Metabolomic characterisation of acidosis in severe falciparum malaria



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Abstract

Severe falciparum malaria is a potentially lethal parasitic infection for which the treatment remains suboptimal as the pathophysiology is incompletely understood. Acidosis is the strongest predictor of death, caused by an accumulation of acids of which some are known, such as L-lactate, while others remain unidentified. The aim of this thesis was to further characterise metabolic acidosis in severe falciparum malaria by establishing the identity and source of the unidentified non-lactate acid load.

A prospective observational study was conducted among patients with falciparum malaria from Bangladesh to establish the identity of metabolites associated with acidosis through a metabolomic analysis of plasma of 152 participants (Chapter 3). Patients with acidosis had significantly elevated levels of organic acids in their plasma that could be traced to a potential microbial source, either parasitic or bacterial. Additionally, dysregulation of plasma free amino acids was observed. The clearance of newly identified microbial acids was impaired in fatal cases.

Follow-up studies were conducted by performing an *in vitro* metabolomic analysis of the liquid culture medium of *Plasmodium falciparum* 3D7 strain parasites with the aim to determine if parasites produce any of the microbial acids identified in patients with severe malaria (Chapter 4). There was no evidence for the release of microbial acids by *Pf* 3D7 *in vitro* suggesting their source is unlikely to be parasitic.

Alongside of this, a prospective study was done to determine factors related to plasma free amino acid dysregulation (Chapter 5). Amino acids were simultaneously quantified in the plasma of 295 participants in relation to clinical syndromes. It was observed that amino acid abnormalities were part of widespread fluctuations in nitrogen balance, whereby the direction of change was related to the severity of malarial disease and the presence of hyperlactataemia, reflecting underlying tissue hypoxia.

Finally, the role of the gut microbiota was further investigated as a potential source of microbial acids in 86 participants, including cases with severe falciparum malaria acidosis, by measuring soluble markers of gut integrity in plasma and sequencing the V4 region of the 16S rRNA gene in faecal DNA (Chapter 6). Patients with malaria had signs of impaired gut barrier function with elevated levels of plasma D-lactate linked to an enrichment of lactate-producing species in the gut microbiota.

In conclusion, acidosis in severe falciparum malaria is associated with microbial acids and plasma free amino acid derangements. Microbial acids are not produced by *Pf* 3D7 *in vitro* but instead might derive from the gut microbiota. These findings point towards a leaky gut as a possible source of previously unidentified acids in severe falciparum malaria acidosis with potential implications for treatment.

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Nomenclature

Analytes

BH_4	Tetrahydrobiopterin
ATP	Adenosine triphosphate
Ca^{2+}	Calcium
Cl^-	Chloride
CO_2	Carbon dioxide
H^+	Hydrogen
H_2CO_3	Carbonic acid
H_3O^+	Hydronium
HCO_3^-	Bicarbonate
K^+	Potassium
LDH	L-lactate dehydrogenase

Mg^{2+} Magnesium

Na^{+} Sodium

NAD^{+} Nicotinamide adenine dinucleotide

NADH Nicotinamide adenine dinucleotide (Reduced)

NO Nitric oxide

PO_4^{3-} Phosphate

*Pf*EMP1 *P. falciparum* Erythrocyte Membrane Protein 1

*Pf*HRP2 *P. falciparum* Histidine Rich Protein 2

*Pf*LDH *P. falciparum* Lactate Dehydrogenase

Abbreviations

hpi Hours post invasion

IDC Intraerythrocytic developmental cycle (of *P. falciparum*)

MCT Monocarboxylate transporter

PDC Pyruvate dehydrogenase complex

TCA Tricarboxylic acid cycle (also known as Krebs' cycle)

ACT Artemisinin combination therapy

AKI Acute kidney injury

ARDS Acute respiratory distress syndrome

ASV Amplicon sequence variants

BCS Blantyre Coma Scale (ranges 0-5)

BH Benjamini-Hochberg

CFH Cell free haemoglobin

CM Cerebral malaria

CMCH Chittagong Medical College Hospital

CRF Case record form

GABA γ-Aminobutyric acid

GCS Glasgow Coma Scale (ranges 3-15)

Hb Haemoglobin

HbAS Sickle cell trait

HE Healthy controls

HIV Human immunodeficiency virus

HMDB Human Metabolome Database (https://www.hmdb.ca/)

HPLC-MS/MS-MRM High-performance liquid-chromatography tandem mass-spectrometry with multiple reaction monitoring

ICF Informed consent form

IVC Inferior vena cava

LCMS Liquid-chromatography mass-spectrometry

MABP Mean arterial blood pressure

MRI Magnetic resonance imaging

PADH Post-artesunate delayed onset haemolysis

PCR Polymerase chain reaction

Pf *P. falciparum*

PFAA Plasma free amino acid

PIS Patient information sheet

QC Quality control

QC-RLSC Quality-control robust LOESS signal correction

RCT Randomised controlled trial

SE Sepsis

SIRS Systemic inflammatory reaction syndrome

SM Severe falciparum malaria

UHPLC-Orbitrap-MS Ultra-high-performance liquid-chromatography Orbitrap mass-spectrometry; also referred to as 'high-resolution mass spectrometry'

UM Uncomplicated falciparum malaria

WHO World Health Organisation

Parameters

$K_{[a]}$ Dissociation constant

pCO_2 Partial pressure of carbon dioxide

AG Anion gap

AGc Anion gap corrected for albumin and phosphate

BE base excess

SBD Standard base deficit (the negative transformation of standard base excess)

SBDc Standard base deficit corrected for albumin and phosphate

SBE Standard base excess

SIDa Apparent strong ion difference

SIDe Effective strong ion difference

SIG Strong ion gap

Chapter 1

Introduction

1.1 The impact of severe malaria on global health

Malaria is the most important human parasitic disease and a major burden to global health. Severe infections are predominantly caused by the malaria parasite *Plasmodium falciparum*. Transmission of malaria occurred in 91 countries and caused an estimated 216 million new infections in 2016 (95% confidence interval between 196-263 million) (WHO, 2017), the overwhelming number of patients (90%) lived in a country in sub-Saharan Africa, Figure 1.1.

Over the past decades the global incidence of malaria has decreased, creating new enthusiasm to eradicate malaria entirely (Murray et al., 2014). Nevertheless, up to 445,000 patients still died from malaria in 2016 (WHO, 2017). Epidemiological models project that malaria will remain an important cause of pre-mature death in endemic countries in the coming decades (Norheim et al., 2015). Furthermore, efforts to eliminate malaria could be

undermined by the spread of antimalarial resistance and new outbreaks driven by population movement and the breakdown of health systems (Tanner et al., 2015).

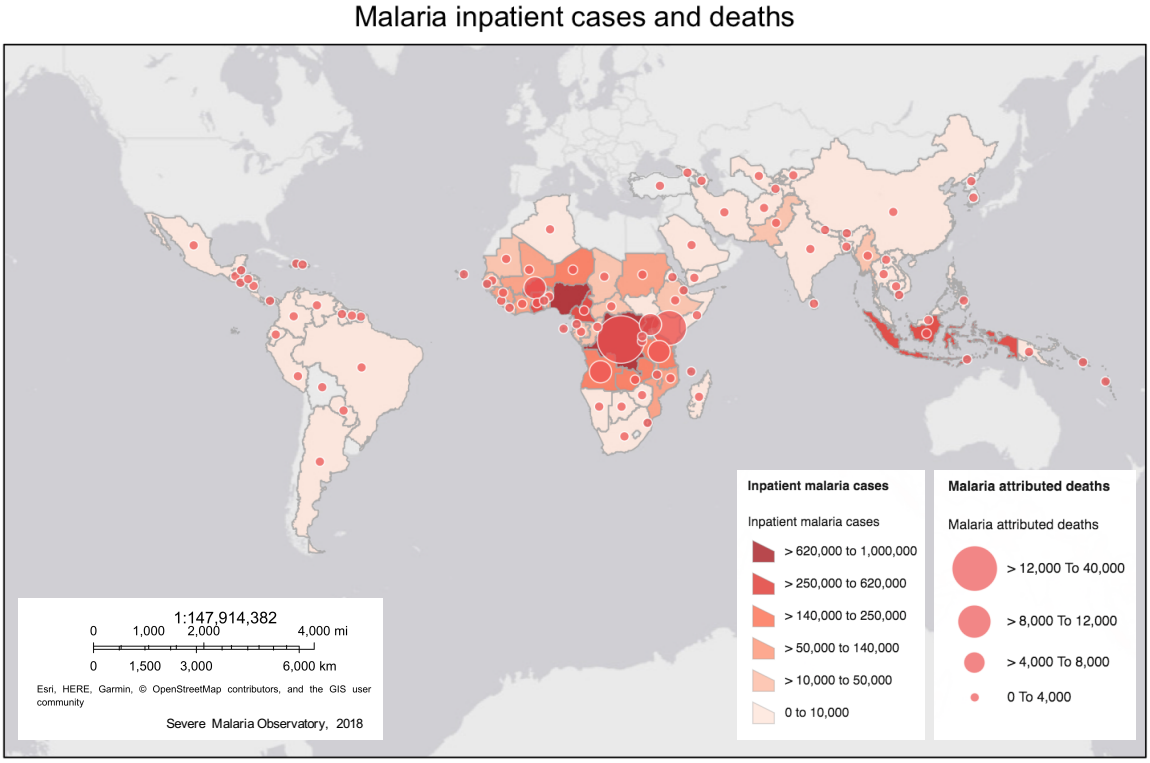


Fig. 1.1 **Global burden of severe malaria.** (Map data © 2018 Severe Malaria Observatory).

The major impact of *P. falciparum* malaria on global health is related to severe disease and mortality. The mortality burden of malaria is highest among young non-immune children living in sub-Saharan Africa (WHO, 2017). In pregnancy, malaria can cause spontaneous abortion and intrauterine growth retardation (Nosten et al., 2007). In patients who survive cerebral malaria, neurological sequelae can persist long after an infectious episode (Van Hensbroek et al., 1997). Hospitalisation due to malaria infection places a burden on household finances, and on the economy of endemic countries (Sachs and Malaney, 2002).

The mortality from severe malaria ranges between 10-30% despite optimal antimalarial treatment with parenteral artesunate and case fatality rates increase depending on the number of clinical complications (WHO, 2014). Major complications of severe malaria are metabolic acidosis, coma and convulsions, acute renal impairment, and severe anaemia (WHO, 2014). Improving the supportive care for critically ill patients and developing effective adjunctive therapies that target pivotal pathophysiological pathways might improve treatment outcomes.

Metabolic acidosis is the strongest predictor of mortality, across all ages and ethnicities (Day et al., 2000b; Dondorp et al., 2008b; English et al., 1997a; Hanson et al., 2012; Jeeyapant et al., 2017; Krishna et al., 1994b; Marsh et al., 1995; Newton et al., 2005; Sasi et al., 2007; Taylor et al., 1993; von Seidlein et al., 2012; White et al., 1985). Acidosis is a common feature of severe malaria and is associated with hyperlactataemia (Day et al., 2000b; English et al., 1997a; Jeeyapant et al., 2017; White et al., 1985). However, the pathophysiology of acidosis in severe falciparum malaria remains incompletely understood. An important proportion of the metabolic acidosis observed in patients with severe malaria is not explained by L-lactate alone (Day et al., 2000b; Dondorp et al., 2004a; Hanson et al., 2012; Herdman et al., 2015; Sasi et al., 2009), indicating there are other unidentified acids. The unidentified non-lactate acid load has previously been found to be an independent predictor of mortality (Dondorp et al., 2004a).

The focus of this thesis lies on the unidentified non-lactate acid load in severe malaria. Establishing the identity and source of unidentified acids in patients with severe falciparum malaria can have important implications for the treatment of acidosis.

