acute cholangitis secondary to hepatobiliary ascariasis contributed to an extended hospital admission and 16 days of IHD. The patient left the hospital against medical advice with a creatinine of 4.7 mg/dL and did not respond to phone calls for ongoing follow up. When hemodialysis patients were excluded, the 72-hour creatinine in the paracetamol group remained lower compared to the control arm in the subgroup of patients with CFH > 48,000 ng/ml (median (IQR): -37 (-48 to -22) versus -24 (-30 to -7), *P* =0.033). The per-protocol analysis showed the same results where proportional improvement in creatinine at 72 hours remained larger in the paracetamol group compared to control (*P* = 0.043) and when adjusted for enrolment CFH and paracetamol 72-hour AUC (*P* = 0.016). The 24-hour fluid balance was similar between the paracetamol and control groups (median, IQR: 2,625 ml, 2,200-3,450 ml versus 2,308 ml, 1,485 – 3,000 ml; *P* = 0.21). Longitudinal creatinine and CFH concentrations for each patient are shown in Supplementary Figures 6.1 to 6.5. The median peak percent change was reduced in the paracetamol arm compared to the control group in the subgroup of patients with CFH > 48,000 ng/ml (median (IQR): -8 (-10 to 0) versus 5 (-8 to 71), *P* =0.032) (Supplementary Tables 6.1 – 6.5).

The mean percent change creatinine from baseline every 12 hours for 72 hours showed that the group treated with paracetamol had lower percent change creatinine concentrations at 48, 60 and 72 hours (*t* test: P = 0.036, P = 0.028, and P = 0.026, respectively) (Figure 6.5A, Supplementary Table 6.1). When stratified by CFH of > 48,000 ng/ml the proportional improvement creatinine from baseline was larger with paracetamol at 24, 36, 48, 60 and 72 hours compared to the control group (*P* = 0.024, *P* = 0.006, *P* = 0.009, *P* = 0.012, *P* = 0.013, respectively) (Figure 6.5B, Supplementary Table 6.1). Mean percent peak creatinine compared to baseline was also lower with paracetamol in patients with CFH > 48,000 ng/ml (*P* = 0.026) (Supplementary Table 6.1). There were no differences in the percent change in creatinine between groups in the individuals with CFH ≤ 48,000 ng/ml (Figure 6.5C, Supplementary Table 6.1). Similar trends were observed when comparing the median percent change creatinine from baseline (Supplementary Table 6.2, Supplementary Figure 6.6). Mixed effects modeling of the entire cohort using the interaction term of treatment arm with time showed that time modifies the effect of paracetamol on creatinine and the slopes were not parallel; specifically the rate (slope) of creatinine change decreased with time in the paracetamol group and increased in the control group (coefficient (95%CI): -3.4 (-4.0 to -2.1) versus 1.7 (0.4 to 3.0), *P* < 0.001) (Table 6.4, Model 1). When stratified by CFH > 48,000 ng/ml on admission, the rate of creatinine changes with time declined with a steeper slope compared to the increasing creatinine in the control group (coefficient (95%CI): -5.7 (-7.7 to -3.7) versus 3.8 (2.0 to 5.6), *P* < 0.001). Among those

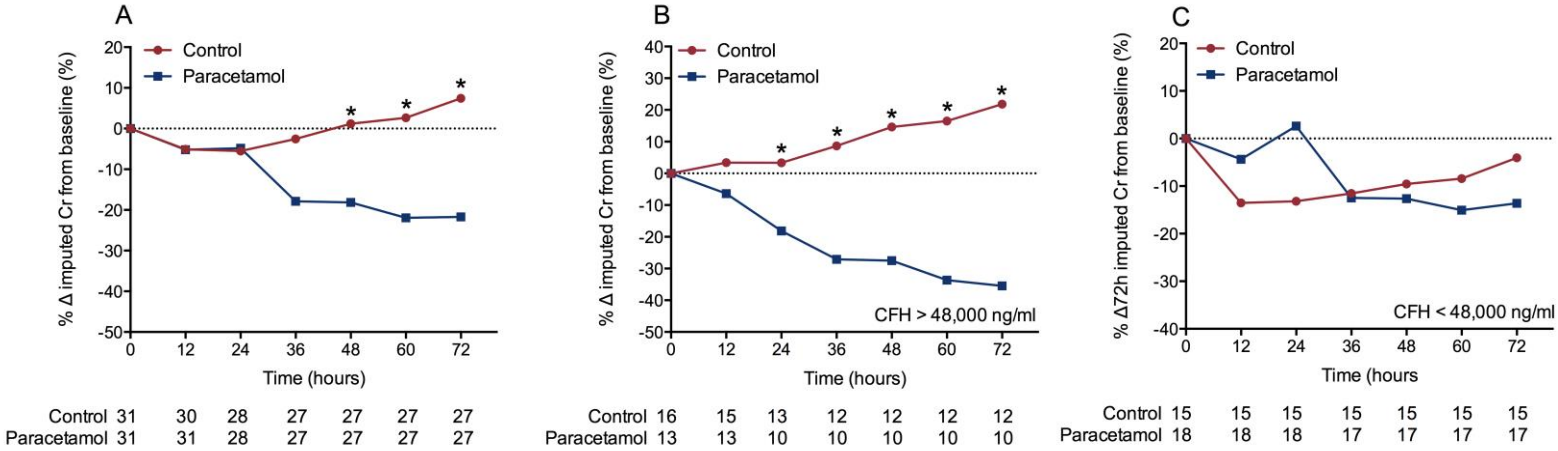


Figure 6.5. Effect of paracetamol on creatinine stratified by intravascular haemolysis. Creatinine mean percent change from baseline at 12, 24, 36, 60, 48, and 72 hours, of (A) entire cohort, (B) patients stratified by level of intravascular haemolysis with plasma CFH > 48,000 ng/ml, and (C) patients stratified by level of intravascular haemolysis with plasma CFH < 48,000 ng/ml. Asterisks represent significant differences between paracetamol and control groups (*t* test *P* <0.05). Across time points there were missing sample collections (median, range; 1 (0,5)), approximately 8% of total creatinine sampling time points. In addition to the complete case analysis (see results), multiple imputation procedure with five rounds was employed. The number of imputed creatinine observations at each time point is detailed in Supplementary Table 1. Frequencies in rows below figures represent number of patients (n) at each time point. *Abbreviations*: Cr, creatinine.

Table 6.4. Mixed effects models of percent change creatinine over time per treatment group

Variable	Study arm	Coefficient	95% CI	*P*
MODEL 1				
Entire cohort				
Treatment group	Paracetamol	3.8	-9.6 to 17.4	0.573
Time		1.7	0.4 to 3.0	0.011
Treatment x time		-5.1	-6.9 to -3.2	< 0.001
CFH > 48,000 ng/ml				
Treatment group	Paracetamol	-1.0	-19.3 to 17.2	0.911
Time		3.8	2.0 to 5.6	< 0.001
Treatment x time		-9.5	-12.2 to -6.8	< 0.001
CFH ≤ 48,000 ng/ml				
Treatment group	Paracetamol	9.0	-9.4 to 27.3	0.337
Time		0.1	-1.7 to 1.8	< 0.001
Treatment x time		-2.1	-4.5 to 0.3	0.088
MODEL 2				
Entire cohort				
Treatment group	Paracetamol	111.7	23.6 to 200	0.013
Time		1.7	0.43 to 3.0	0.009
Treatment x time		-5.2	-7.0 to -3.3	< 0.001
CFH		5.9	0.3 to 11.5	0.038
CFH x treatment		-9.8	-17.8 to -1.8	0.016

Model 1 mixed effects model with treatment*time interaction term assessing if effect of paracetamol on percent change creatinine is modified by time. Model 2: Mixed effects model with addition of cell-free haemoglobin*treatment group interaction term assessing is the dependence of percent creatinine change on paracetamol depends on the concentration of cell-free haemoglobin (CFH) on enrolment.

with admission CFH <48,000, there was no difference in rates of creatinine change between the paracetamol and control groups (*P* = 0.09) (Table 6.4, Model 1). Since the ROC cut-off point for CFH was based on this data, which is not yet validated with an external dataset, it was then assessed whether there was a differential effect of paracetamol on percent change creatinine that depended on enrolment CFH. The mixed effects model including the interaction term CFH and treatment group, in addition to the previously fitted treatment-time interaction term, showed that the effect of paracetamol varied (interacts) with the baseline CFH, interaction test *P* value = 0.016 (Table 6.4, Model 2). Specifically, higher values of CFH on enrolment were associated with benefit from paracetamol, while this was not the case with lower values of CFH (Figure 6.6). Lower mean and median creatinine percent change from baseline in the paracetamol group compared to control group were similarly observed in the per-protocol analysis (Supplementary Tables 6.4-6.5). When haemodialysis patients were excluded, the mean and median percent change in creatinine was lower in the paracetamol group compared to the control at 36 hours, 48 hours, 60 hours and 72 hours in patients with enrolment CFH > 48,000 ng/ml (*P* <0.05 for each time point).

Of the 62 recruited patients, 17 (27%) developed AKI, as defined by the KDIGO criteria of an increase of 0.3 mg/dL. Of the patients that developed AKI, 12/17 (71%) were oligo-anuric, and 4/17 (24%) were non-oliguric; urine output was not recorded from one patient. The 6-hour fluid balance was similar between those that developed AKI and those that did not (median,

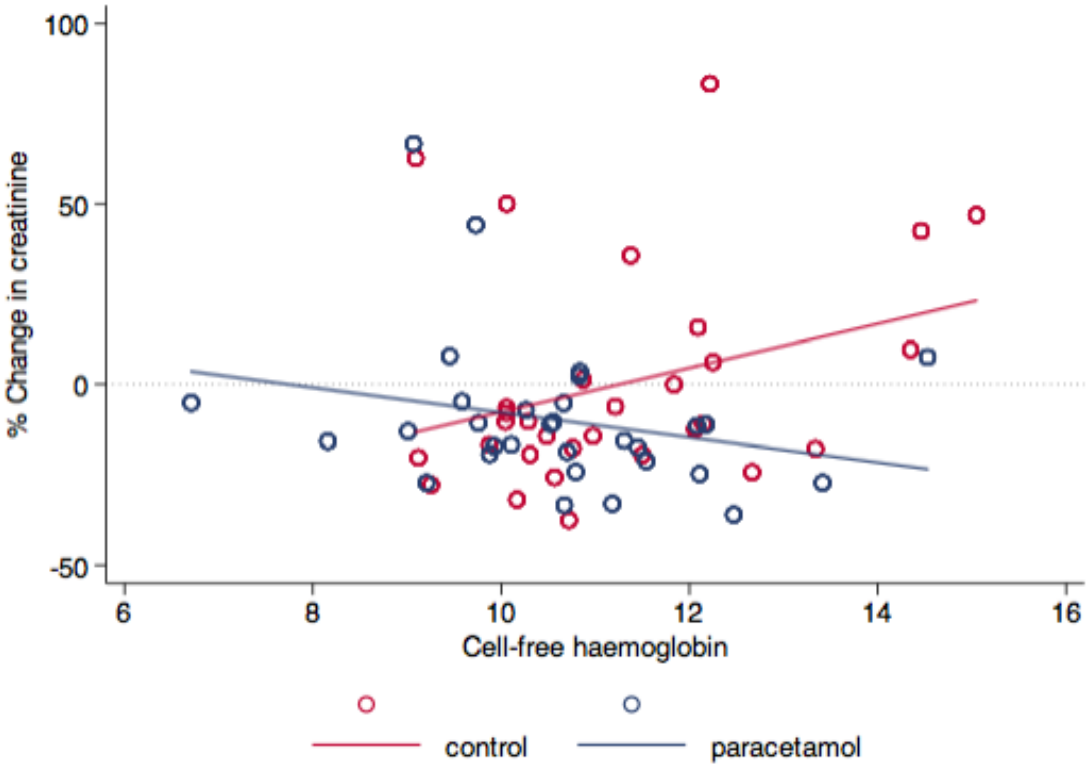


Figure 6.6. Mixed effects model of percent change creatinine accounting for cell-free haemoglobin. Interaction terms included cell-free haemoglobin (log transformed) with treatment group and treatment group with time.

IQR: 750ml, 300 – 940 ml versus 650ml, 400 – 1,180 ml; *P* = 0.67). Patients with oliguric AKI had a median urinary protein of 0.3 g/L (IQR; 0.3 – 1 g/L) and those with non-oliguric AKI had a median urinary protein of approximately 0.1 g/L (IQR: 0 – 60 mg/dL). In the paracetamol group, 5/31 (16%) patients developed AKI versus 12/31 (39%) in the control group (odds ratio 0.26, 95% CI: 0.07-0.94), in favor of the paracetamol group; *P* = 0.039). Risk of developing AKI during admission was analysed by competing-risk regression to account for the presence of a competing event (death) that prevented the event of primary interest (AKI) from occurring. Patients in the control group had a higher riskfor developing AKI, according to KDIGO criteria, compared to the paracetamol group (HR stratified by study site: 3.0, 95% CI: 1.1-8.5; *P* = 0.034) (Figure 6.7). In the per-protocol analysis, 2/26 (8%) patients developed AKI versus 10/26 (38%) in the control group (odds ratio 0.12, 95% CI: 0.02-0.66), in favor of the paracetamol group; *P* = 0.015). Per-protocol competitive risk regression supported that patients in the control group had a higher risk for developing AKI compared to the paracetamol group (HR stratified by study site: 6.3, 95% CI: 1.4-29.4; *P* = 0.018).

10/23 (44%) patients admitted with AKI recovered with steady decrease in creatinine without haemodialysis; of whom 7/10 (70%) were in the paracetamol group and 3/10 (30%) in the control group (*P* = 0.11). Among the patients that had an increase in creatinine of ≥ 0.3 mg/dL during admission (Figure 6.7), 8/17 (47%) required dialysis; of whom 2/8 (25%) were in the paracetamol arm and 6/8 (75%) in the control arm (Table 6.5).

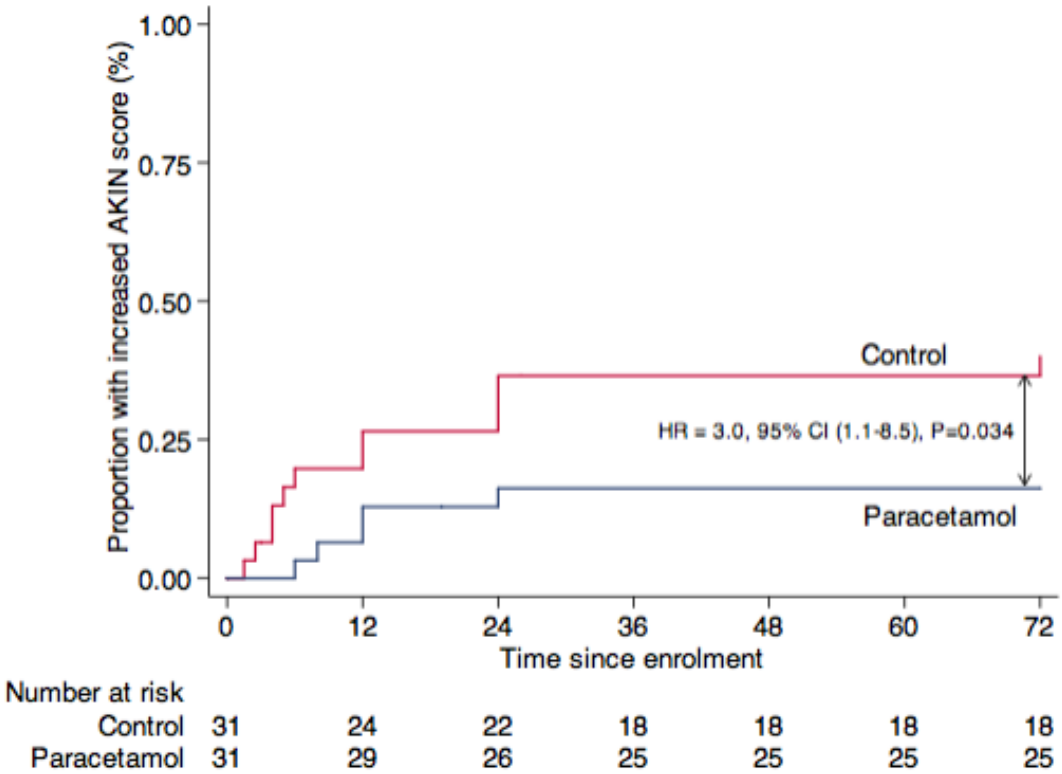


Figure 6.7. Kaplan-Meier comparing in-hospital AKI development in adults with severe and moderately severe malaria treated with either paracetamol or no paracetamol (control). Patients were classified as developing AKI if they met criteria for stage 1 KDIGO during admission (≥ 0.3 mg/dL in creatinine from admission within 48 hours). Patients were censored at the time of creatinine rise meeting KDIGO criteria, with death censored as a competing risk preventing the primary event of interest from occurring. Competing risks regression was used to assess sub-hazard ratio (HR).

Table 6.5. Outcomes by treatment group

	Paracetamol	N	Control	N	*P*
Renal					
Recovery of enrolment AKI without RRT	7 (58%)	12	3 (23%)	13	0.11
Haemodialysis	2 (7%)	31	6 (19%)	31	0.26
Time to haemodialysis, hours (median, range)β	6 (6-8; 6,8)	2	4 (3-5; 2,8)	6	0.16
Number of dialysis cycles (median, range)	4 (1-6; 1,6)	2	5 (3-6; 1,11)	6	0.61
Paracetamol					
Area under the curve 0-72 hours (mg*h/L)	385.9 (273.7-478.7; 37.5,761.0)	28	6.7 (0.4-44.5; 0,292.4)	28	**<0.001**
Hepatotoxicity					
ALT at 0 hours (enrolment) (U/L)	19 (13-28; 6,162)	31	23 (18-37; 9,201)	31	0.11
ALT at 72 hours (U/L)	31 (15-61; 6,98)	26	23 (16-38; 4,109)	25	0.28
% Δ ALT 72-0 hours	32 (-9 to 171; -65, 717)	26	-11 (-34 to 57; -71,264)	25	**0.030**
72h ALT >3-fold ULN+total bilirubin >2mg/dL¶	1 (6%)	18	0 (0%)	21	0.27
72h ALT >3-fold ULN+indirect bilirubin ≥ 2mg/dL	1 (6%)	18	0 (0%)	21	0.27
AST (0 hours)	42 (29-81; 2,579)	31	52 (29-112; 16,483)	31	0.32
AST (72 hours)	60 (20-81; 13,260)	21	36 (25-57; 15,149)	20	0.25
% Δ AST 72-0 hours	0.0 (-27 to 107; -68,371)	21	-18 (-40 to 0; -75,82)	20	0.059
72h AST >3-fold ULN+total bilirubin >2mg/dL¶	2 (18%)	11	0 (0%)	12	0.12
72h AST >3-fold ULN+indirect bilirubin ≥ 2mg/dL	2 (18%)	11	0 (0%)	12	0.12
Survival					
Outcome (died, %)	4 (13%)	31	5 (16%)	31	0.72
Time to death (hours)β	19 (17-20; 17,29)	4	16 (7-26; 3,197)	5	0.47

Data are number (%) or median (IQR; range), unless otherwise indicated. Fisher's exact test and Mann Whiney-U were calculated for categorical and continuous data. P values for time to event outcomes were calculated using log rank test (ζ) or Wilcoxon-Breslow-Gehan test if lines crossed (β). ALT and AST upper limit of normal (ULN) for males and females was 50 U/L and 35 U/L. ¶ Hy's law requires (i) drug causes hepatocellular injury, shown by more frequent 3-fold or greater elevations above the ULN of ALT or AST, than control or placebo, (ii) among those with ALT or AST > than 3x, may show elevation in serum total bilirubin to >2x ULN, without initial findings of cholestasis, and (iii) no other reason to explain the combination of ALT/AST and bilirubin increases. *Abbreviations:* CFH, cell-free haemoglobin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; RRT, renal replacement therapy.

Those that received dialysis had a KDIGO score of ≥ 2 on admission. Plasma CFH on enrolment (pre-dialysis) was higher in those who received hemodialysis compared to those who did not (median, IQR: 144,767 (48,718-1,973,049) versus 42,930 (20,511-102,969), *P* =0.044). Overall 9/62 (15%) patients died; 4/31 (13%) in the paracetamol group and 5/31 (16%) in the control group (*P* = 0.72) (Table 6.5). Of the patients who died, 8/9 (89%) had either AKI on enrolment (2/9) or developed AKI (6/9) during admission, and 2/8 (25%) died despite dialysis (one patient in each treatment group).

Paracetamol effect on temperature

There was a trend to shorter fever clearance time, defined as the first temperature below 37.5°C, in the paracetamol arm compared to the control arm in the intention-to-treat analysis (median, IQR: 9.7h, 5.8h – 30.1h versus 18.5h, 7.0h- 37.0h; *P* = 0.11) (Table 6.6) (Figure 6.8). In the per protocol analysis, which excluded patients who died before receiving paracetamol, the time to fever clearance was shorter in the paracetamol group compared to controls (median, IQR: 7.9h, 5.8h – 25.2h versus 18.5h, 7.0h – 37.0h; *P* = 0.045) (Table 6.6). Further, the paracetamol AUC correlated with the fever clearance time (r_s = - 0.32, *P* = 0.017; N=56). When analysed by competing-risk regression, the fever clearance time remained shorter in the paracetamol group compared to controls in the per protocol analysis (HR: 2.0, 95% CI 1.0-3.7; *P* = 0.036). Given that 16 patients did not have a calculable fever clearance time since they were afebrile on enrolment but 81% (13/16) of these patients subsequently spiked

Table 6.6 Secondary outcomes by treatment group

	Paracetamol	N	Control	N	*P*
Parasite clearance					
Parasite clearance time, hours: ITT β	54.0 (40.1-63.1; 2.6,501.7)	30	42.1 (36.0-55.3;18.0,120.0)	31	0.101
Parasite clearance time, hours: PP β	54.0 (47.6-63.1; 2.6,501.7)	25	42.1 (36.0-55.3;18.0,120.0)	26	0.069
Fever clearance					
Fever clearance time A, hours: ITTζ	9.7 (5.8-30.1; 0,39)	22	18.5 (7.0-37.0; 0,74.3)	24	0.106
Fever clearance time A, hours: PPζ	7.9 (5.8-25.2; 0,39)	19	18.5 (7.0-37.0; 0,74.3)	20	**0.045**
Fever clearance time B, hours: ITTζ	37.0 (24.7-61.1; 0,306.6)	29	60.3 (30.9-86.0; 0,344.5)	30	0.050
Fever clearance time B, hours: PPζ	31.0 (18.3-44.1; 0,306.6)	25	59.8 (30.9-86.0; 0,344.5)	25	0.056
Area above 37.5°C: ITT	16.1 (3.8-24.6; 0,104)	31	21.8 (12.0-33.8; 0,134.8)	31	0.095
Area above 37.5°C: PP	18.5 (6.6-24.6; 0,104)	26	24.3 (14.1-35.5; 0,134.8)	26	0.090
Central nervous system outcome					
Coma recovery time, hours- ITTβ	26.2 (23.0-67.8; 0,115.4)	11	56.5 (31.3-224.7; 0,260.3)	16	0.102
Coma recovery time, hours- PPβ	25.6 (22.3-67.8;0,115.4)	7	49 (31.1-115.2;0,260.3)	12	0.056

Data are number median (IQR; range), unless otherwise indicated. Mann Whiney-U was calculated for area above the curve. P values for time to event outcomes were calculated using log rank test (ζ) or Wilcoxon-Breslow-Gehan test if lines crossed (β). Fever clearance time A defined as time to first temperature below 37.5°C; fever clearance time B defined as time to first temperature that remained below 37.5°C for 24 hours; area above the 37.5°C temperature versus time curve was calculated using the trapezoidal rule. *Abbreviations:* ITT, intention-to-treat; PP, per protocol.

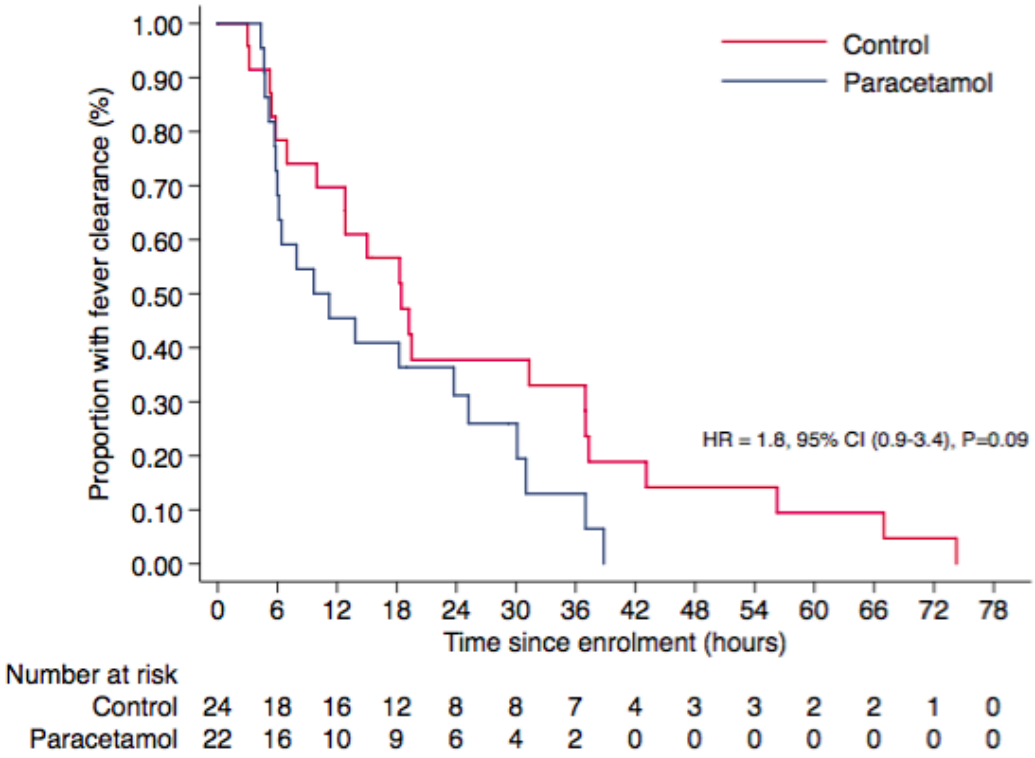


Figure 6.8. Kaplan-Meier comparing fever clearance in patients with severe and moderately severe malaria treated with either paracetamol or no paracetamol (control). Fever clearance defined as time to first aural temperature below 37.5°C from enrolment (FCTA). Patients were censored at the time of first aural temperature measuring less than 37.5°C or time of death. Sixteen patients were afebrile on admission. P value, Cox regression Wilcoxon-Breslow-Gehan test adjusted for baseline temperature.

a temperature after enrolment, the pre-specified area above the 37.5°C temperature versus time curve was calculated (Table 6.6). The time to fever clearance defined as the first time the temperature remained below 37.5°C for 24 hours was shorter in the paracetamol group compared to controls in the intention-to-treat (median, IQR: 37.0h, 24.7h – 66.1h versus 60.3h, 30.9h – 86.0h; *P* = 0.050) (Figure 6.9), and a similar trend was observed in the per protocol analysis (median, IQR: 31.0h, 18.3h – 44.1h versus 59.3h, 30.9h – 86.0h; *P* = 0.056) (Table 6.6). Excluding two patients who had a fever clearance time of greater than 300 hours due to likely secondary bacterial infection (one patient in each group), the fever clearance time remained shorter in the paracetamol arm compared to controls (median, IQR: 31.0h, 18.3h – 44.1h (n=28) versus 59.8h, 30.9h – 85.3h; *P* = 0.043).

Paracetamol effect on parasite clearance

There was a trend of a longer parasite clearance time, defined as the first of two consecutively negative thick smears (per 500 white blood cells), in the paracetamol arm compared to the control arm (median, IQR: 54.0h, 40.1h – 63.1h versus 42.1, 36.0h – 55.3h; *P* = 0.101) (Table 6.6) (Figure 6.10). When adjusted for baseline parasitaemia and disease severity the parasite clearance time was longer in the paracetamol group compared to controls (HR 1.9, 95% CI: 1.0-3.7, *P* = 0.040). One asplenic patient in the paracetamol group with a parasite clearance time of 509 hours was excluded from this analysis given the inherent inability to clear infected

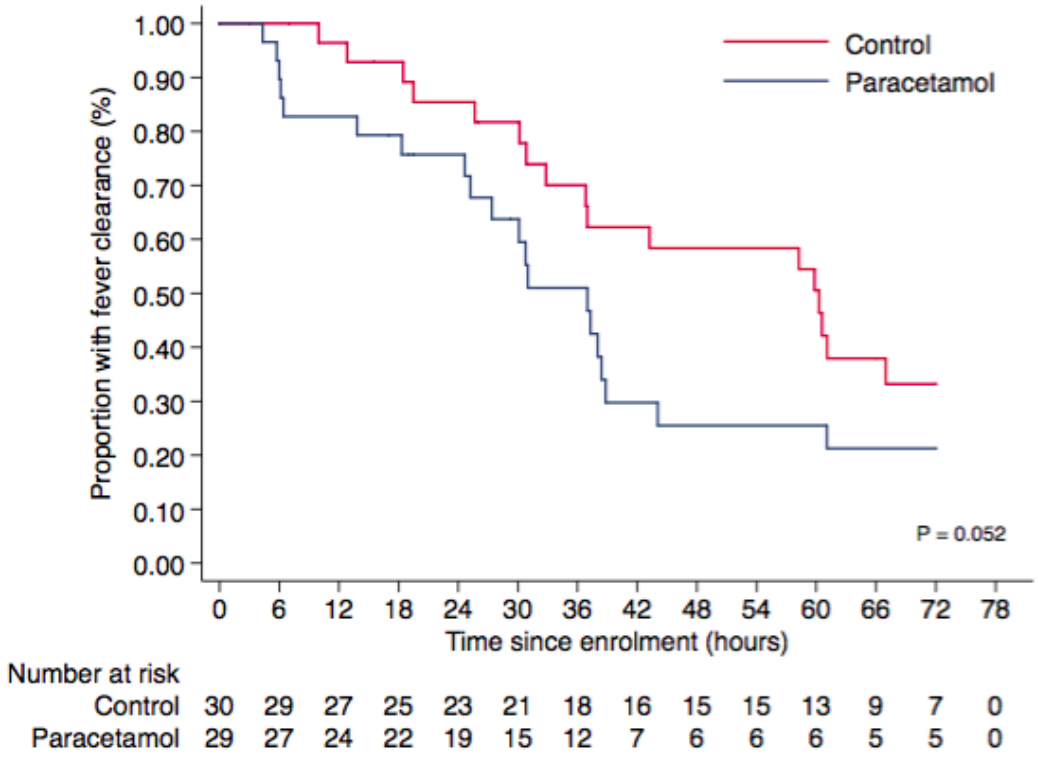


Figure 6.9. Kaplan-Meier comparing fever clearance in patients with severe and moderately severe malaria treated with either paracetamol or no paracetamol (control). Fever clearance defined as time to first aural temperature remaining below 37.5°C for 24 hours since enrolment (FCTB). Patients were censored at the time of first aural temperature measuring less than 37.5°C for a 24-hour duration or time of death. P value, Wilcoxon-Breslow-Gehan test.

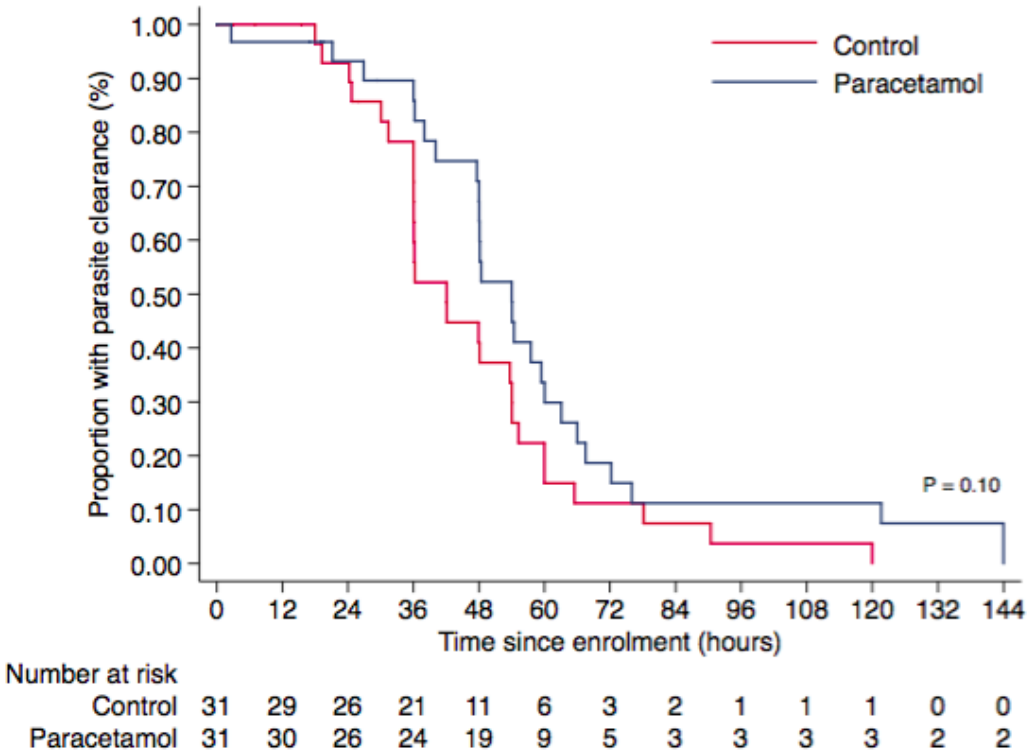


Figure 6.10 Kaplan-Meier comparing parasite clearance time in patients with severe and moderately severe malaria treated with either paracetamol or no paracetamol (control). Parasite clearance defined as time to two consecutive thick smears (per 500 white blood cells) since enrolment. Patients were censored at the time of first negative smear or time of death. P value Wilcoxon test excluding asplenic patient in paracetamol arm with PCT > 500 hours.

RBCs [533]. No infecting parasite strain had a mutation in the *Kelch 13* resistance gene.

Paracetamol effect on coma recovery

There was a trend of shorter coma recovery times in the paracetamol group compared to controls in the intention-to-treat analysis (median, IQR: 26.2h, 23.0h – 67.8h versus 56.5, 31.3h – 224.7h; P = 0.102) and per-protocol analysis (median, IQR: 25.6h, 22.3h – 67.8h versus 49.0, 31.3h – 115.2h; P = 0.056) (Table 6.6) (Figure 6.11). CFH inversely correlated with GCS (r_p = -0.61, P <0.001) and independently predicted GCS while adjusting for enrolment *Pf*HRP2 as an estimate of sequestration (R^2 = 0.44) (Supplementary Table 6.6).