1.2 Aims and objectives

The primary aim of this thesis was to investigate the determinants of life-threatening acidosis in patients with severe falciparum malaria using clinical, parasitological, and biochemical investigations. The scope of this thesis was limited to adult patients with severe *P. falciparum* malaria from Bangladesh. The specific objectives were:

1. To establish the identity, source, and temporal kinetics of circulating organic acids in patients with severe falciparum malaria in Bangladesh using ultra-high-performance liquid-chromatography Orbitrap mass-spectrometry (UHPLC-Orbitrap-MS) based metabolomics (Chapter 3).

2. To characterise the release of organic acids by *P. falciparum* 3D7 strain parasites *in vitro* using UHPLC-Orbitrap-MS based metabolomics (Chapter 4).

3. To determine the relationship between acidosis and plasma free amino acid derangements in adult patients with severe falciparum malaria from Bangladesh (Chapter 5).

4. To describe the role of the 16S rRNA gut microbiota composition in the pathogenesis of metabolic acidosis in adult patients with severe falciparum malaria from Bangladesh (Chapter 6).

1.3 Pathophysiology of severe malaria

1.3.1 Natural history of infection

Malaria is caused by protozoan parasites of the phylum Apicomplexa and genus *Plasmodium*. There are six species that are known to infect humans: *P. falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale curtisi*, *Plasmodium ovale wallikeri*, and *Plasmodium knowlesi* (Sutherland et al., 2010). In sub-Saharan Africa, over 99% of the cases are caused by *P. falciparum* alone (WHO, 2017). Outside of the African sub-continent, *P. vivax* is also an important cause of malaria infection, contributing to approximately 30% of malaria cases in South-East Asia and 64% of cases in South-America.

A human malaria infection begins with a bite from a female Anopheles mosquito that introduces an inoculum of around ten *Plasmodium* sporozoites into the skin while taking a blood meal, Figure 1.2. These sporozoites subsequently migrate to the liver, where they multiply over a period of six to eight days before they are released into the blood stream. Hypnozoites may be left dormant in the liver in *P. vivax* and *P. ovale* infections, responsible for subsequent relapsing infections. Following the liver stage, approximately 10^4-10^5 parasites are released into the blood stream, where they invade red blood cells and undergo growth and asexual replication inside the erythrocyte. During human malaria infections, the blood stage is clinically the most relevant phase of the parasite life cycle, as this is when infections start to cause symptoms and complications. The intraerythrocytic cycle of *P. falciparum* lasts $\sim$48 hours. Under the microscope, the intraerythrocytic parasite can be roughly differentiated into three stages: a ring stage (0-24 hours post invasion), a

trophozoite stage (24-36 hpi) and a schizont stage (36-42 hpi), Figure 1.3 (Silamut et al., 1999). The transition of a young ring form to a schizont is characterised by extensive remodelling of the red blood cell as the parasite consumes the red cell haemoglobin to acquire nutrients and gain space for growth. During this process, parasite derived adhesion proteins attach to the surface of the host red blood cell in the early trophozoite stages after $\sim$16 hpi (Mcmillan et al., 2013). The appearance of these parasite adhesion proteins coincides with the disappearance of trophozoites from the blood circulation as they adhere to the vascular endothelium of capillaries and post-capillary venules. This phenomenon is called sequestration and plays a key role in the pathophysiology of severe falciparum infection. Hidden inside the microvasculature, parasites undergo the last phase of their asexual transition into schizonts. Completion of intraerythrocytic development is marked by the rupture of the red cell releasing 16-32 new merozoites back into the circulation, and leading to multiplication rates of $\leq$20 times per cycle in a susceptible individual (Gravenor et al., 1998).

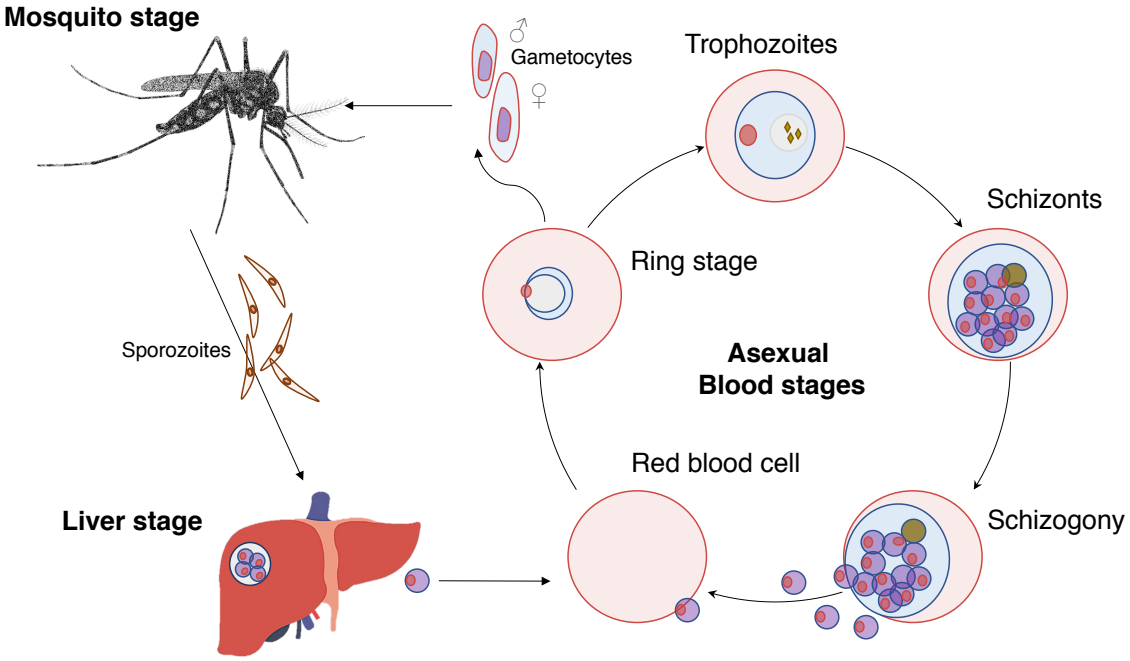


Fig. 1.2 ***P. falciparum*** **life cycle in the human and mosquito.**

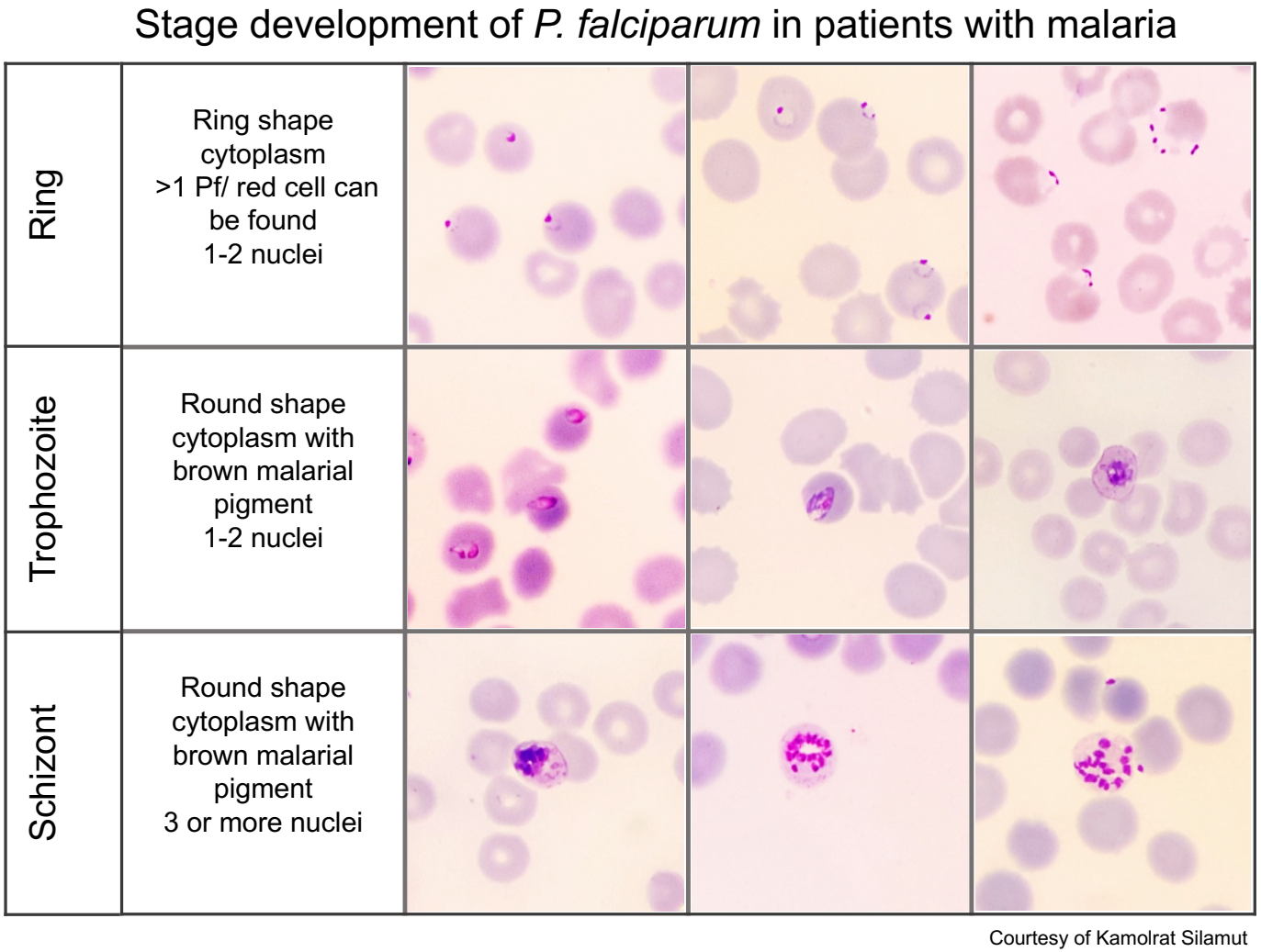


Fig. 1.3 **Intaerythrocytic developmental stages of *P. falciparum* from clinical isolates**. (Courtesy and permission of Dr. Kamolrat Silamut).

1.3.2 Parasite density and severity of disease

The severity of malaria infection is proportional to the pathogen biomass. In other words, disease progression correlates with the number of parasites in the body. Circulating parasite densities can be estimated by determining the parasitaemia in a venous or capillary blood sample. However, the circulating parasitaemia poorly reflects the total number of parasites in the body, as sequestered stages are removed from the circulation leading to a potential underestimation of the parasite burden. *P. falciparum* Histidine Rich Protein 2 (*Pf*HRP2) is a parasite-derived surface protein and can be used to mathematically model the total parasite biomass (Dondorp et al., 2005; Hendriksen et al., 2012). Hospitalised patients with uncomplicated malaria usually have an estimated total body parasite biomass between 10^{11} and 10^{12} parasites. Patients with severe malaria may reach a total body parasite biomass between 10^{12} and 10^{13}. A parasite biomass exceeding 10^{13} is strongly associated with fatal malaria (Dondorp et al., 2005).

The size of the pathogen biomass becomes relevant when malaria is compared to other severe disease states such as bacterial sepsis. In sepsis, a small bacterial load of around 1-10 colony forming units (CFU) per mL can lead to severe symptoms (Wain et al., 1998) and the pathogenesis of sepsis is typically characterised by damage related to a pro-inflammatory innate immune response (Wiersinga et al., 2014). In contrast, severe malaria starts to occur when parasitaemia reaches over a 100,000 parasites per μL. To emphasize this point: imagine an adult male patient with severe falciparum malaria with an estimated total blood volume of 5L and a haematocrit of 30%, where a 10% parasitaemia can lead to a volume of around 150mL of parasitised red blood cells (a glass full of parasites). The pathophysiology of

severe malaria crucially differs from severe bacterial sepsis with regard to the immune response to the pathogen biomass. Current management guidelines for the treatment of severe sepsis overlook the quantitative aspects of the causative pathogen and therefore cannot be generalised to the treatment of patients with severe malaria (Dondorp et al., 2017).