Paracetamol effect on the liver

Patients in the paracetamol group showed increased serum ALT and AST at 72 hours from baseline compared to the control group (P = 0.030 and (P = 0.059, respectively) (Table 6.5), with a maximum concentration of ALT of 98 U/L and AST 260 U/L. No patient met criteria for Hy's Law of hepatotoxicity as the two patients in the paracetamol arm with a concomitant elevation in bilirubin was accounted for by elevated indirect bilirubin, in conjunction with additional laboratory parameters consistent with ongoing haemolysis.

Paracetamol pharmacokinetics

Of the 31 patients in the paracetamol arm, a total of 571 plasma paracetamol concentrations from 28 patients were included in the pharmacokinetic analysis, as described in detail in Chapter 5.1.2. In brief, the median C_{MAX} and T_{MAX} were 13.5 (4.67-24.5) mg/L and 0.50 (0.03-1.94)

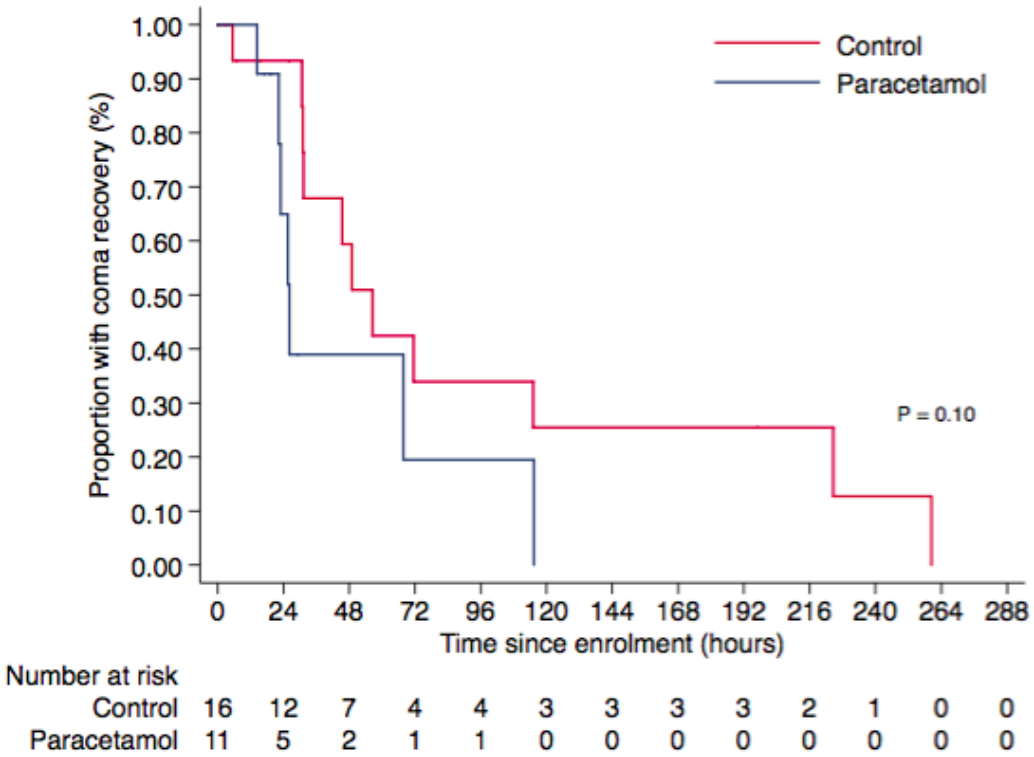


Figure 6.11. Kaplan-Meier comparing coma recovery time in patients with severe and moderately severe malaria treated with either paracetamol or no paracetamol (control). Coma recovery defined as time to Glasgow Coma Score (GCS) recovery of 15 since enrolment. Patients were censored at the time of first Glasgow Coma Score of 15 or time of death.

hours, respectively. The terminal half-life was 2.61 (1.79-3.54) hours and the AUC_{0-24} was 209 (80 - 548) mg*h/L. Simulations from the final population pharmacokinetic model showed that the steady state concentration of paracetamol was 9.0 mg/L when administered orally and approximately 15.1 mg/L when administered by NG tube (Figure 6.12). The AUC of paracetamol of each patient is shown in Supplementary Figures 6.7 – 6.10. The paracetamol AUC in the paracetamol group was higher compared to the control group (median, IQR: 385.9 (273.7-478.7) mg*h/L versus 6.7 (0.4-44.5) mg*h/L, *P* <0.001) (Table 6.5).

6.5 Discussion

This randomised open-label, controlled trial showed that paracetamol reduces kidney dysfunction and the odds of developing AKI during admission in patients with severe and moderately severe malaria, compared to controls. The protective effect of paracetamol was more pronounced in patients with high plasma concentrations of CFH caused by intravascular haemolysis, which is an inherent part of malaria pathophysiology and related to kidney injury. The beneficial effect of paracetamol on kidney function started to appear after 24 hours of treatment, and the effect was especially prominent in patients with high CFH concentration on enrolment.

The mechanism of paracetamol in reducing kidney dysfunction is likely through inhibition of haemoprotein-mediated lipid peroxidation by reduction of the higher oxidative state of iron in haemoproteins [66]. In an experimental

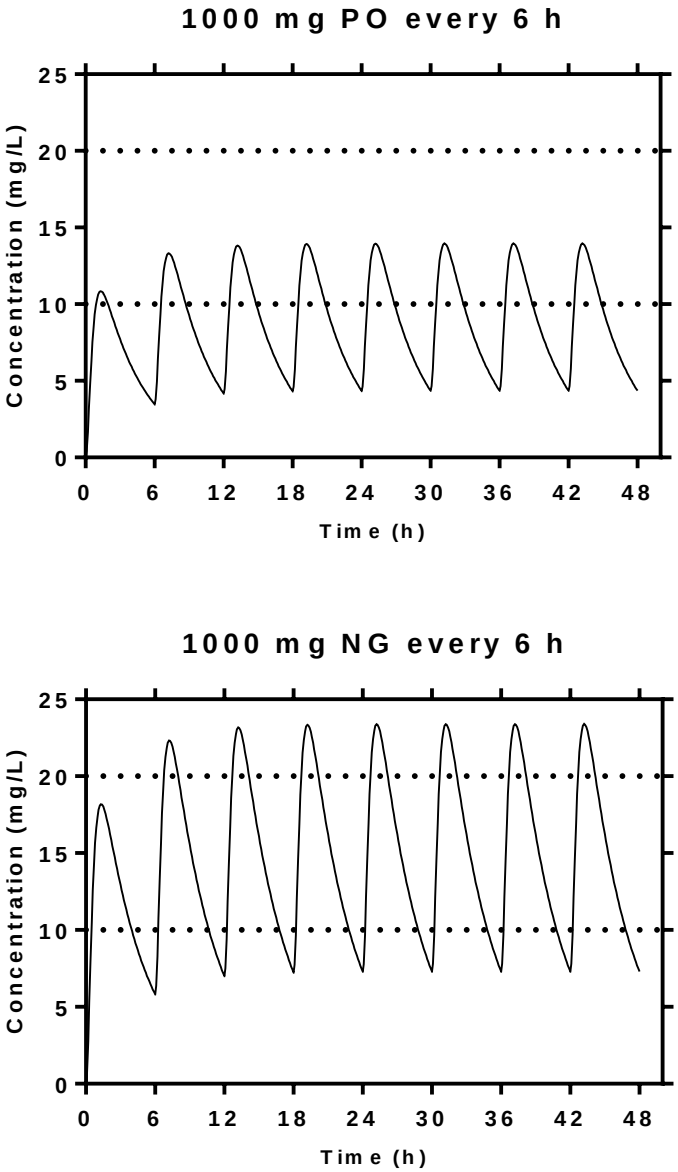


Figure 6.12. Simulations from the final population pharmacokinetic model for paracetamol. The lower and upper dashed lines represent the therapeutic plasma concentration range of paracetamol for fever control (10 – 20 mg/L).

model, inhibition of lipid peroxidation was shown to attenuate reductions of GFR and renal blood flow in rats infused with methaemoglobin [511]. Paracetamol has been shown to reduce a marker of lipid peroxidation, F2-isoprostanes, and kidney injury, and improve kidney function in a rat model of rhabdomyolysis [66]. In two RCTs in adults and children undergoing cardiopulmonary bypass surgery associated with high CFH, paracetamol reduced the intraoperative lipid peroxidation marker, plasma isofurans, compared to placebo [335, 336]. In an observational study of septic adults with high CFH, paracetamol was associated with lower plasma F2-isoprostanes and lower mortality [337]. The current study further supports that paracetamol reduces renal dysfunction in severe and moderately severe malaria with high CFH likely through reduction of oxidative iron in CFH. Although this mechanism was not directly assessed, it has been shown that elevated plasma F2-isoprostanes are associated with high CFH and malaria-associated AKI (Chapter 4.2). There was no difference in mortality or haemodialysis requirement between the paracetamol and control group, however this study was not powered to detect an effect on these endpoints.

By inhibiting the CFH-mediated oxidative damage and improving GFR, it is possible that the observed delay in creatinine reduction may be related to the time-dependent decrement in plasma creatinine [534]. Also, mixed effects regression showed that admission number of severity criteria and fractional sodium excretion were independently associated with the increase in creatinine from 0-24 hours. High fraction sodium excretion suggests

established ATN, which will not be preventable by treatment with paracetamol.

Despite the recommendation of paracetamol as an antipyretic in the WHO guidelines for the treatment of malaria, the routine use and antipyretic effect of paracetamol in malaria has been questioned [21, 535]. Limited prospective studies report that paracetamol is not an effective antipyretic in UM [324, 325]. A randomised study of paracetamol in 50 Gabonese children with UM, found that fever clearance time was shorter in the paracetamol group but this was not significantly different from controls [324]. The rectal paracetamol dose used in this study (50 mg/kg/day) has been shown to result in paracetamol concentrations below therapeutic antipyretic concentrations (10-20 μg/ml). Since no plasma paracetamol concentrations were assessed in the Gabonese study, it is possible that sub-therapeutic concentrations could explain why the reduced fever clearance time was not significant as shown in Chapter 5.1. A more recent randomised controlled study of oral paracetamol in uncomplicated paediatric malaria reported a non-significant fever reduction on day 1 of treatment, however this outpatient study of unobserved paracetamol only recorded temperatures twice daily and plasma paracetamol concentrations were not measured [325]. A study of oral paracetamol in Thai adults with UM showed that a single dose of paracetamol had a temperature lowering effect supported by a 2 and 3 hour post-dose plasma paracetamol concentration just below therapeutic range as these concentrations fall after the expected time to maximum concentration (1-2 hours post-dose). The current study is the first to report

the effect of paracetamol on fever in severe malaria patients and is supported by pharmacokinetic data. The fever clearance time, defined as the time to the first temperature below 37.5°C, was reduced by nearly 50% in the paracetamol group compared to controls in the per protocol analysis, which excluded patients that did not actually receive paracetamol. The time to fever clearance defined by time to temperature below 37.5°C for 24 hours was also shorter in the paracetamol group compared to the control group (*P* = 0.05). The pharmacokinetic simulations show that paracetamol administered orally at 1g every 6 hours resulted in a steady state drug level below 10 μg/ml whereas those that received paracetamol via nasogastric tube did achieve a steady state within the antipyretic range, thus the borderline significance of the reduction in fever clearance times may be due to those with a sub-therapeutic antipyretic concentration despite administering the maximum recommended dose. This further suggests that while a plasma concentration of 10-20 μg/ml may be necessary for an effective antipyretic effect, the renoprotective effect of paracetamol is achieved at lower plasma concentrations.

Paracetamol was shown to be associated with a prolonged parasite clearance in the Gabonese study in UM [324]. However, a meta-analysis of antipyretics in UM concluded that there is insufficient evidence to confirm an effect on parasitaemia [334]. *In vitro* studies have shown that febrile temperatures accelerate and increase cytoadherence of infected RBCs [331]. One explanation for an increased parasite clearance time is that by reducing fever with paracetamol there is less sequestration, resulting in an

increase in the peripheral blood parasitaemia, which would manifest as a prolonged parasite clearance time. The current study showed that there was a trend to a reduced parasite clearance time in the paracetamol group compared to controls. The effect of paracetamol on coma recovery time was explored since increased F2-isoprostanes have been shown to constrict retinal and brain microvessels *in vitro* [536] and increased levels in the cerebrospinal fluid in patients with subarachnoid haemorrhage are associated with cerebral vasospasm [537]. Further, other markers of lipid peroxidation have been shown to elevated in the CSF of patients with cerebral malaria [538]. While there were only 27 patients with an enrolment GCS of less than 15 in this study, there was a trend to a shorter coma recovery time in the paracetamol group compared to controls.

Studies evaluating paracetamol in UM have not reported serious adverse events or hepatotoxicity attributable to paracetamol [284, 326]. The current study shows that the maximum recommended daily dose of paracetamol in severe and moderately severe malaria patients results in a moderate an increase of aminotransferases, but no patient met the criteria for Hy's Law of hepatotoxicity, defined as aminotransferase elevation above 3-times the upper limit accompanied by increased total bilirubin and not explained by another cause. Hy's law is a specific predictor for severe hepatotoxicity used by the Food and Drug Administration to identify the potential for a drug to cause severe liver injury [539, 540]. The 2 patients with both an aminotransferase rise greater than 3 times the upper limit of normal and a total bilirubin > 2 mg/dl had alternative etiologies for the rise in total bilirubin.

In both cases, haemolysis was a cause of the elevated 72-hour total bilirubin as evidenced by the indirect bilirubin > 2 mg/dl with concomitant elevated LDH and decreased haematocrit, with one patient requiring three blood transfusions. Toxicity from paracetamol is unlikely to result from a single dose of < 150 mg/kg in a child, or 7.5 g-10 g in an adult [541]. The pharmacokinetic simulations show that the maximum peak paracetamol concentration of 23 µg/ml observed over 72 hours of regular dosing is far below the 150 µg/ml hepatotoxicity threshold four hours post-ingestion that necessitates N-acetylcysteine treatment [367, 368, 542].

There were limitations in this study. While the primary endpoint was prospectively defined to be stratified by intravascular haemolysis, the ROC analysis was performed on the same data set and not validated on external data. However, the mixed effect model adjusting for CFH concentration on enrolment clearly showed that the effect of paracetamol is dependent on enrolment CFH concentration. As the protocol required intense blood sampling to closely monitor kidney function, 8% of the total creatinine samples were not collected therefore missing creatinine values were assigned by multiple imputation. However, the complete case analysis was in agreement with the overall findings and no imputation was used for the primary endpoint analysis. The mechanism of CFH-mediated lipid peroxidation inhibition was not assessed in this study to support the action of paracetamol effect on kidney function. The analysis in Chapter 4.2 supports that CFH-mediated oxidative stress is associated with AKI in SM. Patients that received haemodialysis had artificially lowered creatinine values thus

patients were assigned an anephric rate of creatinine rise [532]. This is an accepted prediction of creatinine in haemodialysis patients.

6.6 Conclusions

In conclusion, this study showed that paracetamol improves kidney function and reduces the odds of developing AKI in severe and moderately severe malaria complicated by intravascular haemolysis. While this study was conducted in patients aged over 12 years, these findings may potentially have broader application since there are numerous reports of increased incidences of AKI in paediatric malaria [111, 144, 149], particularly in those with haemoglobinuria [84, 86, 88, 543]. Further, paracetamol may be beneficial in other human plasmodium infections, particularly severe knowlesi malaria, and other diseases that are characterised by high CFH and a high incidence of AKI, where a common mechanism of AKI may involve CFH-mediated lipid peroxidation [3, 65, 163, 164, 376, 446]. Paracetamol is inexpensive, safe and widely used, thus implementation of regularly dosed paracetamol as a preventative therapy to reduce kidney dysfunction in severe and moderately severe malaria would be achievable. Since renal dysfunction is a prognostic factor for death in SM, paracetamol has the potential to reduce mortality by preventing this serious complication. A larger trial of the use of paracetamol to reduce renal dysfunction in SM is warranted.

7.0 Summary

7.1 Clinical aspects of AKI and haemolysis in falciparum malaria

In chapter 3, malaria-associated AKI was characterised using the consensus KDIGO and WHO definitions, and G6PD deficiency as a host factor influencing haemolysis in adult Bangladeshi patients with falciparum malaria was assessed. It was shown that the incidence of AKI on enrolment was 44% compared to 16% using the KDIGO and WHO criteria, respectively. This suggests that malaria-associated AKI is under-reported and possibly under-diagnosed in adults. The fact that 47% of KDIGO defined AKI patients who were undiagnosed as AKI using the WHO criteria had a fatal outcome highlights the importance of early recognition of 'mild' AKI in severe malaria. The current WHO malaria guideline AKI definition consisting of a single cut-off creatinine level of 3 g/dl may be too strict as a definition, limiting early detection and treatment of early 'subclinical' deteriorating renal function in at risk patients. The use of other measures of renal function and testing of urinary or blood biomarkers of early AKI would therefore be an important area of future research to target this patient group.

Further, there was a strong association between AKI defined by KDIGO and fatal outcome with an odds ratio of 2.89 (1.76-4.73; $P < 0.001$), whereas AKI defined by the WHO criteria did not show this association. The applicability of the KDIGO criteria to both paediatric and adult populations would provide a harmonised definition of malaria-associated AKI for the WHO malaria guidelines that is aligned with the '0 by 25' initiative to reduce preventable

deaths due to AKI by 2025. KDIGO should be evaluated in paediatric malaria and be considered to replace the current criteria used to define malaria-associated AKI in the WHO malaria guidelines.

In a small cohort of Bangladeshi patients with falciparum malaria (n=62), 4.8% were found to be G6PD deficient by molecular analysis. The observed proportion agrees with the phenotypically assessed prevalence of G6PD deficiency in the same region. This study is first to report the G6PD deficiency variants present in Bangladesh, namely the Orissa, Kerala-Kalyan and Mahidol variants. This knowledge is important in the context of primaquine use for malaria control and elimination programs to reduce transmission since these variants have been shown elsewhere to result in 10-20% of normal G6PD enzyme activity. However, further studies are required in this population to determine the haemolytic risk of primaquine use in patients with these G6PD variants. An interesting observation in this study was the low estimated total parasite burden in the G6PD deficient patients, despite a long duration of illness before admission.

7.2 Pathophysiology of acute kidney injury in malaria

In chapter 4, it was shown that plasma suPAR and *Pf*HRP2 were independently associated with kidney injury and kidney dysfunction, as measured by tubular injury marker urine NGAL and RRT requirement, respectively. Since *Pf*HRP2 is an estimate of sequestered parasite biomass and suPAR is a measure of mononuclear cell activation, this study suggests

that both mechanisms contribute to the pathophysiology of malaria-associated AKI.

It was also shown that cell-free haemoglobin-mediated oxidative stress, as measured by plasma F2-isoprostanes and isofurans, was independently associated with AKI and RRT requirement in patients with severe falciparum malaria. Further, the association of decreased RCD with oxidative stress and kidney dysfunction suggested a mechanism whereby lipid peroxidation may have a dual adverse effect on the kidney. Firstly, the lipid peroxidation metabolites, plasma F2-isoprostanes, may cause direct renal vasoconstriction. Secondly, lipid peroxidation of RBC membranes may result in decreased RCD, which potentially exacerbates renal ischaemia, as rigid RBC perfusion through narrowed vessels containing sequestered infected RBCs would be reduced. These theories require confirmation in models of AKI in malaria, to determine their suitability as therapeutic targets.

As per the KDIGO guidelines, after risk stratification, early diagnosis and staging of AKI, patients should be managed according to cause [103]. Thus, these results suggest that a targeted intervention to reduce renal dysfunction in malaria includes prompt antimalarial treatment with artemisinins to rapidly eliminate ring-staged parasites to prevent their microvascular sequestration in vital organs including the kidney. Further study of the immunopathological mechanisms underlying malaria-associated AKI may reveal novel treatment options targeted to this contributing mechanism. Research into establishing a treatment to reduce renal injury secondary to effects of oxidative stress and haemolysis was assessed in this thesis.

7.3 Paracetamol as an adjunctive therapy in malaria

In chapter 5, the pharmacokinetics of paracetamol was assessed in uncomplicated, moderate and severe malaria, and the potential interaction between artesunate and paracetamol was evaluated. In uncomplicated malaria patients, the population pharmacokinetic properties of paracetamol showed that the bioavailability of oral suspension was 84.4% relative to intramuscular administration. Population pharmacokinetic modeling and simulations showed that paracetamol administered by intramuscular route and oral syrup at a repeated dosing interval of 1 g every 6 hours predicted therapeutic steady state concentration-time profiles. However, therapeutic steady state levels were reached more rapidly by adding a loading dose (total: 4.5 g/day). In severe falciparum malaria patients, the relative bioavailability of paracetamol was 67.6% higher when administered via nasogastric tube as compared to oral administration of tablets, which was likely due to the syrup formation as opposed to altered pharmacokinetics in severe disease. Dosing simulations showed that oral paracetamol tablets administered regularly at the maximum recommended dose (4 g/day) resulted in sub-therapeutic steady state concentrations. This may explain its purported lack of antipyretic effect in uncomplicated malaria studies. Based on this more robust model, simulations showed that higher regular doses of oral paracetamol tablets (1,500 mg every 6 hours; total: 6 g/day) would be required to achieve therapeutic steady state. Although these higher dosing regimens studied in different populations have not shown hepatotoxicity, the safety and efficacy of these higher doses in patients with severe malaria needs to be assessed carefully before these recommendations can be

made. However, paracetamol syrup administered 1g every 6 hours achieves therapeutic steady state in adults with both severe and uncomplicated malaria, and may thus be the most practical regimen that could most readily be incorporated as the formulation of choice into the WHO malaria guidelines. Pharmacokinetic studies of paracetamol in paediatric malaria are lacking and are required for dose optimisation.

A small preliminary *in vitro* drug susceptibility study in both laboratory and field isolates of *P. falciparum* showed no interaction between paracetamol and artesunate with respect to its parasitocidal effect. These results provide reassurance for the use of paracetamol as an antipyretic in severe malaria and for the RCT of paracetamol to assess its potential renoprotective effect in patients with severe and moderately severe malaria. They also suggest that any pharmacodynamic effect of paracetamol on parasite clearance is unlikely to be related to a drug-interaction.

7.4 Randomised controlled trial of paracetamol to prevent AKI in severe and moderately severe falciparum malaria

In chapter 6, it was shown that paracetamol improved kidney function where the beneficial effect was pronounced and increasingly sustained with time after 24 hours of treatment in patients with high CFH concentrations on enrolment ($P < 0.001$). It was also shown that paracetamol reduced the odds of developing AKI during hospital admission (odds ratio 0.26, 95% CI 0.07-0.94). Paracetamol reduced fever clearance time by 50%, which was supported by the inverse correlation between paracetamol AUC and fever

clearance time. It was also shown that the maximum recommended daily dose of paracetamol (4 g/day) administered to patients with severe and moderately severe malaria resulted in a moderate an increase of aminotransferases, but no patient met the criteria of Hy's Law of hepatotoxicity.

These results suggest that further studies are required to examine paracetamol use in paediatric falciparum malaria complicated by haemoglobinuria-associated kidney injury and in *P. knowlesi* malaria, which is also characterised by high CFH and AKI. Since renal dysfunction is a prognostic factor for death in severe malaria, paracetamol has the potential to reduce mortality by preventing this serious complication of severe malaria. A larger trial of the use of paracetamol to reduce renal dysfunction in severe malaria is warranted.

7.5 General conclusions

AKI is a serious complication of *P. falciparum* malaria and is associated with a high mortality. Accurately estimating the true magnitude and spectrum of kidney injury would facilitate research and implementation of preventative and treatment measures. Adopting the consensus KDIGO classification of AKI as a unified definition for both adult and paediatric malaria would permit consistent incidence data in addition to earlier recognition and treatment, which may improve patient outcomes.

The dose and route of paracetamol administration may need to be optimized for the desired therapeutic effect, which necessitates further

pharmacokinetic studies that include toxicity outcome data. The latter would be vital to ensure the safest and most effective dose reaches therapeutic concentrations in settings where drug monitoring for potential toxicity would be unavailable.

Cell-free haemoglobin-mediated kidney toxicity contributes to renal dysfunction in adult severe malaria, but could also be a common mechanism of renal injury in paediatric severe malaria and other human plasmodium infections (*P. knowlesi* and *P. vivax*). The finding that paracetamol reduces kidney dysfunction in adult falciparum malaria warrants larger clinical trials assessing the pathophysiology of AKI and potential use of paracetamol to reduce kidney injury in these populations.

8.0 Related publications

Aspects of this thesis were published and incorporated into the thesis with permission from the journals, including: Malaria Journal.

1. **Plewes K,** Royakkers AA, Hanson J, Hasan MMU, Alam S, Ghose A, Maude RJ, Stassen PM, Prakaykaew C, Lee SJ, Turner GDH, Dondorp AM, Schultz, MJ. *Correlation of Biomarkers for Parasite burden and Immune Activation with Acute Kidney Injury in Severe Falciparum. Malaria.* Malaria J, 2014;13:91.

2. **Plewes K,** Haider MS, Kingston HWF, Yeo TW, Ghose A, Hossain MA, Dondorp AM, Anstey NM. *Severe falciparum malaria treated with artesunate complicated by delayed onset haemolysis and acute kidney injury.* Malaria J, 2015; 14:246.

3. **Plewes K,** Maude RJ, Ghose A, Dondorp AM. Severe malaria complicated by prolonged haemolysis and rhinomaxillary mucormycosis after parasite clearance. *BMC Infect Dis* 2015; 15(1):555.

4. **Plewes K,** Kingston HWF, Ghose A, Maude RJ, Herdman MT, Uddin Hasan MU, Haider MS, Alam S, Piera K, Charunwatthana P, Yeo TW, Faiz MA, Lee SJ, Turner GDH, Anstey NM, Roberts LJ 2nd, White NJ, Day NPJ, Hossain MA, Dondorp AM. *Relationship of cell-free hemoglobin and oxidative stress to disease severity and acute kidney injury in falciparum malaria.* BMC Medicine, submitted.

5. Wattanakul T, **Plewes K**, Teerapong P, Newton P, Chierakul W, Ruangveerayuth R, White NJ, Dondorp AM, Tarning J. *Pharmacokinetic properties of intramuscular versus oral paracetamol in Plasmodium falciparum malaria.* Malaria J, submitted

Other publications during the period of this thesis:

1. Ishioka H, Ghose A, Charunwatthana P, Maude R, **Plewes K**, Kingston H, Intharabut B, Woodrow C, Chotivanich K, Sayeed AA, Hasan MU, Day NP, Faiz A, White NJ, Hossain A, Dondorp AM. Sequestration and red cell deformability as determinants of hyperlactatemia in falciparum malaria. *J. Infect Dis* 2015; Epub ahead of print.

2. Herdman MT, Sriboonvorakul N, Leopold SJ, Douthwaite S, Mohanty S, Hassan MU, Maude RJ, Kingston HWF, **Plewes K**, Charunwatthana P, Silamut K, Woodrow CJ, Chotinavich K, Hossain MA, Faiz MA, Mishra S, Leepipatpiboon, N, White NJ, Day NPJ, Tarning J, Dondorp AM. The role of previously unmeasured organic acids in the pathogenesis of severe malaria. *Critical Care* 2015; 19:317.

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4. Hanson J, Phu NH, Hasan MU, Charunwatthana P, **Plewes K**, Maude RJ, Prapansilp P, Kingston HW, Mishra SK, Mohanty S, Price RN, Faiz MA, Dondorp AM, White NJ, Hien TT, Day NPJ. The clinical implications of thrombocytopenia in adults with severe falciparum malaria: a retrospective analysis. *BMC Medicine* 2015; 13(1): 97.

5. Tun KM, Imwong M, Lwin KM, Win AA, Hlaing, TM, Hlaing T, Lin K, Kyaw MP, **Plewes K**, Faiz MA, Dhorda M, Cheah PY, Pukrittayakamee S, Ashley EA, Anderson TJ, Nair S, McDew-White M, Flegg JA, Grist EP, Guerin P, Maude RJ, Smithius F, Dondorp AM, Day NP, Nosten F, White NJ, Woodrow CJ. Spread of artemisinin-resistant *Plasmodium falciparum* in Myanmar: as cross-sectional survey of the K13 molecular marker. *Lancet Infectious Diseases* 2015; 15(4):415-421.

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Bangladesh

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Appendix A – Supplementary tables

Supplementary Table 1.4.1A Overview of observational studies of AKI prevalence in adult falciparum malaria cohorts in South Asia

LOCATION	YEAR	POPULATION	N	AKI	AKI CRITERIA	DIED	MAKI DIED	RRT	Reference
SOUTH ASIA									
Bikaner, Rajasthan	1994	Adults admitted with SM	532	2.1%	SCr >3mg/dL+ UO <400ml/d	11.1%	---	---	Kochar et al. 1997
Bikaner, Rajasthan	2001	Adults admitted with SM	192	6.3%	SCr >3mg/dL+ UO <400ml/d	10.9%	---	---	Kochar et al. 2006
Bikaner, Rajasthan	2003	Adults admitted with AM	214	28.0%	SCr >3mg/dL+ UO <400ml/d	---	30.0%	10.8%	Gupta et al. 2012
Bikaner, Rajasthan	2014	Adults admitted with AM	191	27.2%	SCr >3mg/dL+ UO <400ml/d	28.9%	27.8%	2.1%	Nayak et al. 2014
Rourkela, Odisha	1995-1998	All ages admitted with SM	452	18.6%	SCr >3mg/dL	4.5%	---	---	Mohanty et al. 2003
Rourkela, Odisha	2006	>12y admitted with SM	212	16.5%		16.0%	62.9%	---	Mishra et al. 2010
Bhubaneshwar, Odisha	1999-2008	>14y admitted with SM	301	41.9%	SCr >3mg/dL+ UO <400ml/d	35.4%	20.6%	--	Sahu et al. 2010
Guwahati, Assam	2006-2007	15-60y admitted with AM	100	28.0%	SCr >3mg/dL	1.8%	---	---	Saya et al. 2012
Mumbai, Maharashtra	1999	Adults admitted with AM	147	10.9%	SCr >5mg/dL	---	33.3%	--	Mehta et al. 2001
Mumbai, Maharashtra	2009	Adults admitted with AM	64	62.5%	SCr >3mg/dL+ UO <400ml/d	31.3%	--	--	Limaye et al. 2012
Mumbai, Maharashtra	2010-2011	>12y admitted with SM	223	55.2%	SCr >3mg/dL+ UO <400ml/d	16.1%	--	--	Nadkar et al. 2012
Mumbai, Maharashtra	--	>12y admitted with SM	301	30.2%	SCr >3mg/dL or UO<400ml/d	24.6%	52.8%	36.3%	Krishnan et al 2003
Satara, Maharashtra	2011	>18y admitted with SM	47	12.8%	SCr >2mg/dl	10.6%	16.7%	100.0%	Patil et al. 2012
Manipal, Karnataka	2007-2011	>18y admitted with AM	300	12.3%	SCr >3mg/dL	2.7%	16.2%	8.1%	Saravu et al. 2014
Chittagong, Bangaldesh	2003-2010	>14y admitted with SM	289	18.0%	SCr >3mg/dL	32.2%	40.4%	28.6%	Plewes, K et al. unpub
Chittagong, Bangladesh	2011-2014	>12y admitted with SM	119	28.6%	SCr >3mg/dL	---	---	---	Plewes, K et al. unpub

Abbreviations: AKI, acute kidney injury, MAKI, malaria-associated acute kidney injury; RRT, renal replacement therapy; SM, severe malaria; AM, acute malaria;SCr, serum creatinine; UO, urine output; y, years old.

Supplementary Table 1.4.1B *Overview of observational studies of AKI prevalence in adult falciparum malaria cohorts in Southeast Asia and Africa*

LOCATION	YEAR	POPULATION	N	AKI	AKI CRITERIA	DIED	MAKI DIED	RRT	Reference
SOUTH EAST ASIA									
Bangkok,Thailand	1991-2003	15-74y admitted with SM	915	21.3%	SCr >3mg/dL+ UO <400ml/d	3.8%	6.2%	87.7%	Vannaphan et al. 2010
Mae Sot, Thailand	2004-2008	>15y admitted with SM	262	43.9%	SCr >3mg/dL	21.0%	31.3%	46.1%	Win et al. 2012
Northern Thailand	1986-2002	>15y admitted with SM	315	18.1%	SCr >3mg/dL	24.4%	52.6%	45.6%	Newton et al. 2013
Mae Sot, Thailand	2004-2008	>15y admitted with SM	247	44.7%	SCr >3mg/dL	21.4%	31.3%	45.2%	Thanachartwet et al. 2013
Mae Sot, Thailand	2004-2008	>15y admitted with SM	247	73.9%	RIFLE	21.4%	25.8%	27.9%	Thanachartwet et al. 2013
Myanmar/Indonesia India/Bangladesh	2003-2005	>2y admitted with SM	1461	20.4%	BUN >47mg/dL	19.5%	45.0%	38.3%	Dondorp et al. 2005
Ho Chi Minh, Vietnam	1987-1988	Adults admitted with AM	346	30.1%	SCr >3mg/dL+ UO <400ml/d	38.2%	75.0%	100.0%	Trang et al. 1992
Ho Chi Minh, Vietnam	1991-1996	>14y admitted with SM	561		SCr >3mg/dL+ UO <400ml/d	14.8%	59.0%	76.0%	Hien et al. 1996
Sarawak, Malaysia	1996 - 2004	Adults admitted with AM	40	47.5%	SCr >3mg/dL+ UO <400ml/d	35.0%	42.1%	57.9%	Koh et al. 2006
Sabah, Malaysia	2010-2011	≥12y admitted with AM	122	23.1%	SCr >3 mg/dl	0.0%	0.0%	--	Barber et al. 2013
Sabah, Malaysia	2010-2011	≥12y admitted with AM	130	23.7%	SCr >3 mg/dl	0.0%	0.0%	22.2%	Barber et al. 2013
Sabah, Malaysia	2010-2011	≥12y admitted with AM	43	0.0%	SCr >3 mg/dl	0.0%	0.0%	--	Barber et al. 2013
Phenom Pehn, Cambodia	1985	Adults admitted with AM	373	16.6%	NR	--	--	--	Bouth et al. 1987
Battambang, Cambodia	2006-2009	>15y admitted with SM	217	7.4%	UO<400mg/d	--	--	0.0%	Lon et al. 2013
NCD, Papua New Guinea	1990-1992	>12y admitted with SM	79	22.9%	SCr>565 µmol/l	18.6%	37.5%	18.8%	Lalloo et al. 1996
Timika, Indonesia	2005-2006	>18y admitted with SM	51	33.3%	SCr>3 or UO<400ml/d	13.7%	--	--	Yeo et al. 2007
AFRICA									
Moshi, Tanzania	1998-2001	>15y admitted with SM	82	48.8%	SCr>3mg/dl	15.2%	35.0%	0.0%	Robinson et al. 2006
Bujumbura, Burundi	1991-1992	Adult admitted with CM	31	12.9%	SCr>3mg/dl	22.6%	75.0%	0.0%	Niyongabo et al. 1994
Kassala, Eastern Sudan	2011-2012	Adults admitted with SM	113	0.9%	SCr >3mg/dL+ UO <400ml/d	2.7%	100%	0.0%	Abdallah et al. 2013
Dakar, Senegal	2007-2008	Adults admitted with SM	87	28.7%	SCr >3mg/dL+ UO <400ml/d	33.3%	48.0%	---	Wade et al. 2012