As the malaria parasite biomass expands inside the bloodstream, a sequence of clinical events typically follows: starting with asymptomatic parasitaemia, followed by symptomatic uncomplicated malaria, and finally progressing into severe malaria with a potentially fatal outcome, Figure 1.4. The progression of this clinical sequence depends on the speed at which the parasite replicates within the host, which is largely determined by the parasite species and immunity of the host, and on the timing and efficacy of treatment, which depends on the access to safe and effective antimalarial therapy, and supportive and intensive care.

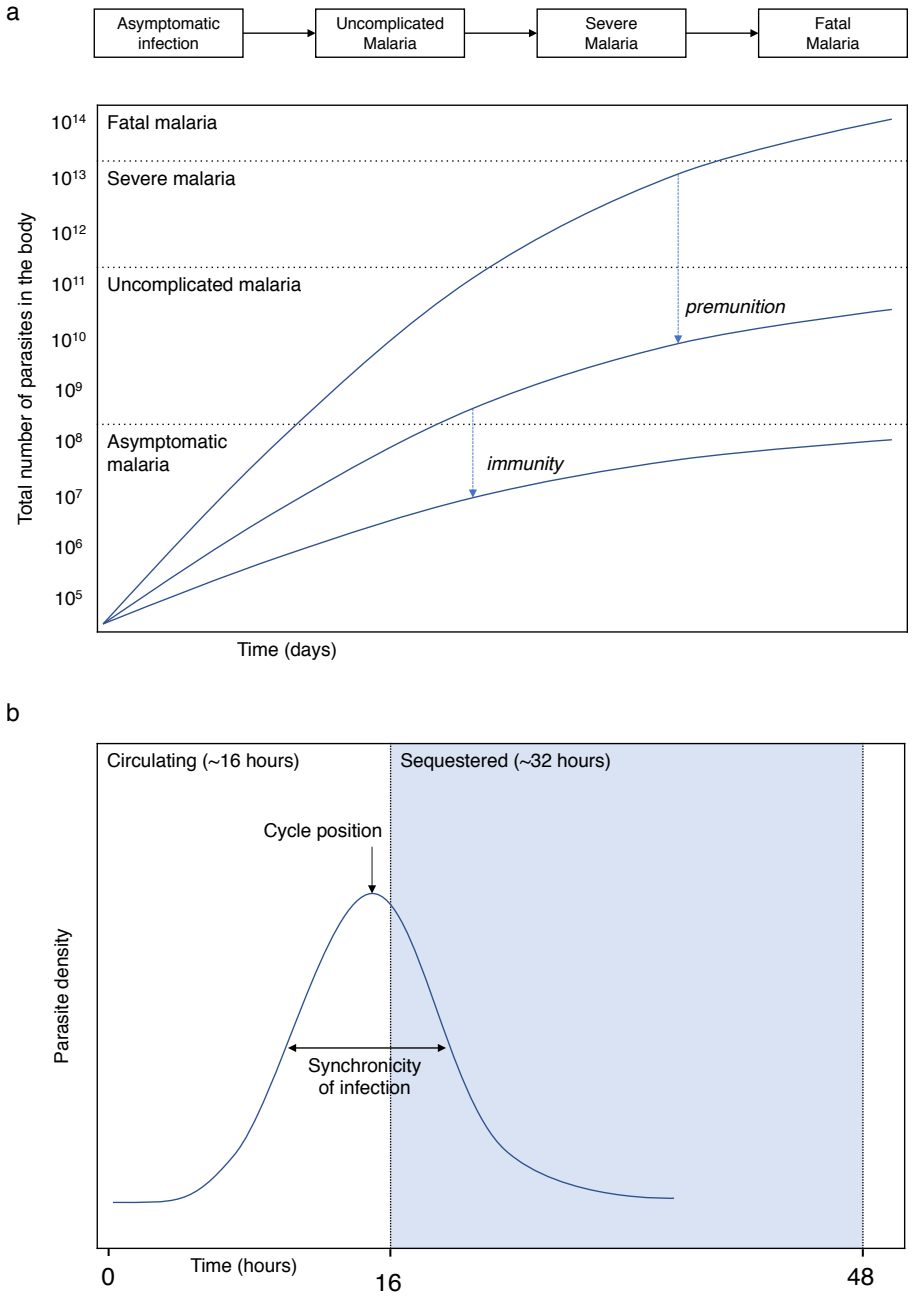


Fig. 1.4 **Disease dynamics of human *P. falciparum* infection.** (**a**) schematic of the relationship of total parasite biomass and severity of disease in *P. falciparum* malaria. (**b**) schematic of the circulating and sequestered intervals. The synchronicity of infection determines the spread of the distribution of circulating and sequestered stages. The cycle position refers to the average stage of the parasites.

1.3.3 Microvascular sequestration

The clinical manifestations of severe malaria result from tissue damage by *P. falciparum* parasites sequestered in the microcirculation. Some of the first observations of sequestration in vital organs were made in 1892 by the Italians Marchiafava and Bignami and in their seminal work 'On summer-autumn malarial fevers' they write: 'In the comatose malignant fevers the capillaries of the brain, and especially the grey matter, are found to be injected with an immense quantity of red blood-corpuscles loaded with *Plasmodia*', (Marchiafava and Bignami, 1894, p109).

Cytoadherence of parasitised red blood cells is still considered to be a key process whereby malaria infection inflicts organ damage (White et al., 2013). Adhesion is mediated by antigenically variant adhesive proteins which are exported to the surface of the red cell, including *P. falciparum* Erythrocyte Membrane Protein 1 (*Pf*EMP1) (Mcmillan et al., 2013). These knob-like parasite-derived surface proteins mediate adherence of the red cell to other infected red cells (agglutination) (Bull et al., 2000), to uninfected red cells (rosetting) (David et al., 1988), and most importantly, to the vascular endothelium of capillaries and post-capillary venules (sequestration) (MacPherson et al., 1985). Host endothelial ligands include ICAM1 and Endothelial Protein C in the brain (Moxon et al., 2013), E-selectin and chrondroitin sulphate A in the placenta, and CD36 in other tissues (Rowe et al., 2009).

1.3.4 Endothelial dysfunction

Sequestration and microvascular obstruction are worsened by endothelial dysfunction. As parasitised red blood cells adhere to the vascular wall, endothelial activation occurs. This

process induces the release of Weibel-Palade bodies containing Von Willebrand factor and angiopoietin2, which activate coagulation and platelet aggregation. Thrombocytopenia is commonly observed in severe malaria as platelets are consumed in this process, and splenic sequestration and dysthrombopoiesis also contribute (Hanson et al., 2015b). Sequestered inside the microcirculation, parasites mature and eventually release merozoites back into the circulation, which leads to intravascular haemolysis and the release of cell free haemoglobin and haem. Circulating nitric oxide (NO) is depleted, reducing vascular responsiveness and limiting vasodilation (Yeo et al., 2009). Furthermore, oxidative stress stiffens the circulating red cells making them less deformable and more likely to sequester (Dondorp et al., 2004b).

1.4 Diagnosis

Early species-specific diagnosis of malaria is necessary for optimal case management. The reference standard for the diagnosis of malaria is light microscopy of thick and thin blood smears. Through microscopic analysis of a thick smear parasitaemia can be quantified at low parasite densities. Depending on the conditions under which blood slides are prepared and the expertise of the microscopist, the limit of detection of light microscopy of thick blood smears may range between 10-100 parasites per μL (Joanny et al., 2014; Zimmerman and Howes, 2015). Analysis of the thin smear allows parasite speciation, staging, and inspection of the proportion of malaria pigment-containing neutrophils. The finding of mature parasite stages or >5% pigmented neutrophils is predictive of mortality (Phu et al., 1995; Silamut and White, 1993).

Point-of-care diagnosis of *P. falciparum* may include the use of rapid diagnostic tests based on circulating *Pf*HRP2 or *Pf*LDH. The detection limits vary depending on the manufacturer. In general, if the instructions are followed, the detection limit of *Pf*HRP2 is around 200 parasites per µL (Jimenez et al., 2017). Recently, high-sensitivity RDTs were introduced for the detection of *P. falciparum*, mainly useful for their application in elimination efforts, which could detect a parasitaemia as low as 4 parasites per µL (Das et al., 2018). Quantitative *Pf*HRP2 RDTs are currently lacking. Approximately half of the *Pf*HRP2 rapid tests are still positive at two weeks post-treatment (and can be up to a month), as *Pf*HRP2 remains on the surface of initially parasitised red cells (Dalrymple et al., 2018).

Molecular diagnostics are available for the diagnosis of *Plasmodium* spp. at lower detection thresholds (Imwong et al., 2014). Polymerase chain reaction (PCR) methods provide relevant methods for the epidemiological study of asymptomatic carriers with low circulating parasitaemia and to determine the extent of the asymptomatic malaria reservoir, but are not directly relevant for the diagnosis of *Plasmodium* spp. in patients with severe malaria since severe infection is associated with high peripheral parasitaemia.

1.5 Clinical management

1.5.1 Definitions

Following the diagnosis of asexual-blood stages of *P. falciparum*, clinical and laboratory criteria are used to classify patients who are at increased risk of a poor outcome. In general, patients can be classified as having uncomplicated or severe malaria. This classification is useful for decision-making with regard to the administration of parenteral artesunate and escalation of care. Severe malaria refers to the presence of asexual-stage *P. falciparum* in the blood together with signs of organ dysfunction. Definitions are available for epidemiological and research purposes and are summarized in Table 1.1. In practice, clinicians may decide to use lower thresholds for the administration of parenteral treatment based on their clinical judgement; certainly in patients who cannot take medication orally.

The first symptoms of acute *P. falciparum* malaria are usually non-specific. There may be 'flu-like' symptoms such as generalised malaise, fever, dizziness, headache, nausea, anorexia, vomiting and sometimes diarrhoea (WHO, 2014). Clinical findings may include fast breathing, anaemia, jaundice, and an enlarged liver and spleen. Postural-hypotension occurs in both uncomplicated as well as severe malaria. These initial signs and symptoms of malaria have limited value in differentiating malaria from other infectious diseases. Therefore, a clinical suspicion of malaria should be confirmed by a haematological blood test.

Severe malaria is diagnosed based on the presence or absence of clinical and laboratory signs indicating potentially life-threatening organ involvement. The major clinical manifestations of severe falciparum malaria at hospital admission include metabolic aci-

dosis (40-50%), cerebral malaria (40-50%), renal impairment (20-30%), severe anaemia (10-40%) and concomitant sepsis (6-16%) (Dondorp et al., 2008b). The distribution of severe malaria manifestations depends on geography and age - in high stable transmission areas nearly all young children have severe anaemia (Dondorp et al., 2010). These major clinical manifestations of severe malaria are determinants of outcome and can be used for clinical prognosis. The epidemiology, pathophysiology and complication-specific treatment of the major determinants of outcome in severe malaria are discussed further in Section 1.5.3.

Table 1.1 **Epidemiological classification of severity of disease in patients with *P. falciparum* malaria.**

WHO classification of severity of disease in patients with *P. falciparum* malaria	
Uncomplicated Malaria	
A patient who presents with symptoms of malaria and a positive parasitological test (microscopy or RDT) but with no features of severe malaria	
Severe Malaria	
Any of the following signs or symptoms in the absence of an identified alternative cause and in the presence of *P. falciparum* asexual parasitaemia:	
Impaired consciousness	GCS <11 in adults or BCS <3 in children.
Prostration	Generalized weakness; unable to sit, stand or walk without assistance.
Multiple convulsions	>2 episodes within 24 hours.
Acidosis	Base deficit >8 mEq/L or a plasma bicarbonate level of <15 mmol/L or venous plasma L-lactate >5 mmol/L.
Hypoglycaemia	Blood or plasma glucose <2.2 mmol/L (<40 mg/dL).
Severe malarial anaemia	Hb <5 g/dL or a Hct of <15% in children <12 years of age (<7 g/dL and <20%, respectively, in adults) with a parasite count >10 000/μL.
Renal impairment	Plasma or serum creatinine >265 μmol/L (3 mg/dL) or blood urea >20 mmol/L.
Jaundice	Plasma or serum bilirubin >50 mmol/L (3 mg/dL) with a parasite count >100 000/μL.
Pulmonary oedema	Radiologically confirmed or SpO2 <92% on room air with a respiratory rate >30/min.
Significant bleeding	Recurrent bleeding from the gums, nose, haematemesis or melaena.
Compensated Shock	CRT >3 sec or temperature gradient on leg, but no hypotension.
Decompensated Shock	SBP <70 mm Hg in children or <80 mm Hg in adults, with evidence of impaired perfusion. SBP <70 mm Hg in children or <80 mm Hg in adults, with evidence of impaired perfusion.
Hyperparasitaemia	*P. falciparum* parasitaemia >10%.

Adapted from World Health Organization (2015).
RDT = rapid diagnostic test. GCS = Glasgow coma scale. BCS = Blantyre coma scale. Hb = haemoglobin. CRT = capillary refill time (in seconds). SBP = systolic blood pressure.

1.5.2 Antimalarial treatment

Patients with severe malaria require prompt and effective antimalarial treatment. Untreated severe falciparum malaria likely reaches 100% mortality. Optimal and timely treatment with parenteral artesunate and supportive care reduces case fatality to 10-30%. Multiple landmark randomized trials provide strong evidence for the superiority of artesunate over quinine in the treatment of severe falciparum malaria (Dondorp, 2005; Dondorp et al., 2010; Hien et al., 1992a, 1996, 1992b; Newton et al., 2003). A randomized controlled trial of 1461 adults (and children) in South-East Asia, referred to as the SEAQUAMAT study, showed a reduction in mortality by 35% (95% CI, 18.5-47.6) (Dondorp, 2005). Following, a randomized controlled trial of 5425 children living in sub-Saharan African, referred to as the AQUAMAT study, confirmed the efficacy and safety of artesunate, with a mortality reduction of 22% (95% CI, 8.1-36.9) (Dondorp et al., 2010). The mortality benefit of artesunate is mostly explained by the more rapid parasitocidal action of parenteral artesunate against the early intraerythrocytic parasite stages, which also kills the younger ring stage parasites (as opposed to quinine which only acts on the mature stages) and thereby prevents their impending sequestration. Therefore, there is potentially a subgroup benefiting most from artesunate constituted by hyperparasitaemic patients with synchronous infections in the ring stage cycle position. Injectable artesunate comes in 60 mg vials and follows weight based dosing with 2.4 mg/kg body weight, given at 0, 12, 24 hours, then daily. In children below 20 kg, the dose is 3.0 mg/kg body weight, to ensure similar drug exposure in these small children. Pre-referral treatment with rectal artesunate might further reduce hospital mortality in children under 6 years (Gomes et al., 2009). Following parenteral treatment (at least after 24 hours), a

full course of artemisinin combination therapy (ACT) must be completed. The choice of ACTs depends on regional resistance patterns. Ongoing trials are evaluating the use of triple combination therapies (TACT) in Asia and sub-Saharan Africa.

Post-artesunate delayed onset haemolysis (PADH) has been described in non-immune travellers treated with artesunate for malaria (Jauréguiberry et al., 2014, 2015; Rolling et al., 2014). The younger ring stage parasites killed by artesunate become pyknotic and are removed from the red cell by a process called 'pitting' in the spleen. Once-infected pitted red cells re-enter the circulation, yet, their longevity is reduced. Haemolysis is reprieved to two or three weeks following treatment with artesunate and is higher in patients with hyperparasitaemia. The plasma concentration of *Pf*HRP2 following completion of antimalarial treatment predict the extent of PADH (Ndour et al., 2017). However, a recent study in 214 children with acute uncomplicated malaria in a high transmission area in the Democratic Republic of Congo randomised between artesunate and quinine treatment showed that late anaemia was rare and not more frequent with artesunate in patients with hyperparasitaemia (>100 000 parasites/μL) (Fanello et al., 2017).

1.5.3 Determinants of outcome

The majority of deaths from severe malaria occur within the first 24 hours of hospital admission, in particular in children (Keene et al., 2018; Kendjo et al., 2013). A considerable number of prognostic studies have been done, aimed to identify the determinants of outcome in severe malaria in endemic countries (Allen et al., 1996; Argy et al., 2017; Beare et al., 2004; Cserti-Gazdewich et al., 2013; Day et al., 2000b; Dondorp et al., 2008b; English et al., 1997a; Evans et al., 2006; Hanson et al., 2010; Jallow et al., 2012; Kendjo et al., 2013; Krishna et al., 1994a; Manning et al., 2011; Marsh et al., 1995; Molyneux et al., 1989b; Newton et al., 2005, 2013; Sypniewska et al., 2017; Taylor et al., 1993; von Seidlein et al., 2012; White et al., 1985) and Western intensive care units (Bruneel et al., 2003; Marks et al., 2013; Santos et al., 2012; van Genderen et al., 2006). Many of these prognostic studies are based on multivariate logistic regression models including clinical and laboratory measures taken on admission. These studies consistently find three major independent predictors of death: fast breathing or metabolic acidosis, uraemia or renal impairment, and cerebral malaria. Together, these three complications accurately predict mortality and have therefore been included in composite prognostic scores such as the Coma Acidosis Malaria (CAM) score (Hanson et al., 2010). The prognostic value of acidosis and renal failure is strong as these complications are on the causal pathway of death. In severe malaria, multiple clinical syndromes correlate and overlap, and where these three clinical syndromes overlap the risk of death is increased (WHO, 2014).

Metabolic acidosis

Acidosis is a common complication in severe falciparum malaria. Acidosis was diagnosed in 43% of children enrolled in the AQUAMAT trial and 48% of children and adults enrolled in the SEAQUAMAT trial. Acidosis is consistently found to be one of the strongest independent predictors of mortality from severe malaria in all ages and geographical regions (Day et al., 2000b; Dondorp et al., 2008b; English et al., 1997a; Hanson et al., 2010; Krishna et al., 1994a; Marsh et al., 1995; Newton et al., 2005; Sasi et al., 2007, 2009; Sypniewska et al., 2017; Taylor et al., 1993; von Seidlein et al., 2012; White et al., 1985). Case fatality increases stepwise with increasing levels of metabolic acidosis, Figure 1.5. Before blood gas analysis was regularly performed, clinical studies identified fast and deep breathing patterns to be predictive of mortality (English et al., 1996b; Marsh et al., 1995). Rapid breathing can indicate a respiratory compensation mechanism for underlying acidosis. Following the introduction of quantitative measure of acidosis, such as standard base deficit, bicarbonate and L-lactate, these became the strongest prognostic indicators of death (Bhaskaran et al., 2013; Cserti-Gazdewich et al., 2013; Day et al., 2000b; English et al., 1997b; Krishna et al., 1994c; Mtove et al., 2011; Newton et al., 2005; Sasi et al., 2007, 2009; von Seidlein et al., 2012).

The typical acid-base disorder found in patients with severe falciparum malaria is a respiratory compensated metabolic acidosis with an elevated anion gap. An elevated anion gap observed in patients with severe falciparum malaria is primarily caused by an increase in L-lactate. Hyperlactataemia in severe malaria is caused by hypoxia following microcircu-

latory hypoperfusion from diffusely sequestered parasitised red blood cells in combination with an increased basal oxygen consumption.

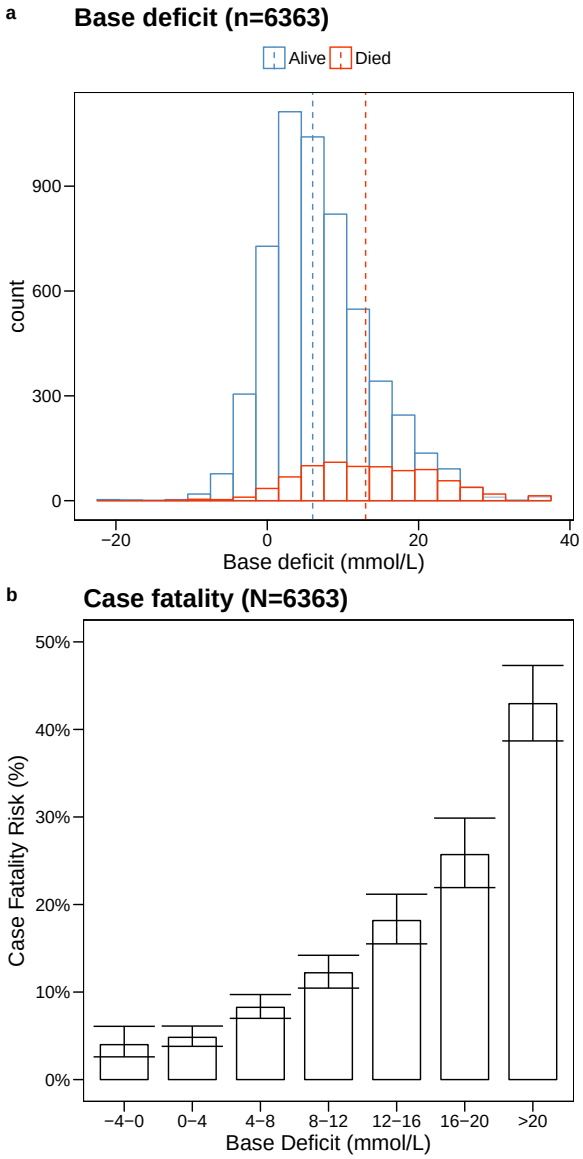


Fig. 1.5 **Base deficit in relation to outcome measured in 6,363 patients with severe falciparum malaria.** (Unpublished data, severe malaria legacy dataset 1986-2016, Mahidol-Oxford Tropical Medicine Research Unit).

In addition to microvascular obstruction, multiple other clinical complications may coincide that could cause a high L-lactate: shock, severe anaemia, renal failure, thiamine deficiency, parasite L-lactate metabolism, and stress or adrenaline induced glycolysis (Planche et al., 2005; Planche and Krishna, 2006). The causes of hyperlactataemia in severe malaria are discussed in more detail below, Section 1.8.1. Importantly, L-lactate correlates strongly with microvascular obstruction directly visualised by video microscopy (Dondorp et al., 2008a), retinal blood flow abnormalities (Abu Sayeed et al., 2011), reduced red cell deformability, and parasite biomass (Ishioka et al., 2016). In contrast, macrovascular shock is uncommon and therefore less likely to contribute significantly to the high incidence of hyperlactataemia (Jarvis et al., 2006) and fluid resuscitation does not significantly alter the observed acidosis (Hanson et al., 2013b).

L-lactate does not explain the full extent of acidosis and there are other unidentified acids elevated in blood (Dondorp et al., 2004a; English et al., 1997b; Herdman et al., 2015; Sasi et al., 2009). Using the so called 'Stewart approach', an elevated strong ion gap was observed in children and adults with severe malaria who died, suggesting unidentified acids are present in blood and prognostic of outcome (Dondorp et al., 2004a; Sasi et al., 2009). Further characterisation of metabolic acidosis in patients with severe falciparum malaria by direct quantification of organic acids showed ketones were slightly elevated in children (Sasi et al., 2009). Ketones were not an important contributor to acidosis in a study in adult patients with severe malaria (Herdman et al., 2015). Establishing the identity of currently unidentified acids can provide new insight into the pathophysiology of metabolic acidosis with potentially important therapeutic implications.

Cerebral malaria and convulsions

Cerebral malaria (CM) refers to unrousable coma observed in patients with *P. falciparum* malaria. Coma is defined as a Blantyre Coma Scale <3 out of 5 (BCS) in pre-verbal children or a Glasgow Coma Scale <11 out 15 (GCS) in all other patients (WHO, 2014; World Health Organization, 2015). The prevalence of coma on hospital admission is similar among children and adults (30-40%) (Dondorp et al., 2008b). Notably, a history of two or more generalized convulsions in the 24 hours prior to admission is much more common in children (80%) than in adults (15%) (Dondorp et al., 2008b). In a large randomized controlled trial the risk of death among adult patients treated with artesunate was increased if they had coma (30%) compared to those without coma (6%) (Dondorp, 2005).