Abbreviations: AKI, acute kidney injury, MAKI, malaria-associated acute kidney injury; RRT, renal replacement therapy; SM, severe malaria; AM, acute malaria;SCr, serum creatinine; UO, urine output; y, years ol

Supplementary Table 1.4.2 Overview of observational studies of malaria-associated AKI prevalence in paediatric falciparum malaria cohorts by region

LOCATION	YEAR	POPULATION	N	MAKI	AKI CRITERIA	DIED	MAKI DIED	RRT	Reference
SOUTH ASIA									
Berhampur, Odisha	1989-1991	<12y with AM	50	6.0%	Oliguria	16.0%	66.7%	33.3%	Bag *et al.* 1994
Berhampur, Odisha	2001-2002	Children with SM	216	14.8%	SCr >3mg/dL	9.3%	---	---	Mohanty *et al.* 2004
Berhampur, Odisha	2001-2003	<14y with SM	242	14.5%	SCr >3mg/dL	9.9%	11.4%	---	Satipathy *et al.* 2004
Rourkela, Odisha	1995-1998	<15y with SM	156	0.6%	SCr >3mg/dL	4.5%	---	0.0%	Mohanty *et al*. 2003
Rourkela, Odisha	2010	Children with SM	303	0.8%	SCr >3mg/dL	7.2%	---	---	Nanda *et al.* 2011
Bikaner, Rajasthan	2007-2008	<15y with SM	79	30.4%	SCr >3mg/dL+UO <400ml/d	7.6%	---	---	Kochar *et al.* 2010
Aligarh, Uttar Pradesh	1987	2-12y with AM	35	20.0%	CrCl <29.3ml/min	0.0%	0.0%	---	Ahmad *et al.* 1989
Bankura ,West Bengal	2010-2011	<8y with SM	47	8.5%	SCr > 2.5mg/dL	21.3%	50.0%	---	Ganguly *et al.* 2012
Cuttack, Odisha	2006-2008	<14y with AM	108	50.9%	SCr >1.5 mg/dL or SCr >0.3 mg/dL from bl	---	---	---	Padhi *et al.* 2013
Mumbai, Maharashtra	2009	1m-12y with AM	94*	9.6%	SCr >3mg/dL+UO <400ml/d	---	22.2%	33.3%	Zaki *et al.* 2013
AFRICA									
Kinshasa, DRC	2005-2010	<15y with SM	410	48.0%	BUN > 20 mg/dL	--	6.6%	--	Dondorp *et al.* 2010
Biera, Mozambique	2005-2011	<15y with SM	651	25.8%	BUN > 20 mg/dL	--	31.0%	--	Dondorp *et al.* 2010
Banjul,The Gambia	2005-2012	<15y with SM	424	24.3%	BUN > 20 mg/dL	--	23.3%	--	Dondorp *et al.* 2010
Kumasi, Ghana	2005-2013	<15y with SM	301	26.2%	BUN > 20 mg/dL	--	6.6%	--	Dondorp *et al.* 2010
Kilifi, Kenya	2005-2014	<15y with SM	354	19.5%	BUN > 20 mg/dL	--	27.5%	--	Dondorp *et al.* 2010
Tanga, Tanzania	2005-2015	<15y with SM	1355	19.3%	BUN > 20 mg/dL	--	31.4%	--	Dondorp *et al.* 2010
Ilorin, Nigeria	2005-2016	<15y with SM	331	18.4%	BUN > 20 mg/dL	--	24.6%	--	Dondorp *et al.* 2010
Mbarara, Uganda	2005-2017	<15y with SM	626	31.9%	BUN > 20 mg/dL	--	13.5%	--	Dondorp *et al.* 2010
Kigali, Rwanda	2005-2018	<15y with SM	169	63.9%	BUN > 20 mg/dL	--	8.3%	--	Dondorp *et al.* 2010
Kinshasa, DRC	2008	<14y with SM	378	23.5%	SCr ≥0.3mg/dl, or SCr >50% or UO<12ml/kg/d	12.6%	12.6%	25.8%	Kunuannuna *et al.* 2013
Lome, Togo	2000-2002	0-15y with SM	361	3.0%	SCr >3mg/dL+UO <400ml/d	9.7%	27.3%	N/A	Gbadoe *et al.* 2006
Banjul, The Gambia	1997-2009	4m-14y with SM	2901	0.6%	UO <12ml/kg/d	13.4%	*52.9%*	N/A	Jallow *et al.* 2012
Banjul, The Gambia	1992-1993	1-9y with CM	80	31.3%	SCr >62µmol/L	26.0%	28.0%	---	Weber *et al.* 1999
Kilifi, Kenya	1992-2004	<14y with SM	3659	18.2%	SCr >80µmol/L	---	---	---	Idro *et al.* 2007
Dakar, Senegal	1997-1999	<16y with SM	311	2.3%	SCr >3mg/dL+UO <400ml/d	14.0%	42.9%	---	Imbert *et al.* 2003
Dakar, Senegal	1990-1996	<15y with SM	161	2.5%	SCr >3mg/dL	---	---	---	Imbert *et al.* 1997

Supplementary Table 1.4.3 Overview of observational studies of malaria-associated AKI prevalence in tropical AKI population by region

LOCATION	YEAR	POPULATION	N	MAKI	AKI CRITERIA	DIED	MAKI DIED	MAKI RRT	Reference
ADULTS									
SOUTH + SOUTHEAST ASIA									
Ahmadabad, Gujarat	2008-2009	Adm w AKI	958	8.9%	SCr >3mg/dL+UO <400ml/d	---	21.0%	78.0%	Kanodia *et al.* 2010
Varanasi, Uttar Pradesh	1984-1994	15-85y Adm w AKI	540	4.8%	NR	---	30.8%	57.7%	Prakash *et al.* 1996
Varanasi, Uttar Pradesh	1995-2000	Adm with AKI	577	12.8%	NR	---	---	---	Prakash *et al.* 2003
Varanasi, Uttar Pradesh	2010-2011	>18y adm w AKI	85	--	SCr >2mg/dL for >24 hours	---	9.9%	36.6%	Shukla *et a*l. 2013
Lucknow, Uttar Pradesh	2005-2009	Adm w AKI	186	15.1%	SCr >2mg/dL	--	20.0%	88.6%	Kaul *et al.* 2012
Chennai, Tamil Nadu	1995-2004	Adm w AKI	1112	4.1%	SCr >2mg/dL	19.6%	26.5%	77.5%	Jayakumar *et al.* 2006
Karachi, Pakistan	1990-1999	Transplant referals	2098	5.8%	SCr >2mg/dL	---	25.8%	79.8%	Naqvi *et al.* 2003
Karachi, Pakistan	2003-2004	Adm to nephrology	237	19.4%	SCr >3mg/dL+UO <400ml/d	---	23.9%	78.3%	Manan *et al.* 2006
Bangkok, Thailand	1991-1997	>16y adm w AKI	112	--	SCr >3mg/dL+UO <400ml/d	---	10.7%	89.8%	Wilairatana *et al.* 1999
CHILDREN									
INDIA									
Pondicherry, Tamil Nadu	2010-2011	1m-13y adm w AKI	2161	0.6%	AKIN criteria	46.2%	--	--	Krishnamurthy *et al.* 2013
AFRICA									
Brazaville, DRC	1989-1994	1m-17y adm w AKI	105	12.4%	SCr >15 +normal size kidneys	37.1%	---	---	Assounga *et al.* 2000
Kinshasa, DRC	2000-2007	<13y adm to ICU	56	42.9%	SCr incr for age & height UO <1ml/kg/h	25.0%	---	---	Aloni *et al*. 2012
Kinshasa, DRC	1994-2003 2004-2007	<14y adm w AKI	63	69.8%	CrCl <50 ± UO < 0.5ml/kg/h	22.2%	18.2%	57.0%	Bodi *et al*. 2014
Port Harcourt, Nigeria	1985-2003	<16y adm w AKI	211	13.7%	SCr or urea inc ± UO <1ml/kg/h	40.3%	---	---	Anochie *et al.* 2005
Osun State, Nigeria	1994-2003	<15y adm w AKI	143	25.9%	UO <300ml/m2/d + abn urea/chem UO >300ml/kg/d + abn chem UO < 1ml/kg/d + abnchem	46.2%	24.3%	32.0%	Olowu *et al.* 2004
Lagos State, Nigeria	2010-2012	1m-16y adm w AKI	70	11.4%	pRIFLE	28.4%	---	38.0%	Esezobor *et al.* 2012
Accra, Ghana	--	1m-8y adm w AKI	13	38.5%	Oliguria+uremia+low SG/Osm	23.1%	20.0%	---	Adu *et al.* 1984
Abidjan, Ivory Coast	2008-2013	1m-15y adm w AKI	88	31.8%	SCr ± 200µmol/l	36.0%	---	---	Diarrassouba *et al.* 2015

Abbreviations: AKI, acute kidney injury, MAKI, malaria-associated acute kidney injury; RRT, renal replacement therapy; SCr, serum creatinine; UO, urine output.

Supplementary Table 4.2.1. Percent change from baseline at 24, 48, and 72 hours of oxidative stress measures, lactate dehydrogenase and creatinine clearance by clinical course of renal function during admission

Groups	N	24 hours			48 hours			72 hours		
		Median (IQR; range)		*P*	Median (IQR; range)		*P*	Median (IQR; range)		*P*
1. AKI on admission	**26**									
% Δ CrCl (ml/min)		-9	(-33 to 1;-65,113)	0.214	-11	(-44 to 27;-73,115)	0.715	-4	(-22 to 16;-76,70)	0.068
% Δ LDH (U/L)		14	(-16 to 64;-39, 294)	0.208	5	(-23 to 20;-48, 79)	0.715	-3	(-32 to 4;-54,35)	0.285
% Δ pF2-IsoP (pg/ml)		1	(-12 to 28;-45,192)	0.917	-6	(-20 to 43;-57,146)	0.500	-10	(-32 to 36;-54,98)	0.715
% Δ pIsoF (pg/ml)		8	(-28 to 97;-47, 244)	0.463	36	(-27 to 177;-46, 640)	0.686	-29	(-46 to 7;-55, 411)	0.068
uF2-IsoP (ng/24h)[v]		0.9	(0.7 to 2.5;0.1,4.4)	-		--			--	
uIsoF (mg/24h)[v]		9.4	(-4.4 to 17.3;0.2,66.1)	-		--			--	
2. Developed AKI	**8**									
% Δ CrCl (ml/min)		-28	(-53 to -14;-69,-8)	**0.043**	-31	(-61 to -14;-83,-10)	**0.043**	-27	(-52 to -6;-60,0)	0.095
% Δ LDH (U/L)		61	(58 to 105;18, 127)	**0.043**	33	(17 to 205;-31,99)	0.109	4	(-10 to 38;-10,38)	0.593
% Δ pF2-IsoP (pg/ml)		13	(5 to 65;-0.4,114)	0.144	94	(-3 to 134;-2.7,134)	0.285	45	(10 to 89;-6, 102)	0.068
% Δ pIsoF (pg/ml)		47	(38 to 242;33, 434)	0.068	48	(14 to 123;14,123)	0.109	38	(15 to 65;-9, 92)	0.144
uF2-IsoP (ng/24h)[v]		4.1	(1.4 to 5.2;1.4,5.2)	-		--			--	
uIsoF (mg/24h)[v]		30.4	(3.7 to 84.0;2.7,112.0)	-		--			--	
3. Recovered from AKI	**8**									
% Δ CrCl (ml/min)		20	(1 to 80;0, 86)	**0.035**	27	(12 to 86;-19,117)	0.075	70	(17 to 113;13, 157)	**0.028**
% Δ LDH (U/L)		-28	(-51 to -6;-55,-3)	0.068	-30	(-54 to -27;-54,-27)	0.109	-28	(-50 to -8;-68,6)	0.144
% Δ pF2-IsoP (pg/ml)		-26	(-28 to 8;-28,8)	0.285	-19	(-38 to 93;-38,93)	1.000	--	---	--
% Δ pIsoF (pg/ml)		-20	(-76 to 300;-76,300)	1.000	-17	(-66 to 87;-66,87)	1.000	--	---	--
uF2-IsoP (ng/24h)[v]		2.9	(1.9 to 3.4;1.9,3.4)	-		--			--	
uIsoF (mg/24h)[v]		3.9	(1.6 to 6.8;1.6,6.8)	-		--			--	
4. No AKI	**13**									
% Δ CrCl (ml/min)		0	(-8 to 18;-23, 43)	0.275	20	(1 to 47;-23, 73)	0.050	17	(-22 to 103;-25, 122)	0.237
% Δ LDH (U/L)		2.5	(-19 to 127;-24, 152)	0.182	-26	(-31 to 51;-43, 101)	0.767	-32	(-40 to 55;-40, 56)	0.686
% Δ pF2-IsoP (pg/ml)		7	(6 to 14;0.1,16)	**0.043**	21	(21 to 31;-31,100)	0.225	46	(9 to 46;9,46)	0.109
% Δ pIsoF (pg/ml)		51	(-13 to 250;-23, 360)	0.225	85	(43 to 340;-32, 457)	0.080	68	(38 to 771;38, 771)	0.109
uF2-IsoP (ng/24h)[v]		2.0	(1.1 to 3.6;0.9,4.0)	-		--			--	
uIsoF (mg/24h)[v]		9.2	(5.6 to 50.2;-5.5,89.1)	-		--			--	

Supplementary Table 6.1. Mean percent change creatinine from admission of longitudinal creatinine concentrations intention-to-treat analysis

	Paracetamol	N	Control	N	*P*
Mean % change from admission					
SCr adm (µmol/L)	155 (105;80,450)	31	174 (131;88,557)	31	0.51
% peak Δ SCr	2 (33 ;-31,138)	31	18 (52;-32,167)	30	0.17
% Δ SCr 12 h	-5 (17;-31,62)	31	-5 (18;-32,32)	30	0.98
% Δ SCr 24 h	-5 (32;-33,138)	28	-6 (24;-42,56)	28	0.93
% Δ SCr 36 h	-18 (18;-44,44)	27	-3 (36;-47,87)	27	0.05
% Δ SCr 48 h	-18 (20;-48, 59)	27	1 (42;-47,111)	27	**0.036**
% Δ SCr 60 h	-22 (23;-49,74)	27	3 (52;-47,139)	27	**0.028**
% Δ SCr 72 h	-22 (27;-53,88)	27	7 (60;-47,167)	27	**0.026**
Mean % change CFH > 48,000 ng/ml					
SCr adm (µmol/L)	184 (121;80,439)	13	209 (160;88,557)	16	0.64
% peak Δ SCr	-6 (14;-31,19)	13	30 (53;-20,167)	15	**0.026**
% Δ SCr 12 h	-6 (14;-31,19)	13	3 (18;-20,32)	15	0.13
% Δ SCr 24 h	-18 (9;-33,-9)	10	3 (27;-30,56)	13	**0.024**
% Δ SCr 36 h	-27 (12;-43,-11)	10	9 (35;-30,83)	12	**0.006**
% Δ SCr 48 h	-28 (12;-48,-10)	10	15 (44;-30,111)	12	**0.009**
% Δ SCr 60 h	-34 (11;-49,-20)	10	17 (56;-33,139)	12	**0.012**
% Δ SCr 72 h	-35 (12;-53,-20)	10	22 (66;-33,167)	12	**0.013**
Mean % change CFH ≤ 48,000 ng/ml					
SCr adm (µmol/L)	134 (90;80,451)	18	138 (79;88,398)	15	0.89
% peak Δ SCr	9 (40;-20,138)	18	6 (51;-32,152)	15	0.88
% Δ SCr 12 h	-4 (19;-20,62)	18	-14 (13;-32,17)	15	0.12
% Δ SCr 24 h	3 (37;-28,138)	18	-13 (20;-42,33)	15	0.15
% Δ SCr 36 h	-12 (19;-44,44)	17	-12 (35;-47,87)	15	0.93
% Δ SCr 48 h	-13 (22;-43,59)	17	-10 (38;-47,94)	15	0.78
% Δ SCr 60 h	-15 (26;-47,74)	17	-8 (47;-47,120)	15	0.62
% Δ SCr 72 h	-14 (30;-53,88)	17	-4 (55;-47,152)	15	0.54

*Data are means (SD;max,min). Approximately 8% of total creatinine time points were missing sample collections (median, range; 1 (0,5). Multiple imputation procedure with five rounds was employed. The number and time point of each imputed value is detailed in Supplementary Table 1. Significant in bold, *P*<0.05 using Student's *t* test. *Abbreviations*: SCr; serum creatinine; CFH, cell-free haemoglobin.