In patients who are alert, confusion and agitation are prodromal for cerebral malaria. In patients who have developed coma, other underlying causes should be ruled out first, such as hypoglycaemia or a bacterial meningitis. Existing hypoglycaemia should be corrected. A lumbar puncture can provide evidence for underlying meningitis and is considered safe in children even when there is papilloedema or brain swelling on magnetic resonance imaging (MRI) (Moxon et al., 2016). Fundoscopy can reveal malaria specific retinopathy including haemorrhages, vessel discolouration, cotton wool spots, and papilloedema (in children) (Maude et al., 2009).

There is ongoing debate whether the pathophysiology of cerebral malaria differs between children and adults. Given that the causative pathogen is the same (*P. falciparum*) and the pivotal pathogenic mechanisms of malaria are not age-dependent, there is no *a priori* reason to assume a difference in the pathogenesis of cerebral malaria in children and adults. The key

role for sequestration of late stage asexual *P. falciparum* parasites in the microvasculature of the brain has been confirmed in all ages by numerous autopsy studies (Silamut et al., 1999; Taylor et al., 2004). Sequestration of parasitised red cells in the brain microvasculature and endothelial dysfunction may cause microcirculatory blood flow abnormalities and sludging of flow in the brain vessels. Based on the observations of rapid loss of consciousness and fast recovery in patients who are successfully treated (Dondorp et al., 2010) (Time to speak in comatose patients in the AUQUAMAT trial was 18-20 hours), cerebral malaria resembles a neurological complication suggestive of a metabolic encephalopathy. The pathogenic features of cerebral malaria are generally similar in children and adults.

An indication that the pathogenesis of cerebral malaria might be age-specific is that brain swelling is more prominent in children. Paediatric MRI studies suggest increasing brain volumes related to vasogenic oedema and increased red cell mass due to erythrocyte sequestration, in some cases this may result in increased intra-cranial pressure with compression of the brainstem and subsequent neurogenic respiratory failure (Potchen et al., 2012; Seydel et al., 2015). In adults with cerebral malaria, brain swelling is less prominent and not related to depth of coma (Medana et al., 2011; Mohanty et al., 2011). Yet, unpublished MRI studies show that the pathogenesis of cerebral malaria in adults also involves vasogenic oedema, cytotoxic oedema and an increased blood volume related to red cell sequestration (personal communication, Professor A.M. Dondorp, Mahidol-Oxford Tropical Medicine Research Unit).

The current treatment of cerebral malaria aims to provide optimal supportive care to prevent airway blockage, aspiration pneumonia and hypoglycaemia, in order to bridge a

vulnerable period of reduced consciousness until antimalarial treatment starts to act and the patient regains consciousness. Where mechanical ventilation is not available, a patient should be placed in the recovery position with their mouth open and their head tilted back to secure an open airway and allow secretions to drain from the mouth. A nasal airway or a Guedel tube can help to maintain an open airway (note some patients have jaw-lock or bruxism). Although feeding requirements may be increased, naso-gastric feeding increases the risk of aspiration in the acute phase and should be started only after 60 hours from admission (Maude et al., 2011). If seizures occur, benzodiazepines should be given. If these are insufficient, additional anticonvulsive therapy is indicated, which in practice often includes phenobarbital. Since these drugs are respiratory depressants, respiratory monitoring is important, especially where mechanical ventilation is not an option. Adjunctive treatments that have been trialled and found not to be effective or even harmful include mannitol, dexamethasone, corticosteroids, and seizure prophylaxis with high dose phenobarbital (20 mg/kg body weight).

Acute kidney injury

Acute Kidney Injury (AKI) in severe malaria is defined by the World Health Organisation as a serum creatinine >265 μmol/L (3mg/dL) (World Health Organization, 2015). Based on the WHO criteria, AKI is more common in adults (40%) (Dondorp, 2005) than children (around 10%) (Dondorp et al., 2010). However, when using paediatric criteria for acute renal impairment, the incidence of AKI in children may be underestimated and higher (up to 50%) (Conroy et al., 2016). AKI is associated with high mortality in all ages (Dondorp et al.,

2008b), among adults treated with artesunate the mortality is increased (38%) compared to patients without renal failure (9%) (Dondorp, 2005).

Malaria-associated AKI can be due to pre-renal or intra-renal causes. Pre-renal factors include dehydration and hypotension leading to decreased filtration. Microvascular sequestration by parasitised red cells is associated with acute tubular necrosis in malaria (Nguansangiam et al., 2007). The presence of AKI has been related to increased parasite densities and mono-nuclear cell activation (Plewes et al., 2014). Importantly, CFH may induce oxidative stress and intrinsic renal insults (Elphinstone et al., 2016). Following intravascular haemolysis, CFH is scavenged by haptoglobin and haemopexin, and when their scavenging capacities are reached CFH becomes harmful to the kidneys and starts to appear in the urine, causing a dark discolouration of urine responsible for the classical observations of blackwater fever (Elphinstone et al., 2016; Plewes et al., 2017).

To accurately measure a fluid balance, a 'Foley' catheter should be placed. Oliguria (<0.5 mL/Kg/h) is more commonly found in adults than children. If an oliguric patient is also dehydrated, fluid replacement should be given first. Creatinine and urea (blood urea nitrogen) may normalize upon initial fluid resuscitation. If there is still no urine output after fluid replacement nor after adding a loop diuretic and blood markers indicate renal dysfunction, then renal replacement therapy may be considered and fluid therapies should be tailored down. Early referral based on current indications for renal replacement therapy is a priority. If available, haemodialysis or haemofiltration are preferred over peritoneal dialysis (Phu et al., 2002). A recent randomized trial found paracetamol (acetaminophen) was a potential reno-protective agent by preventing haemoprotein-induced renal damage

(Plewes et al., 2018). Currently, studies are being prepared and conducted to evaluate the effect of reno-protective adjunctive therapies using paracetamol in children and patients with *P. knowlesi* infection (Cooper et al., 2018).

Severe anaemia

Severe anaemia attributable to malaria infection is defined as a parasitaemia >10 000/µL with a haemoglobin (Hb) <50 g/L or a haematocrit <15% in children and a haemoglobin <70 g/L or a haematocrit <20% in adults (WHO, 2014). Approximately 30% of children and 24% of South-East Asian adults have severe anaemia on admission to hospital (Dondorp, 2005; Dondorp et al., 2010). A recent systematic review and meta-analysis found anaemia only weakly correlated with increased mortality within the group of patients with severe malaria (Sypniewska et al., 2017). Only when anaemia drops below a critical threshold (Hb <40 g/L) it becomes strongly associated with an increased risk of death (von Seidlein et al., 2012). Severe malaria anaemia is an important and treatable determinant of patient outcome. Pre-existing anaemia can be present in children mostly due to iron-deficiency or chronic inflammation and children with chronic anaemia can rapidly decompensate during an acute severe malaria infection.

Malaria-attributable anaemia is caused by the loss of both infected and uninfected red blood cells, as well as dyserythropoiesis during the acute phase of the disease. Obligatory haemolysis follows schizogony, when the red cell is destroyed by the release of merozoites into the circulation. Thus, each asexual life cycle worsens (existing) anaemia. Reduced red cell deformability is observed in both infected as well as uninfected red cells and associated

with anaemia (Dondorp et al., 1999). Stiffening of the parasitised red blood cells is thought to increase red cell removal by the spleen further contributing to anaemia (Buffet et al., 2011). Non-malaria causes of anaemia may be present due to pre-existing red cell disorders related to nutritional deficiencies, haemoglobinopathies or other inherited disorders, and concomitant infections such as hookworms, HIV, or bacteraemia.

Recommended thresholds for blood transfusion in a moderate to high transmission settings are a Hb < 50 g/L or a haematocrit <15%; In low transmission setting, the recommended threshold is 70 g/L or a haematocrit <20% (WHO, 2014; World Health Organization, 2015). Thresholds also depend on the availability of a safe source of blood for transfusion and patient symptoms. Currently, a multicentre three-way factorial randomized clinical trial is being conducted to evaluate blood transfusion strategies, vitamin supplementation, and Co-Trimoxazole prophylaxis, in a large paediatric population in sub-Saharan Africa (Maitland, 2016) [ISRCTN84086586].

Dehydration and shock

There are few reliable clinical indicators to assess fluid status in critically ill patients with severe falciparum malaria (Hanson et al., 2013a), however patients are nearly always dehydrated. Orthostatic hypotension occurs in pre-treated patients and may be present in uncomplicated malaria (Butler and Weber, 1973; Supanaranond et al., 1993). Ultrasound assessment of the inferior vena cava (IVC) showed high IVC collapsibility indices in an observational study in 30 Kenyan children, while cardiac output was maintained (Yacoub et al., 2010).

Dehydration in severe malaria is not necessarily related to shock. In severe malaria, arterial blood pressure is usually maintained within normal ranges despite very high parasite densities. In children, signs of shock (systolic blood pressure <70 mm Hg with cold peripheries) were found in 12% of cases and associated with a mortality of 19% (von Seidlein et al., 2012). In adults, signs of shock (systolic blood pressure <80 mm Hg with cold peripheries) were found in 11% of patients and associated with a mortality of 16% (Dondorp, 2005).

Fluid management in patients with severe malaria who do not have access to mechanical ventilation poses a risk of overfilling. Because of an increased risk of ARDS due to a combination of increased capillary leakage and fluid overload (Hanson et al., 2013b), there is a general recommendation to "*keep the patient (slightly) dry*" (WHO, 2014). In the absence of hypotensive shock, fluid deficits should be corrected *slowly* using normal saline (0.9%) in children (3-5 mL/Kg/h over 3-4 hours) and adults (3-5 mL/Kg/h over 6 hours), with regular reassessment of chest and vital signs. In a large multicentre randomized controlled study in 1793 African children with compensated shock, defined as signs of hypoperfusion but no severe hypotension, fluid bolus therapy (20 or 40 mL/ kg body weight) was associated with a sharp increase in mortality compared to more conservative fluid management (9.2% vs 5.8%; relative risk 1.59 (95%CI 1.10-2.31) (Maitland et al., 2011). In hypotensive shock, fluid bolus therapy remains indicated, as in other causes of (bacterial) septic shock.

Individualized fluid regimens may prevent adverse outcomes related to fluid therapies. However, it is currently unclear what the best clinical measures are to assess fluid status and fluid responsiveness, what the upper limits of fluid resuscitation are before patients develop

pulmonary oedema, and what the lower limits of restrictive fluid management are before microvascular and kidney perfusion are compromised in patients with severe malaria.

Concomitant sepsis

Malaria infection predisposes children and adults to bacterial sepsis. The estimated incidence of concomitant bacteraemia ranges between 6-16% in children and adults (Nyein et al., 2016; Scott et al., 2011). Bacteraemia may be underestimated because of low yield of microbiological culture methods, partly caused by pre-treatment with antibiotics or limitations in laboratory facilities. An increased incidence of bacteraemia during malaria infection has been described in children (Bassat et al., 2009; Berkley et al., 1999; Bronzan et al., 2007; Enwere et al., 1998; Scott et al., 2011; Takem et al., 2014; Were et al., 2011), as well as in adults (Nyein et al., 2016). In Mendelian randomisation studies, sickle cell trait (HbAS), known to confer protection against severe malaria, also appeared to protect against bacteraemia (Scott et al., 2011). In endemic regions where the incidence of malaria dropped, the protective effect of HbAS against bacterial sepsis disappeared (Scott et al., 2011). Thus, malaria infection poses a higher risk to the development of concomitant sepsis.

Common pathogens that are isolated from blood include Gram-negative enteric bacteria such as non-typhoidal salmonella and *Escherichia coli* (Chau et al., 2013; Nyein et al., 2016; Scott et al., 2011). Malaria may predispose for Gram-negative enteric bacteraemia by increasing the permeability of the intestinal wall through infected red cell sequestration in the mesenteric circulation (Pongponratn et al., 1991, 2003; Seydel et al., 2006; Taniguchi et al., 2015). Microvascular obstruction has been directly visualised *in vivo* (Dondorp et al.,

2004a) and could potentially lead to intestinal injury with increased intestinal permeability (Molyneux et al., 1989a; Wilairatana et al., 1997). Although leukocytosis might indicate concomitant sepsis, there is no reliable biomarker to predict bacteraemia in the absence of blood culture.

1.6 Knowledge gaps

The case fatality rates of severe malaria range between 10-30% despite optimal treatment, and acidosis is the strongest predictor of death. Acidosis in severe malaria is characterised by a metabolic acidosis with a high-anion gap and an elevated L-lactate. However, L-lactate does not explain the full degree of acidosis observed, indicating the presence of unidentified acids. These currently unidentified acids, quantified using standard base deficit, anion gap, and strong ion gap, are independently associated with mortality. Assuming that most organic acids are metabolised, acidosis may resolve over time if the underlying cause is adequately treated and patients receive optimal supportive care to survive critical illness. Further characterisation of these acids could help identify the underlying source of non-lactate acidosis. Establishing the identity of unknown acids in patients with severe falciparum malaria should be a priority, as knowing the source of these acids can have important implications for treatment.

1.7 Acid-base physiology

1.7.1 Definitions

Background

To determine the causes of acid-base derangements in severe falciparum malaria it is important to establish what constitutes normal acid-base homeostasis and which outcome measures are useful for the quantification of metabolic acidosis. Acid-base balance refers to the regulation of the concentration of hydrogen ions [H^+] in a physiological aqueous solution. Hydrogen ions are among the most strictly regulated ions in the human body because they have a high propensity to bind proteins, which can transform their charge distribution, configuration, and ultimately their function. Compared to other ions such as sodium or potassium, which occur in millimolar ranges, hydrogen ions are regulated in a nanomolar range (around 40 nmol/L). Uncontrolled accumulation of [H^+] can disrupt normal cell metabolism.

Acid-base theory has developed mainly over the past two centuries and included different perspectives on what constitutes an 'acid' or a 'base'. Reviewing the literature on acid-base theory, I noted there are still differences in opinion over what is considered to be the best measure of the metabolic component of acidosis. The debate centres around the use of the 'classical' Henderson-Hasselbalch approach versus the 'modern' Stewart approach in describing the metabolic components of acidosis, which are discussed in more detail below. Although these approaches are theoretically different, they often have the same practical implications. Below, I will attempt to to summarise prevailing acid-base theories in a chronological order.

Historical perspective

In 1831, a cholera pandemic made its way from Russia into Great Britain. At the very start of the outbreak in Newcastle upon-Tyne, observations in patients with cholera were made by W.B. O'Shaughnessy. In a letter to the Lancet he wrote: "of the free alkali contained in healthy serum, not a particle is present in some cholera cases", and he described the loss of "carbonate of soda" during cholera disease (O'Shaughnessy, 1831). He associated clinical disease with a reduction of bicarbonate in blood and was thereby one of the first to describe a clinical acid-base disorder. This led him to propose a treatment using intravenous fluid therapy, which he experimented on dogs (O'shaughnessy, 1831). During the cholera outbreak, the intravenous fluid therapy was tested in humans by Thomas Latta (Lewins, 1832), which saved many lives.

Formal definitions of acid-base

A formal definition of acids and bases was lacking until 1884, when Arrhenius introduced an acid-base theory for which he received the Nobel prize in 1903. Before his theory gained acceptance, anions and cations were considered acids and bases. Instead, Arrhenius attributed acidity to the properties of hydrogen ions (H^+). He defined an acid as a substance which increases the concentration of H^+, more accurately hydronium H_3O^+, in water, and a base as a substance that can decrease its concentration (Arrhenius, 1889). Arrhenius' theory was extended by Brønsted and Lowry in 1923. Besides proton reactions increasing H_3O^+ in water, proton transfer reactions in non-aqueous solutions were also considered acid-base reactions (Brønsted, 1923; Lowry, 1923). This led to the currently accepted definition of acid-base:

an acid is a substance which can donate a proton; a base is a substance which can accept a proton. The general formula describing a Brønsted-Lowry acid-base reaction:

$$HA + H_2O \rightleftharpoons A^- + H_3O^- \tag{1.1}$$

The tendency of an acid to lose a H^+ in solution provides a measure of its acidity. The equilibrium constant for the protonated and deprotonated form of an acid quantitatively expresses the strength of an acid:

$$k_a = \frac{[H^+][A^-]}{[HA]} \tag{1.2}$$

The ability to measure H^+ using a glass electrode meter led to an increase in studies on acid-base balance (Cremer, 1906). This was helped by Sørensen, who introduced the concept of pH in 1909 (Sørensen, 1909). The logarithmic transformation of H^+ and Ka made it more practical to describe acid-base reactions.

$$pH = -\log_{10}[H^+] \tag{1.3}$$

$$pK_{[a]} = -\log_{10}[k_{[a]}] \tag{1.4}$$

In 1916, the Henderson-Hasselbalch equation was introduced based on the equilibrium constant of pCO_2 and HCO_3^- (Hasselbalch, 1916; Henderson, 1908). The aim was to describe a relationship to characterise acid-base balance in physiological fluids based on the partial pressure of carbon dioxide (pCO_2) and bicarbonate (HCO_3^-). Here pK is the dissociation

coefficient of carbonic acid (H_2HCO_3) (6.1) and S is the solubility coefficient of CO_2 (0.0307) in water.

$$pH = pK + log10\frac{[HCO_3^-]}{[S_{CO2}][pCO2]} \tag{1.5}$$

When the Henderson-Hasselbalch equation is applied to human physiology the model represents the three main components of acid-base balance in the human body: 1) buffering capacity, 2) pCO_2 regulated by respiration; and 3) HCO_3^- regulated by renal function. For example, a sudden increase of H^+ will be immediately buffered by plasma bicarbonate (HCO_3^-), followed by buffering through haemoglobin, plasma proteins and bone. Following and during this buffering phase, changes in respiration will compensate acidosis by regulating the partial pressure of carbon dioxide (a volatile acid) in blood. Finally, a protracted acid-base disturbance will be compensated by a renal component. The kidneys can increase the re-absorption of bicarbonate and the production of new bicarbonate, increase the excretion of organic acids, increase the excretion of NH_4^+ and acidify the urine to a minimal pH of 4.5. The process of normal daily acid production and clearance is summarised in figure 1.6. The Henderson-Hasselbalch equation reflects the components of normal acid-base physiology in humans as it models the effect of respiration and renal function on pH.

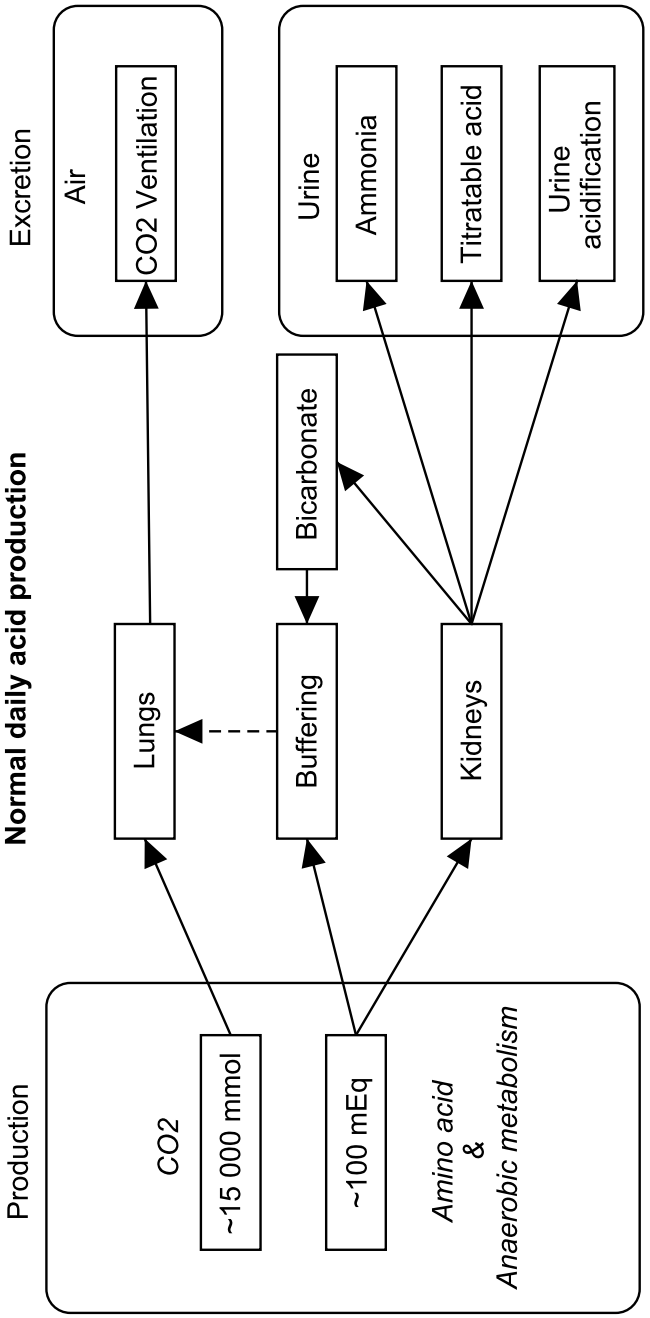


Fig. 1.6 **Normal daily acid production, buffering and excretion in humans.**

1.7.2 Outcome measures

Bicarbonate

A decrease in plasma HCO_3^- can result from a loss in bicarbonate, for example through severe diarrhoea (as in cholera) or an increase in plasma acids (as these are buffered by HCO_3^-). An increase in H^+ shifts the equation below towards the left, generating volatile CO_2, which can be expired in air (respiratory compensation maintaining the acid-base balance).

$$CO_{2(air)} \rightleftharpoons CO_{2(aq)} + H_2O \overset{CA}{\rightleftharpoons} H_2HCO_3 \rightleftharpoons H^+ + HCO_3^- \tag{1.6}$$

While HCO_3^- is generally considered to reflect the metabolic component of acid-base balance, there are some caveats. First, the Henderson-Hasselbalch equation implicitly assumes that pCO_2 and HCO_3^- are independent, which they are not. Carbonic anhydrase (CA) generates carbonic acid (H_2HCO_3), primarily in the kidney, so that retention of CO_2 will increase H_2HCO_3 levels, with a subsequent rise in HCO_3^-. Therefore, both pH and HCO_3^- are dependent on pCO_2. Consequently, the interpretation of an acid-base disorder according to the plasma HCO_3^- concentration and pCO_2 is different in case of a more chronic respiratory acidosis. Secondly, changes in the plasma concentration of HCO_3^- do not fully reflect the accumulation of non-respiratory metabolic acids because there are other important non-HCO_3^- buffers in blood.

$$2\,H^+ + HCO_3^- + \text{non}-HCO_3^-\text{buffers} \rightleftharpoons + H_2HCO_3 + H^+ + \text{non}-HCO_3^-\text{buffers} \tag{1.7}$$

The major non-HCO_3^- buffers are haemoglobin (in whole blood), and albumin and phosphate (in plasma).