Supplementary Table 6.2. Median percent change creatinine from admission of longitudinal creatinine concentrations intention-to-treat analysis

	Paracetamol	N	Control	N	*P*
Median % change from admission					
SCr adm (µmol/L)	106 (97 to 169;80,450)	31	115 (97 to 168;88,557)	31	0.46
% peak Δ SCr	-8 (-10 to 8;-31,138)	31	-2 (-17 to 27;-32,167)	30	0.56
% Δ SCr 12 h	-9 (-15 to 15;-31,62)	31	-7 (-18 to 6;-32,32)	30	1.00
% Δ SCr 24 h	-11 (-20 to 0;-33, 138)	28	-14 (-23 to 12;-42, 56)	28	0.99
% Δ SCr 36 h	-18 (-31 to -9;-44,44)	27	-17 (-29 to 9;-47,87)	27	0.21
% Δ SCr 48 h	-20 (-31 to -10;-48, 59)	27	-15 (-25 to 12;-47,111)	27	0.13
% Δ SCr 60 h	-22 (-38 to -17;-49,74)	27	-20 (-30 to 0;-47,139)	27	0.12
% Δ SCr 72 h	-22 (-37 to -17;-53,88)	27	-14 (-30 to 0;-47,167)	27	**0.043**
Median % change CFH > 48,000 ng/ml					
Scr adm (µmol/L)	141 (97 to 221;80,439)	13	118 (97 to 327;88,557)	16	0.69
% peak Δ SCr	-8 (-10 to 0;-31,19)	13	5 (-8 to 71;-20,167)	15	**0.032**
% Δ SCr 12 h	-8 (-11 to 15;-31,19)	13	4 (-17 to 16;-20,32)	15	0.17
% Δ SCr 24 h	-17 (-23 to -10;-33,-9)	10	-9 (-15 to 24;-30,56)	13	0.07
% Δ SCr 36 h	-28 (-38 to -18;-43,-10)	10	-4 (-19 to 39;-30,83)	12	**0.003**
% Δ SCr 48 h	-29 (-37 to -18;-48,-10)	10	-5 (-17 to 52;-30,111)	12	**0.004**
% Δ SCr 60 h	-33 (-44 to -22;-49,-20)	10	-8 (-25 to 65;-33,139)	12	**0.005**
% Δ SCr 72 h	-37 (-48 to -22;-53,-20)	10	-7 (-29 to 78;-33,167)	12	**0.004**
Median % change CFH ≤ 48,000 ng/ml					
SCr adm (µmol/L)	106 (97 to 115;80,115)	18	115 (97 to 150;88,398)	15	0.55
% peak Δ SCr	-4 (-10 to 9;-20,138)	18	-10 (-20 to 0;-32,152)	15	0.24
% Δ SCr 12 h	-9 (-17 to 0;-20,62)	18	-17 (-23 to -7;-32,17)	15	0.14
% Δ SCr 24 h	-10 (-17 to 9;-28,138)	18	-20 (-26 to 0;-42,33)	15	0.06
% Δ SCr 36 h	-16 (-20 to -8;-44,44)	17	-20 (-35 to -8;-47,87)	15	0.40
% Δ SCr 48 h	-17 (-23 to -9;-43,59)	17	-20 (-30 to -9;-47,94)	15	0.47
% Δ SCr 60 h	-18 (-25 to -11;-47,74)	17	-20 (-33 to -14;-47,120)	15	0.58
% Δ SCr 72 h	-20 (-25 to -8;-53,88)	17	-20 (-30 to -9;-47,152)	15	0.68

*Data are medians (IQR;max, min). Approximately 8% of total creatinine time points were missing sample collections (median, range; 1 (0,5). Multiple imputation procedure with five rounds was employed. The number and time point of each imputed value is detailed in Supplementary Table 1. Significant in bold, *P*<0.05 using Whitney Mann *U* test. *Abbreviations*: SCr; serum creatinine; CFH, cell-free haemoglobin.

Supplementary Table 6.3. Mean percent change creatinine from admission of longitudinal creatinine concentrations intention-to-treat analysis complete case analysis

	Paracetamol	N	Control	N	*P*
Mean % change from admission					
SCr adm (µmol/L)	155 (105;80,451)	31	175 (131;88,557)	31	0.51
% peak Δ SCr	2 (33;-31,138)	30	19 (53;-32,167)	29	0.15
% Δ SCr 12 h	-6 (18;-31,62)	27	-5 (18;-32,32)	27	0.86
% Δ SCr 24 h	-5 (32;-33, 138)	28	-6 (25;-42,56)	27	0.89
% Δ SCr 36 h	-17 (18;-44,44)	19	-3 (36;-47,83)	20	0.15
% Δ SCr 48 h	-18 (21;-48, 59)	26	-2 (39;-47,111)	25	0.08
% Δ SCr 60 h	-22 (24;-47,74)	23	0 (48;-47,139)	24	0.05
% Δ SCr 72 h	-23 (27;-53,88)	26	9 (61;-47,167)	26	**0.018**
Mean % change CFH > 48,000 ng/ml					
SCr adm (µmol/L)	184 (121;80,439)	13	209 (160;88,557)	16	0.64
% peak Δ SCr	-8 (13;-31,15)	12	32 (54;-20,167)	14	**0.018**
% Δ SCr 12 h	-8 (13;-31,15)	11	6 (19;-20,32)	12	0.06
% Δ SCr 24 h	-18 (9;-33,-9)	10	3 (28;-30,56)	12	**0.031**
% Δ SCr 36 h	-23 (11;-38,-10)	7	11 (38;-30,83)	10	**0.034**
% Δ SCr 48 h	-28 (12;-48,-10)	10	15 (44;-30,111)	12	**0.009**
% Δ SCr 60 h	-32 (10;-47,-20)	9	17 (56;-33,139)	12	**0.020**
% Δ SCr 72 h	-35 (12;-53,-20)	10	22 (66;-33,167)	12	**0.013**
Mean % change CFH ≤ 48,000 ng/ml					
SCr adm (µmol/L)	134 (90;80,451)	18	138 (79;88,398)	15	0.89
% peak Δ SCr	9 (40;-20,138)	18	6 (51;-32,152)	15	0.87
% Δ SCr 12 h	-4 (20;-20,62)	16	-14 (13;-32,17)	15	0.14
% Δ SCr 24 h	3 (37;-28,138)	18	-13 (20;-42,33)	15	0.15
% Δ SCr 36 h	-13 (21;-44,44)	12	-18 (28;-47,50)	10	0.64
% Δ SCr 48 h	-12 (23;-43,59)	16	-17 (28;-47,67)	13	0.55
% Δ SCr 60 h	-16 (28;-47,74)	14	-17 (34;-47,83)	12	0.97
% Δ SCr 72 h	-15 (31;-53,88)	16	-2 (56;-47,152)	14	0.43

*Data are means (SD;max,min). Significant in bold, *P*<0.05 using Student's *t* test. *Abbreviations*: SCr; serum creatinine; CFH, cell-free haemoglobin.

Supplementary Table 6.4. Mean percent change creatinine from admission of longitudinal creatinine concentrations per protocol analysis

	Paracetamol	N	Control	N	*P*
Mean % change from admission					
SCr adm (µmol/L)	135 (82;80,451)	26	170 (133;88,557)	26	0.27
% peak Δ SCr	-5 (22;-31,88)	26	20 (56;-20,167)	26	**0.042**
% Δ SCr 12 h	-10 (10;-31,15)	26	-6 (17;-32,28)	26	0.35
% Δ SCr 24 h	-11 (15;-33,29)	26	-5 (25;-42,56)	26	0.31
% Δ SCr 36 h	-19 (18;-44,44)	26	-1 (36;-47,87)	26	**0.030**
% Δ SCr 48 h	-19 (20;-48,59)	26	3 (42;-47,111)	26	**0.022**
% Δ SCr 60 h	-23 (23;-49,74)	26	4 (52;-47,139)	26	**0.019**
% Δ SCr 72 h	-23 (27;-53,88)	26	9 (61;-47,167)	26	**0.018**
Mean % change CFH > 48,000 ng/ml					
SCr adm (µmol/L)	132 (60;80,265)	10	212 (171;88,557)	12	0.17
% peak Δ SCr	-12 (10;-31,0)	10	34 (58;-20,167)	12	**0.021**
% Δ SCr 12 h	-12 (10;-31,0)	10	2 (18;-20,28)	12	**0.041**
% Δ SCr 24 h	-18 (9;-33,-9)	10	3 (27;-30,56)	12	**0.031**
% Δ SCr 36 h	-27 (12;-43,-11)	10	9 (35;-30,83)	12	**0.006**
% Δ SCr 48 h	-28 (12;-48,-10)	10	15 (44;-30,111)	12	**0.009**
% Δ SCr 60 h	-34 (11;-49,-20)	10	17 (56;-33,139)	12	**0.012**
% Δ SCr 72 h	-35 (12;-53,-20)	10	22 (66;-33,167)	12	**0.013**
Mean % change CFH ≤ 48,000 ng/ml					
SCr adm (µmol/L)	137 (95;80,451)	16	133 (80;88,398)	14	0.90
% peak Δ SCr	0 (26;-20,88)	16	8 (53;-32,152)	14	0.62
% Δ SCr 12 h	-9 (10;-20,15)	16	-13 (14;-32,17)	14	0.29
% Δ SCr 24 h	-6 (16;-28,-29)	16	-12 (20;-42,33)	14	0.37
% Δ SCr 36 h	-14 (19;-44,44)	16	-9 (36;-47,87)	14	0.70
% Δ SCr 48 h	-14 (22;-43,59)	16	-8 (39;-47,94)	14	0.59
% Δ SCr 60 h	-16 (26;-47,74)	16	-7 (48;-47,120)	14	0.49
% Δ SCr 72 h	-14 (30;-53,88)	17	-2 (56;-47,152)	14	0.43

*Data are means (SD;max, min). Approximately 8% of total creatinine time points were missing sample collections (median, range; 1 (0,5). Multiple imputation procedure with five rounds was employed. The number and time point of each imputed value is detailed in Supplementary Table 1. Significant in bold, *P*<0.05 using Student's *t* test. *Abbreviations*: SCr; serum creatinine; CFH, cell-free haemoglobin.

Supplementary Table 6.5. Median percent change creatinine from admission of longitudinal creatinine concentrations per protocol analysis

	Paracetamol	N	Control	N	*P*
Median % change from admission					
SCr adm (µmol/L)	106 (97 to 141;80,451)	26	115 (97 to 150;88,557)	26	0.38
% peak Δ SCr	-9 (-11 to 0;-31,88)	26	-4 (-17 to271;-32,167)	26	0.29
% Δ SCr 12 h	-10 (-71 to -8;-31,15)	26	-8 (-20 to 6;-32,28)	26	0.65
% Δ SCr 24 h	-11 (-20 to -9;-33,29)	26	-14 (-21 to 14;-42,56)	26	0.71
% Δ SCr 36 h	-19 (-31 to -10;-44,44)	26	-15 (-24 to 9;-47,87)	26	0.11
% Δ SCr 48 h	-20 (-31 to -11;-48,59)	26	-15 (-24 to 12;-47,111)	26	0.06
% Δ SCr 60 h	-24 (-38 to -18;-49,74)	26	-1 (-29 to 0;-47,139)	26	0.06
% Δ SCr 72 h	-23 (-37 to -18;-53,88)	26	-14 (-29 to 0;-47,167)	26	**0.043**
Median % change CFH > 48,000 ng/ml					
SCr adm (µmol/L)	106 (88 to169;80,265)	10	115 (97 to 332;88,557)	12	0.32
% peak Δ SCr	-9 (-11 to -8;-31,0)	10	11 (-9 to 78;-20,167)	12	**0.025**
% Δ SCr 12 h	-10 (-11 to -8;-31,0)	10	3 (-17 to 15;-20,28)	12	0.10
% Δ SCr 24 h	-17 (-23 to -10;-33,-9)	10	-10 (-65 to 26;-30,56)	12	0.11
% Δ SCr 36 h	-28 (-38 to -18;-43,-10)	10	-5 (-19 to 39;-30,83)	12	**0.003**
% Δ SCr 48 h	-29 (-37 to -18;-48,-10)	10	-5 (-17 to 52;-30,111)	12	**0.004**
% Δ SCr 60 h	-33 (-44 to -22;-49,-20)	10	-8 (-25 to 65;-33,139)	12	**0.005**
% Δ SCr 72 h	-37 (-48 to -22;-53,-20)	10	-7 (-29 to 78;-33,167)	12	**0.004**
Median % change CFH ≤ 48,000 ng/ml					
SCr adm (µmol/L)	106 (97 to 111;80,451)	16	111(97 to 1240;88,398)	14	0.75
% peak Δ SCr	-8 (-13 to 4;-20,88)	16	-9 (-20 to 0;-32,152)	14	0.52
% Δ SCr 12 h	-10 (-17 to-4;-20,15)	16	-13 (-23 to -7;-32,17)	14	0.34
% Δ SCr 24 h	-11 (-17 to 4;-28,29)	16	-19 (-25 to 0;-42,33)	14	0.18
% Δ SCr 36 h	-16 (-22 to -9;-44,44)	16	-18 (-30 to -8;-47,87)	14	0.65
% Δ SCr 48 h	-17 (-24 to -9;-43,59)	16	-20 (-25 to -9;-47,94)	14	0.75
% Δ SCr 60 h	-19 (-26 to -14;-47,74)	16	-20 (-30 to -14;-47,120)	14	0.90
% Δ SCr 72 h	-20 (-25 to -9;-53,88)	16	-17 (-29 to -9;-47,152)	14	0.98

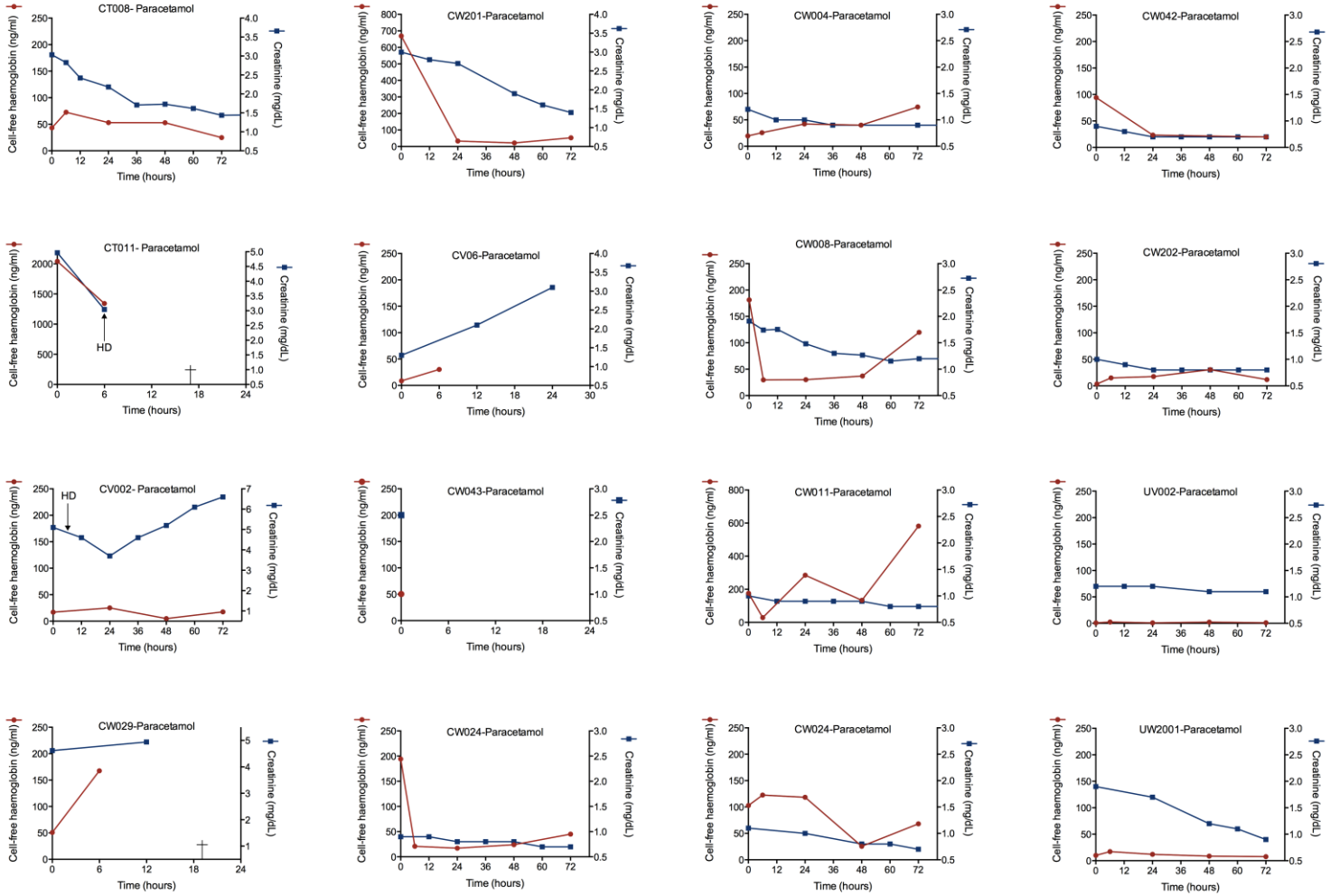
*Data are medians (IQR;max, min). Approximately 8% of total creatinine time points were missing sample collections (median, range; 1 (0,5). Multiple imputation procedure with five rounds was employed. The number and time point of each imputed value is detailed in Supplementary Table 1. Significant in bold, *P*<0.05 using Whitney Mann*U* test. *Abbreviations*:SCr; serum creatinine; CFH, cell-free haemoglobin.