Base excess

Various attempts have been made to identify measures of the metabolic component of acidosis that are robust against changes in pCO_2, including standard bicarbonate (Van Slyke et al., 1922) and buffer base (Singer and Hastings, 1948). In Copenhagen, Siggaard-Andersen advanced a new concept under the name base excess (BE) (Siggaard-Andersen, 1977). In blood or plasma, a deviation away from physiological pH caused by an accumulation of (fixed) acids or bases may be back-titrated by adding or removing hydrogen ions. Base excess quantifies the concentration of titratable hydrogen ions by mathematically titrating a sample to the physiological state of pH 7.4 , pCO_2 5.33 kPa, and a temperature of 37 °C. Siggaard-Andersen named the base excess equation after Van Slyke, as a tribute to his prior work on acid-base physiology (Siggaard-Andersen, 1977).

$$\mathrm{BE}=([\mathrm{HCO_3^-}] + 24.4\,) + (2.3\,[\mathrm{Hb}] + 7.7) \cdot (\mathrm{pH} - 7.4) \cdot (1 - 0.023 \cdot [\mathrm{Hb}]) \qquad (1.8)$$

In the Van Slyke equation, as stated above, base excess, $[HCO_3^-]$, and haemoglobin (Hb) are quantitatively expressed in millimol per litre (mmol/L).

In vitro experiments showed estimation of base excess using the van Slyke equation is robust to large fluctuations in pCO_2 (Morgan et al., 2000). However, *in vivo* studies suggested base excess may still be affected by pCO_2 as variations in the concentration of

haemoglobin could lead to differences in the re-equilibration of CO_2 across the interstitial fluid compartment (Park et al., 2009; Siggaard-Andersen and Fogh-Andersen, 1995). In response, Siggaard-Andersen introduced 'standard base excess', which used a fixed value for Hb and was found less affected by pCO_2 *in vivo* (Siggaard-Andersen and Fogh-Andersen, 1995). The negative transformation of standard base excess is the standard base deficit, which applies more directly to states of metabolic acidosis, and will be used hereafter. Standard base deficit provides an overall estimation of the metabolic acids contributing to acidosis but it does not specify the cause. Partitioning of the standard base deficit shows the main contributing factors are unmeasured acids, albumin and chloride (O'Dell et al., 2005). As a result, the standard base deficit does not represent the unmeasured acids when concentrations of albumin or chloride have changed importantly. Hypoalbuminaemia may have an important alkalinising effect and could lead to an underestimation of the severity of acidosis when the standard base deficit is used for interpretation. A standard base deficit that is corrected for plasma albumin concentrations can circumvent this problem. Severe hyperchloraemia, which is often iatrogenic due to liberal fluid resuscitation with 0.9% w/v NaCl, may artificially increase the standard base deficit.

Stewart approach

The only acid-base theory attempting to quantitatively describe the source of acid-base disturbances has been developed by Peter Stewart, working at Brown University (Stewart, 1978, 1983). The so called 'Stewart approach' emphasizes the importance of the laws of physical chemistry in acid-base physiology: 1) the law of electro-neutrality (positive charges

must be balanced by negative charges); 2) the law of conservation of mass (stoichiometry); 3) the law of mass action (dissociation equilibria must be met). Stewart reasoned all relevant physiological acid-base disturbances occur in solutions predominantly constituted by water. Water is electrically neutral. Negatively charged ions present in water have to be balanced by positively charged ions.

The first independent variable in the Stewart model is called the strong ion difference. In a physiological solution, such as blood plasma, there are completely dissociated ions referred to as 'strong ions', including sodium (Na^+) , potassium (K^+), Calcium (Ca^{2+}), Magnesium (Mg^{2+}) and chloride (Cl^-) (Van Panthaleon Van Eck et al., 1957). These strong ions lead to a charge difference, referred to as the strong ion difference (SID).

$$\text{Strong Ion Difference (SID)}=([Na^+] + [K^+] + [Ca^{2+}] + [Mg^{2+}]) - [Cl^-] \tag{1.9}$$

The second independent variable in the model is the total concentration of weak acids. In physiological solutions, incompletely dissociated weak acids include albumin, phosphate, L-lactate, and other non-volatile acids. These weak acids, with their total concentration expressed as $[A_{tot}]$, are dissociated according to their dissociation constant at physiological pH, similarly as to previously stated in Equation 1.1 above and Equation 1.10 below.

$$[A_{tot}]=[HA] + [A-] \tag{1.10}$$

The third independent variable of the model is the partial pressure of CO_2. Acid-base status, according to Stewart, is determined by the independent effects of SID,$[A_{tot}]$, and

pCO_2. In a physiological solution, these three variables are balanced by the dissociation of HCO_3^- (in water) to accommodate differences in charge imbalances and to maintain electro-neutrality. Since the dissociation constant of water is extremely high ($1.0 \cdot 10^{-14}$), there is a practically unlimited supply of H^+ to balance any charge differences due to strong ions and weak acids. Thus, following Stewart's theory, pH and bicarbonate are dependent variables. In this context, acid base disturbances can be interpreted as a result of water and electrolyte disorders.

Comparison of the classical and modern approaches

The 'classical' Henderson-Hasselbalch model and the 'modern' Stewart approach are conceptually very different. I interpret them as having opposing views on the fundamental nature of H^+ in physiological solutions: the classical approach views H^+ as an independent variable and the modern approach views H^+ as a dependent variable. For example, the classical approach emphasizes that H^+ are produced as a consequence of intracellular metabolism and may lead to a shift of electrolytes in and out of the cell to accommodate electroneutrality. This is contrary to the approach proposed by Stewart, which finds that electrolyte disorders are themselves responsible for an increase or decrease of H^+ as they lead to the dissociation of HCO_3^-. These fundamental (and possibly non-falsifiable) differences are important for the interpretation of acid-base physiology and the behaviour of H^+. They have led to long-standing and still unresolved debates within the acid-base community described as "the Great Trans-Atlantic Acid-Base Debate" (Severinghaus, 1993) (as proponents of the Henderson-Hasselbalch model are predominantly from Europe and proponents of the

Stewart approach from the United States of America). To the reassurance of most clinicians (including myself), both approaches generally come to the same conclusion with regard to the diagnosis of major acid-base derangements, except the Stewart approach claims to provide further insight in the underlying causal mechanisms and quantify better the contribution to the acid load of individual strong anions.

Critique of the Stewart approach

There are major limitations to the Stewart approach. First, the Stewart approach is predominantly based on *in silico* predictions. Second, the theory only considers the plasma compartment. Therefore, the effects of a Gibbs-Donnan equilibrium are unaccounted for, which refers to for the effect of large charged molecules, trapped within a cell and unable to cross over into plasma, which affect the re-equilibration of electrolytes across the (red) cell membrane. Third, the large number of measured variables can lead to cumulative errors and make it impractical to use at the bedside. Furthermore, the error margin of electrolytes measured in the millimolar range cannot account for accurate estimation of $[H^+]$ in the nanomolar range. Finally, Siggaard-Andersen called the Stewart approach 'anachronistic' because of the old definition of acids using anions and cations (Siggaard-Andersen and Fogh-Andersen 1995).

Integration of acid-base physiology and electrolyte disturbances

Although standard base deficit itself does not provide information on the source of a non-volatile acids contributing to an acid-base disturbance, this may be solved by (Siggaard-

Andersen, 2005): 1) determining the causative acid by direct measurement (e.g. lactic acid or ketones), or 2) using the anion gap, which is the gap between the readily measurable sum of cations minus the sum of anions in blood indicates an organic acidosis (Emmett and Narins, 1977; Oh and Carrol, 1977).

$$\text{Anion gap (AG)}=([Na^+] + [K^+]) - ([HCO_3^-] + [Cl^-]) \tag{1.11}$$

To reconcile the 'classical' Henderson-Hasselbalch approach and the 'modern' Stewart approach, the standard base deficit can be partitioned into albumin, chloride and unmeasured acids (O'Dell et al., 2005). Following such partitioning, the remaining unmeasured acid load correlates with the strong ion gap (O'Dell et al., 2005). Furthermore, there is positive correlation between the standard base deficit and the SID (Siggaard-Andersen and Fogh-Andersen, 1995).

Summary of conclusions

The acid-base literature includes an ongoing debate on the determinants of acid-base balance with on the one hand proponents of the Henderson-Hasselbalch model and on the other hand supporters of the Stewart approach. The metabolic (non-volatile) acid load can be accurately accounted for by measurement of the standard base deficit but may require adjustment for changes in plasma albumin in critically ill patients. While the standard base deficit does not reveal the causes of acid-base derangements, the Stewart approach claims to do so. However, the Stewart model has inherent weaknesses as it is dependent on the measurement of a large number of variables with the potential for cumulative errors. In conclusion, the standard

base deficit corrected for albumin and phosphate is a robust overall outcome measure of the metabolic component of acidosis, which I will use as a measure in this thesis. The anion gap, also corrected for albumin, may provide complementary information on the relation with coincidental electrolyte disorders. Only direct measurement and identification of organic acids contributing to standard base deficit may reveal the actual source of acidosis. This is the approach of the studies in this thesis.

1.8 Determinants of acidosis in severe falciparum malaria

To describe the acid-base disturbances specific to patients with severe falciparum malaria, I will summarise the common biochemical findings related to acid-base balance in malaria and then continue to describe the causes of acidosis in more detail below.

Blood gas analysis of patients with severe malaria usually reveals a low to low-normal arterial blood pH with a decrease in HCO_3^- as well as pCO_2, accompanied by an increase in standard base deficit and anion gap (Day et al., 2000b; Dondorp et al., 2004a; English et al., 1997b; Hanson et al., 2013b; Herdman et al., 2015; Krishna et al., 1994d; Taylor et al., 1993). These findings suggest there is an acid-gain type acute metabolic acidosis with respiratory compensation. Coinciding respiratory acidosis is uncommon and patients show in general adequate respiratory compensation (Hanson et al., 2013b). Known textbook causes of a high anion gap metabolic acidosis are summarized in Table 1.2, as adapted from elsewhere (Kraut and Madias, 2010).

Electrolyte disorders are usually moderate. Patients with malaria often have a moderate hyponatraemia (English et al., 1996c; Hanson et al., 2009; Maitland et al., 2005). Hyperchloraemia is uncommon at hospital admission, however iatrogenic hyperchloraemia may be caused by liberal fluid resuscitation with saline (Hanson et al., 2013b). Mild asymptomatic hypocalcaemia occurs frequently in patients with falciparum malaria, regardless of disease severity, and is likely related to a depressed parathyroid hormone response during infection (Davis et al., 1991; St John et al., 1995). Serum phosphate levels can be markedly depleted in patients with severe malaria who do not have renal impairment (Davis et al., 1991; St John et al., 1995), while in cases with acute renal failure (requiring dialysis) phosphate may be elevated (St John et al., 1995). Renal dysfunction plays an important role in the development of metabolic acidosis in patients with acute kidney injury due to severe malaria. However, acidosis also occurs in patients who do not have apparent renal dysfunction (Day et al., 2000b). Because plasma albumin is low (Graninger et al., 1992) a correction of standard base deficit and anion gap are appropriate. Serum osmolality is slightly elevated in patients with severe malaria (English et al., 1997b; Planche et al., 2004).

Table 1.2 **Specific causes of high anion gap metabolic acidosis.**

High anion gap metabolic acidosis
L-Lactic acidosis
D-Lactic acidosis
Fasting ketoacidosis
Diabetic ketoacidosis
Alcoholic ketoacidosis
Salicylate toxicity
Pyroglutamic acid toxicity
Acute kidney injury
Chronic kidney injury

Adapted from Kraut and Madias (2010).

Direct measurement of organic acids reveals an elevated L-lactate (Day et al., 2000b; English et al., 1997a; Hanson et al., 2012; Jeeyapant et al., 2017; Newton et al., 2005; von Seidlein et al., 2012; White et al., 1985). The standard base deficit is usually much larger than the concentration in L-lactate, suggesting there are unaccounted acids (Day et al., 2000b; Dondorp et al., 2004a; Hanson et al., 2012; Herdman et al., 2015; Sasi et al., 2009). No previous publications describe the concentration of D-lactate in patients with severe malaria. Ketones, resulting from fasting, may be present in paediatric patients (Sasi et al., 2009), but they do not contribute significantly to acidosis in adults (Herdman et al., 2015). There have been reports of salicylate toxicity (English et al., 1996a) and paracetamol poisoning may also cause a high anion-gap acidosis through the generation of pyroglutamic acid (5-oxoproline), but the role of these metabolites has not been confirmed in larger cohorts.