Supplementary table 6.6. Association of cell-free haemoglobin with Glasgow Coma Score adjusting for *P. falciparum* histidine rich protein 2 on enrolment in patients with severe and moderately severe malaria

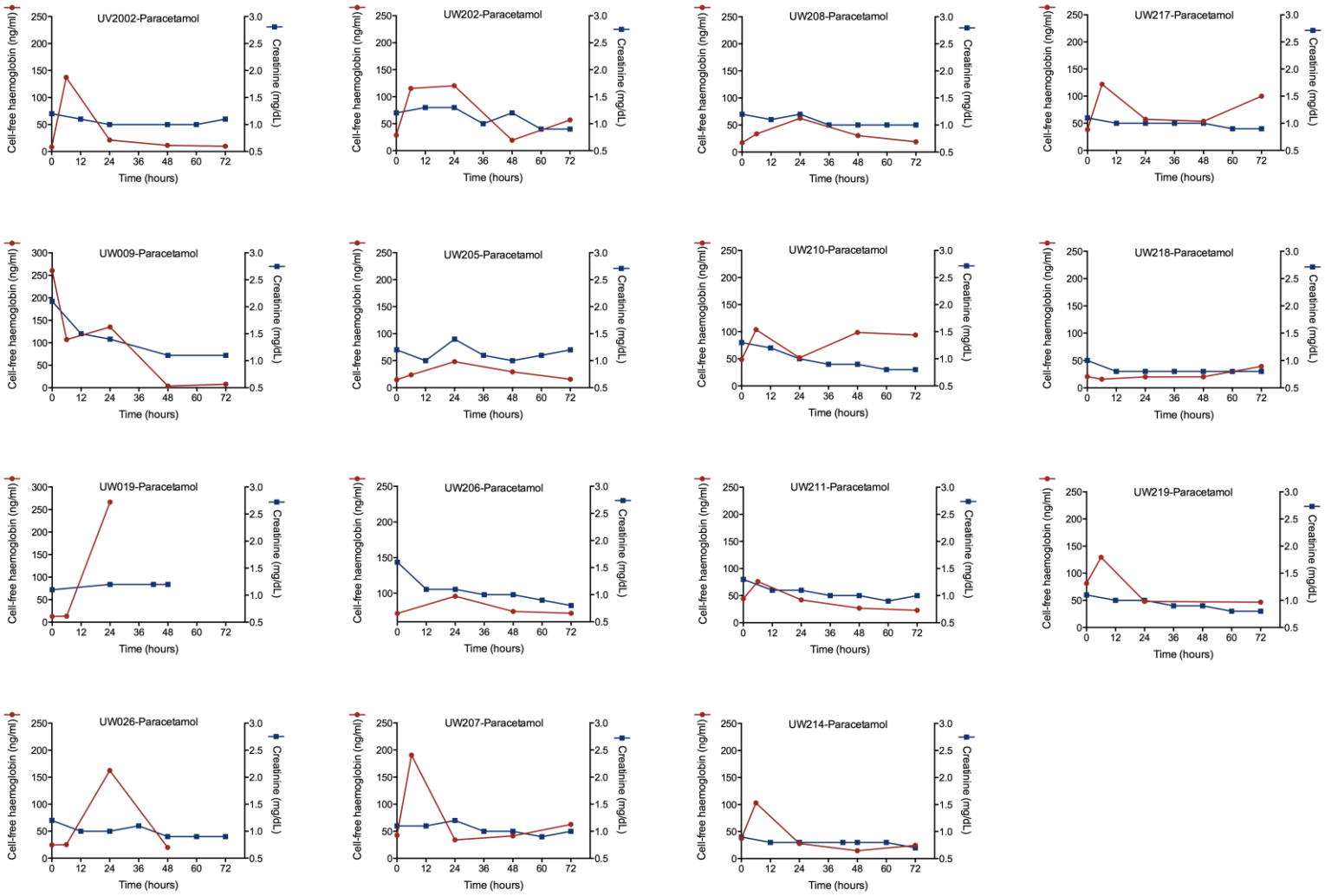
	Univariate analysis		Multivariate model (R^2=0.44)	
Variable	β (95% CI)[a]	*P*	β (95% CI)[a]	*P*
Log CFH	- 1.31 (-1.78 to -0.84)	**< 0.001**	- 0.82 (-1.35 to -0.28)	**0.003**
Log*Pf*HRP2	- 0.78 (-1.06 to -0.49)	**< 0.001**	- 0.51 (-0.83 to -0.18)	**0.003**

[a]Regression coefficient (β) with 95% confidence intervals (CIs) showing the estimated decrease in Glasgow Coma Score predicted by 1-U changes in the independent (predictor) variables. *Abbreviations*: CFH, cell free haemoglobin; *Pf*HRP2, *P. falciparum* histidine rich protein 2.

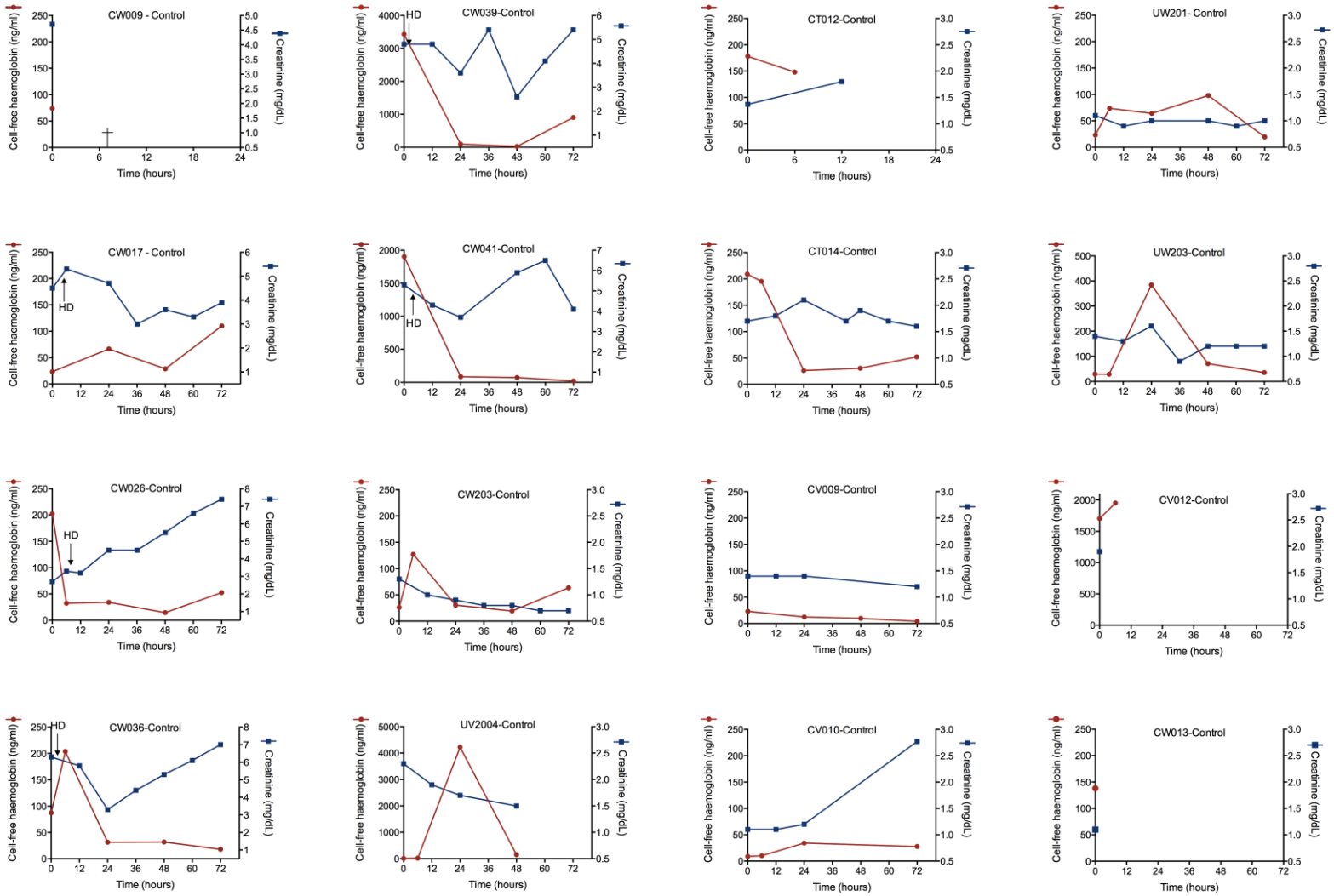
Appendix B – Supplementary figures



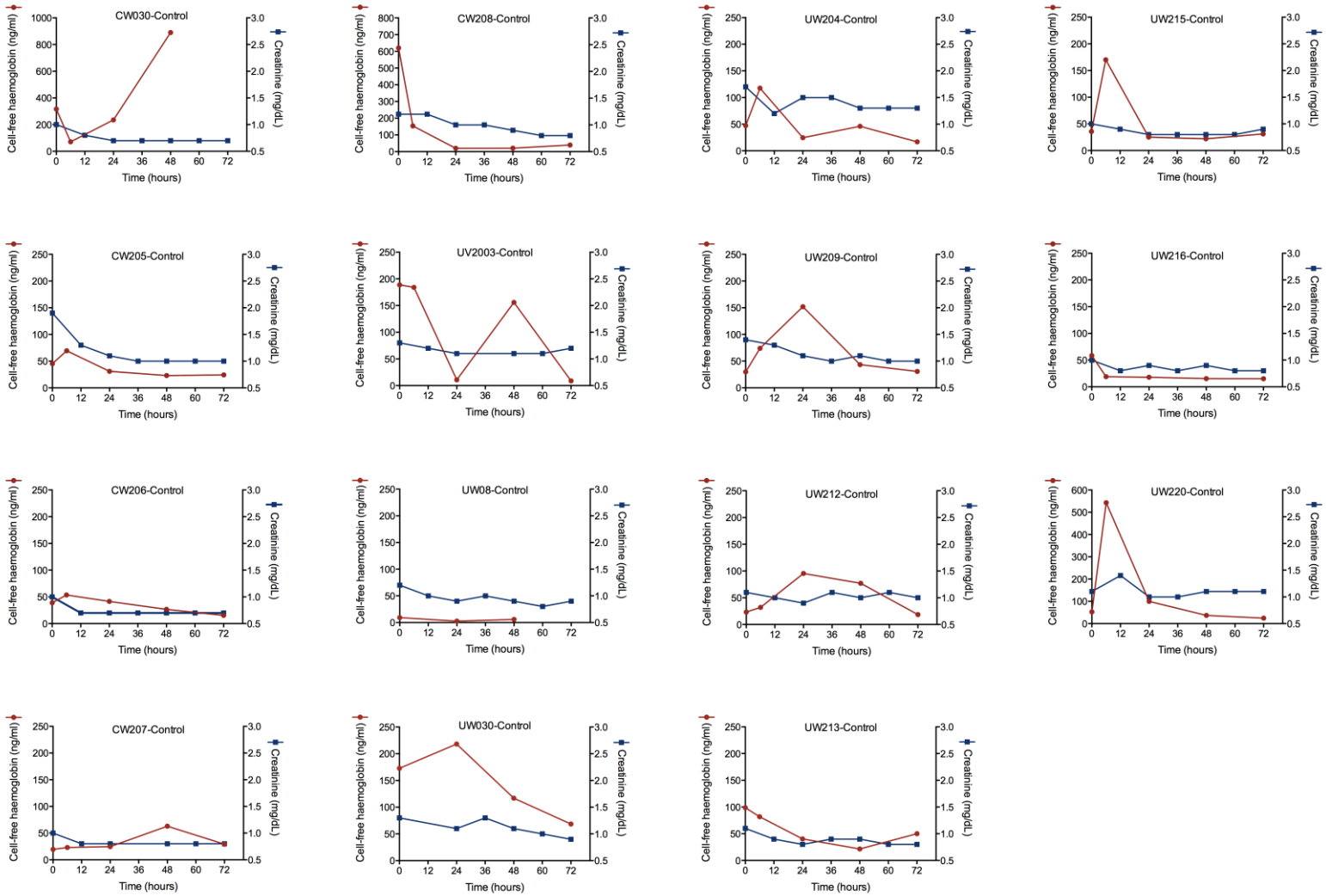
Supplementary Figure 6.1 Longitudinal creatinine and cell-free haemoglobin concentrations in individual paracetamol patients (n=16).



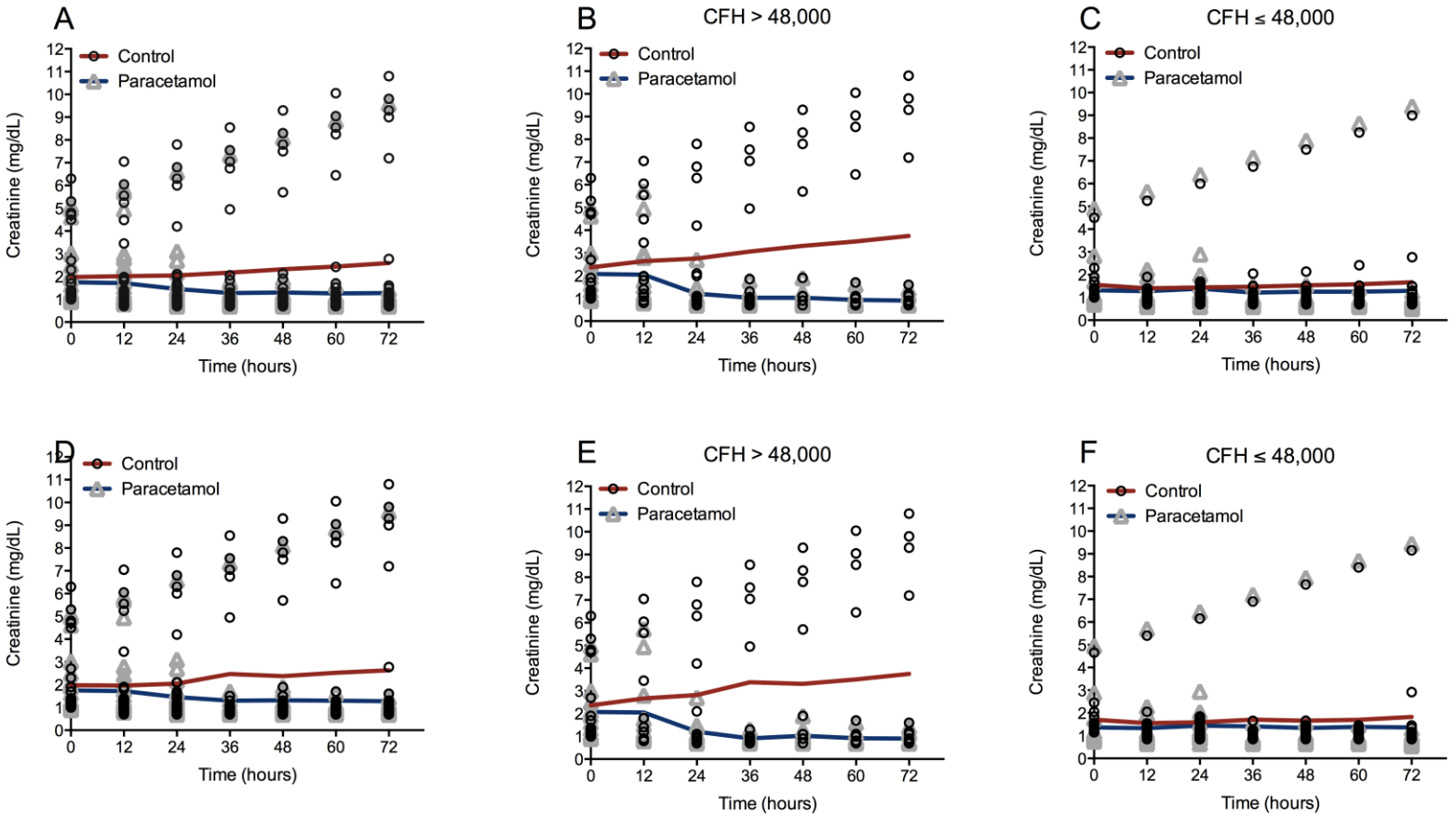
Supplementary Figure 6.2 Longitudinal creatinine and cell-free haemoglobin concentrations in individual paracetamol patients (n=15).



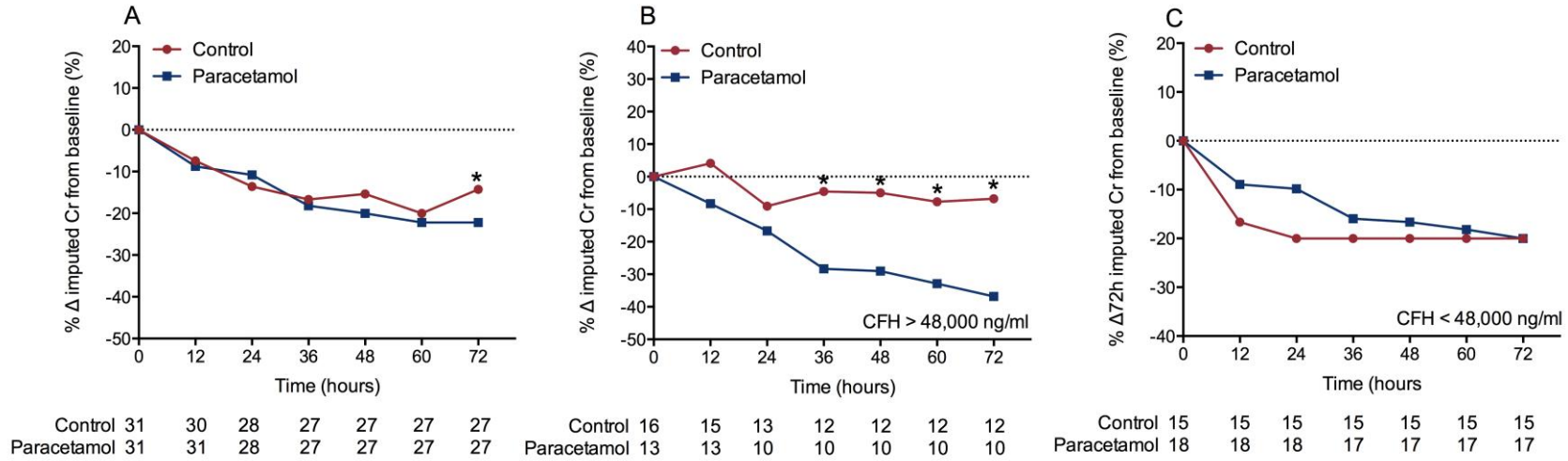
Supplementary Figure 6.3 Longitudinal creatinine and cell-free haemoglobin concentrations in individual control patients (n=16).



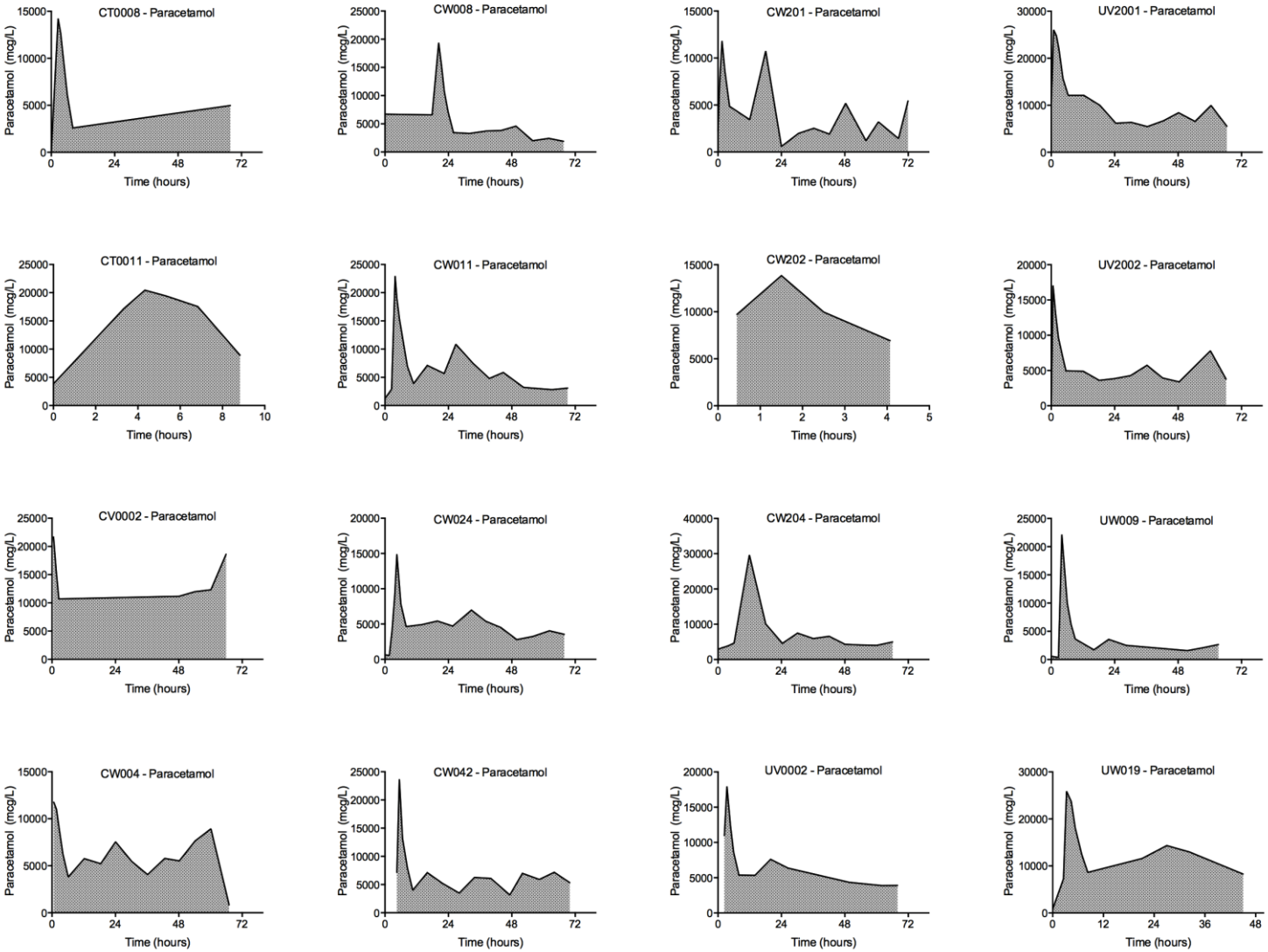
Supplementary Figure 6.4 Longitudinal creatinine and cell-free haemoglobin concentrations in individual control patients (n=15).



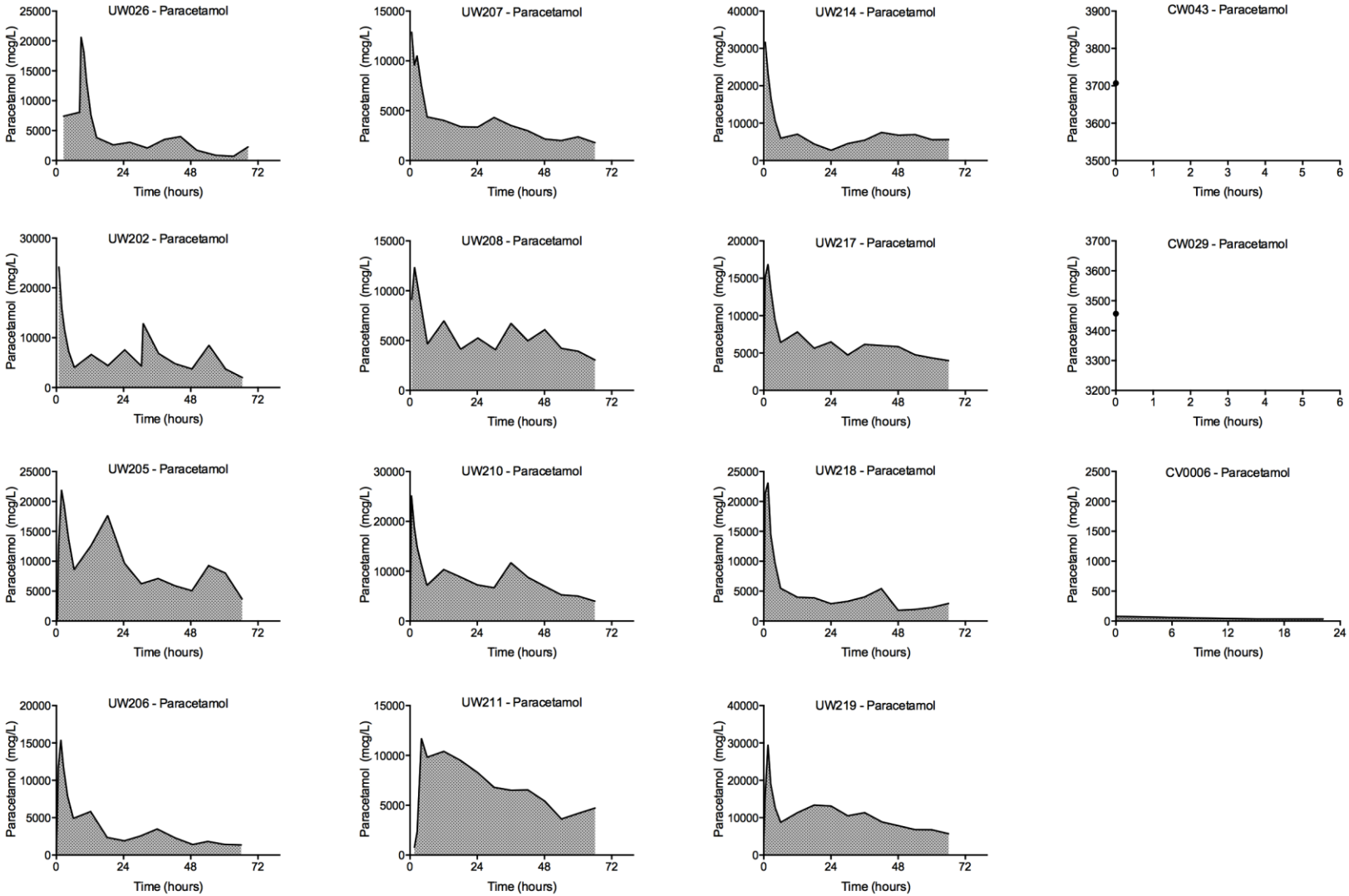
Supplementary Figure 6.5 Scatter plots of individual creatinine concentrations over time with imputation in (A) entire cohort, (B) individuals with enrolment cell-free haemoglobin > 48,000 mg/ml, and (C) individuals with enrolment cell-free haemoglobin ≤ 48,000 mg/ml. Scatter plots of complete case individual creatinine concentrations over time in (D) entire cohort, (E) individuals with enrolment cell-free haemoglobin > 48,000 mg/ml, and (F) individuals with enrolment cell-free haemoglobin ≤ 48,000 mg/ml.



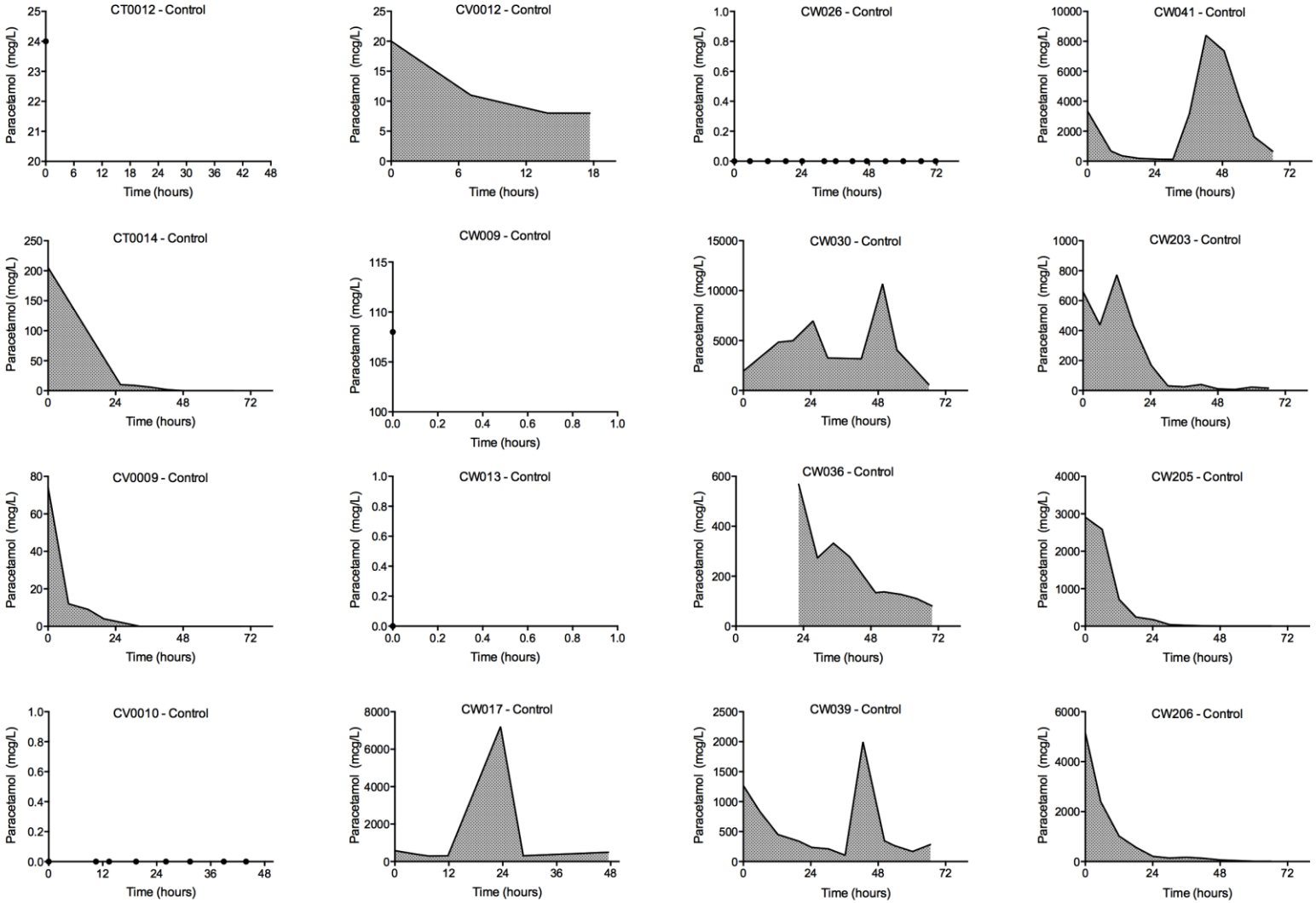
Supplementary Figure 6.6 Effect of paracetamol on median creatinine stratified by intravascular hemolysis. Creatinine median percent change from baseline at 12, 24, 36, 60, 48, and 72 hours, of (A) entire cohort, (B) patients stratified by level of intravascular haemolysis with plasma cell-free haemoglobin > 48,000 ng/ml, and (C) patients stratified by level of intravascular haemolysis with plasma cell-free haemoglobin < 48,000 ng/ml. Asterisks represent significant differences between paracetamol and control groups (Mann U Whitney test, *P* <0.05). Across time points there were missing sample collections (median, range; 1 (0,5)), approximately 8% of total creatinine sampling time points. In addition to the complete case analysis (see results), multiple imputation procedure with five rounds was employed. The number of imputed creatinine observations at each time point is detailed in Table 6.1. Frequencies in rows below figures represent number of patients (n) at each time point. *Abbreviations*: Cr, creatinine; CFH, cell-free haemoglobin.



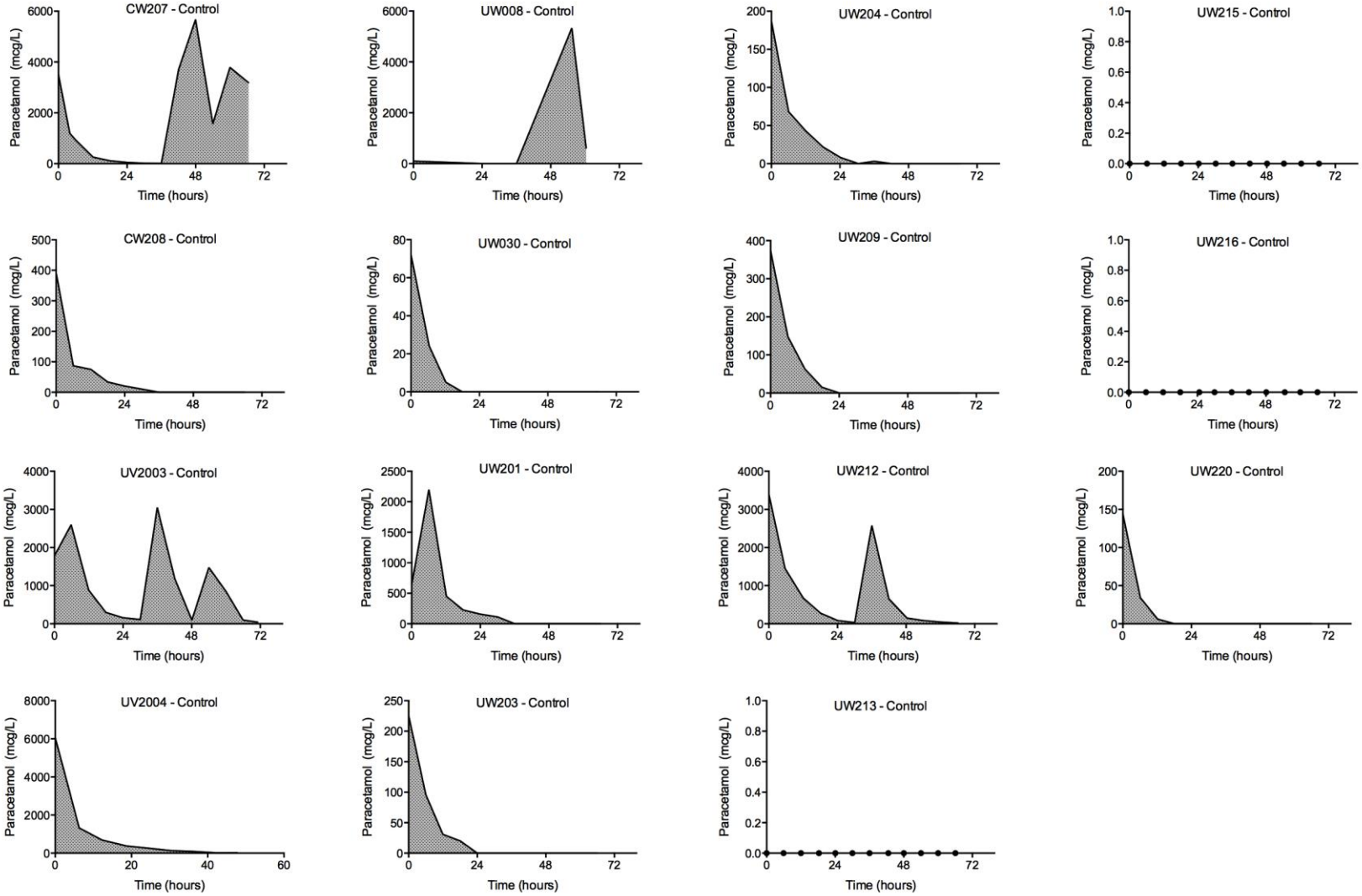
Supplementary Figure 6.7 Area under the curve of paracetamol in individual paracetamol patients (n=16).



Supplementary Figure 6.8 Area under the curve of paracetamol in individual paracetamol patients (n=15).



Supplementary Figure 6.9 Area under the curve of paracetamol in individual control patients (n=16).



Supplementary Figure 6.10 Area under the curve of paracetamol in individual control patients (n=15).

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