Further characterisation of acidosis by analysis of the strong ion gap using the Stewart approach shows there are remaining unidentified anions (Dondorp et al., 2004a; Hanson et al., 2013b; Herdman et al., 2015). The strong ion gap (SIG; derived from the Stewart approach) was independently predictive of mortality and correlated with creatinine and reduced renal clearance may contribute to the accumulation of organic acids (Dondorp et al., 2004a). These findings suggest that besides L-lactate there are other unknown acids that independently may contribute to mortality. Below follows a more detailed discussion of contributors to high anion gap metabolic acidosis in severe falciparum malaria including: 1) L-lactate; 2) unmeasured acids.

1.8.1 L-lactate

L-lactate is the main measured anion known to increase during severe falciparum malaria and the role of L-lactate is well established in the pathogenesis of acidosis. Here follows a discussion of the metabolism of L-lactate and the causes of hyperlactataemia in severe falciparum malaria.

L-lactate metabolism

In healthy individuals the venous L-lactate concentration is around 1 mmol/L. At rest, L-lactate production has been estimated at approximately 20 mmol/kg per day. Major L-lactate producing tissues are skeletal muscle (Brooks, 1986), the brain (Van Hall et al., 2009), adipose tissue (Jansson et al., 1996), and the intestines (Murphy et al., 2001). The relative contribution of each of the tissues to total body L-lactate production is unclear. L-lactate can be recycled within and between cells of the same tissue where compartmentalised pathways simultaneously produce and utilise L-lactate as an energy substrate (Brooks, 2009; Van Hall, 2000). L-lactate released in the bloodstream is cycled and oxidized in the liver ($\sim$70%) or kidneys ($\sim$30%) (Consoli et al., 1990), a process also known as the Cori cycle, Figure 1.7. Cross-membrane flux occurs through one of four currently known proton dependent monocarboxylate transporters (MCT1-MCT4), of which MCT1 is the best characterised and most abundant in skeletal muscle (Brooks, 2009; Gladden, 2004). L-lactate clearance is estimated around $\sim$800-1800 mL/minute (Levraut et al., 1997). Acidosis inhibits oxidation of L-lactate in hepatocytes (Iles et al., 1977) but not in the renal cortex (Alleyne et al., 1973). Renal excretion of L-lactate is around 1-2% and can increase during acidosis (Connor et al.,

1982). Human L-lactate is solely produced from pyruvate. L-lactate dehydrogenase (LDH) catalyses the interconversion between pyruvate and L-lactate. In humans, there are five known LDH isoforms with a different tissue distribution (LDH1 – LDH5) (Wróblewski and Gregory, 1961). LDH5 is the most dominant isoform in skeletal muscle and liver where it is present at high concentrations in the cytosol (Tesch et al., 1978). LDH5 contains four (M) subunits encoded by the LDHA gene.

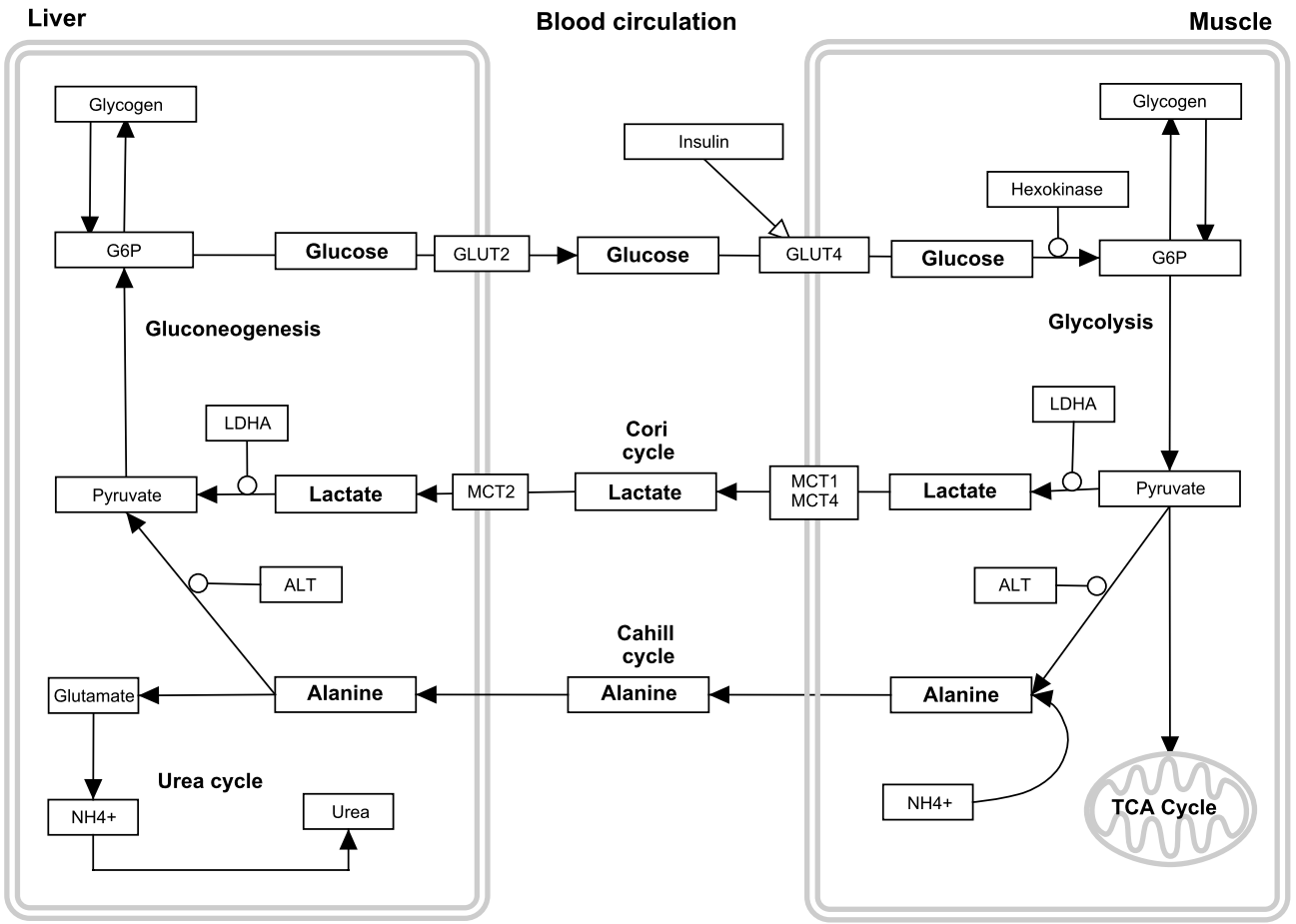


Fig. 1.7 **The 'Cori cycle' and the 'Cahill cycle'.**

L-lactate production

Pyruvate is the sole precursor of L-lactate. However, pyruvate itself has diverse metabolic fates. The intracellular determinants that favour conversion of pyruvate to L-lactate are the concentration of pyruvate, the [NADH]/ [NAD^+] ratio, and [H^+] in the cytosol, Equation 1.12. Any increase in these will shift the balance towards reduction of pyruvate to L-lactate.

$$[\mathrm{L-lactate}] = k_{eq}[\mathrm{Pyruvate}]\frac{[\mathrm{NADH}]}{[\mathrm{NAD^+}]}[\mathrm{H^+}] \tag{1.12}$$

The primary source of pyruvate is the breakdown of glucose. During glycolysis, free energy is released and stored in the form of ATP and reducing equivalents NADH and $FADH_2$. Pyruvate is the final product of glycolysis and onward metabolism usually occurs through the conversion to acetyl-CoA by the pyruvate dehydrogenase complex (PDC). Acetyl-CoA enters the tricarboxylic acid cycle (TCA) providing additional electrons and reducing equivalents for further oxidative phosphorylation of ATP. Under circumstances when oxygen is available, catabolism of the carbon chain of acetyl-CoA into the TCA cycle completes the process of combustion of glucose and oxygen to carbon dioxide and water, with a final net yield of $\sim$32 ATP.

Conversion of pyruvate to L-lactate thus occurs when pyruvate accumulates in the cytosol at a higher rate than it is removed. Accumulation of pyruvate can be due to reduced PDC activity in anaerobic conditions affecting the pyruvate dehydrogenase complex. Alternatively, adrenaline induced accelerated glycolysis can take place at a faster rate than the TCA cycle.

Redox state

If oxygen is not available, PDC reaction rates fall and substrate concentrations of pyruvate increase with lower TCA cycle activity (Kim et al., 2006). To compensate for the energy deficit of incomplete oxidation of glucose, the intracellular rate of glycolysis increases (Robin et al., 1984). However, the energy yields are only ∼2 ATP per molecule of glucose, and thus high glycolytic turnover is required to produce ATP over a sustained period of dysoxia. During intensified anaerobic glycolysis, changes that occur in the cytosol include: 1) formation of pyruvate as an end product; 2) consumption of available NAD^+ in the glycolysis pathway, increasing the NADH/NAD^+ ratio; and, 3) production of H^+ from the use (hydrolysis) of ATP. These are all favourable circumstances for the reduction of pyruvate to L-lactate, which facilitates continued glycolysis by oxidizing NADH, Figure 1.8. The parallel formation of L-lactate and the release of H^+ from the hydrolysis of ATP constitute a lactic acidosis.

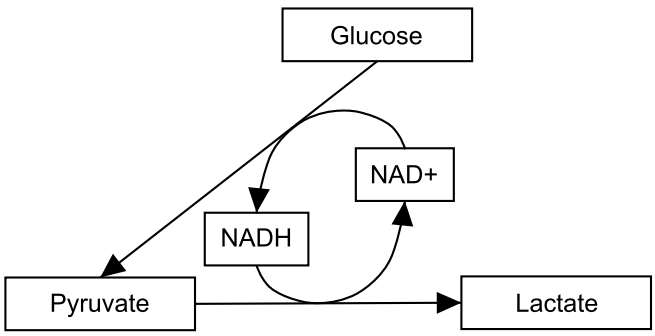


Fig. 1.8 **Cytosolic redox balance during glycolysis.**

In healthy individuals the concentration of L-lactate is, as mentioned before, around 1 mmol/L, and pyruvate is around 0.1 mmol/L. Hence, the lactate-to-pyruvate (L:P) ratio is $\sim$ 10 and may be read as a proxy measure of the intracellular redox state (Williamson et al. 1967). In patients with severe malaria L:P ratios were raised to $\sim$30 and predicted death (Day et al., 2000b; Krishna et al., 1994a). Prior unpublished data shows a significant difference in the L:P ratio in patients with malaria compared to meliodosis (p=0.011), Figure 1.9. This suggests that L-lactate is formed following a shift in the redox state of the cell in line with anaerobic or dysoxic conditions. In comparison, among patients with sepsis without severe haemodynamic shock, overall L:P ratios were below 20 (Kopterides et al., 2011; Levy et al., 1997), similar to a mixed patient cohort admitted to intensive care (Suistomaa et al., 2000). Only in severe haemodynamic shock L:P ratios also tended to increase in sepsis (Levy et al., 1997, 2000; Rimachi et al., 2012).

In septic hyperlactataemia, catecholamine induced acceleration of glycolysis might play a more important role than tissue hypoxia. Stimulation of $\beta 2$-adrenergic receptors in muscle by adrenalin raises intracellular AMP production (Clausen and Flatman, 1980). A subsequent increase in Na+ K+ ATPase generates cytosolic ADP and activates phosphofructokinase, a rate limiting enzyme in glycolysis (Dietz et al., 1979). Adrenergic stimulation thereby accelerates glycolysis and L-lactate formation under aerobic conditions.

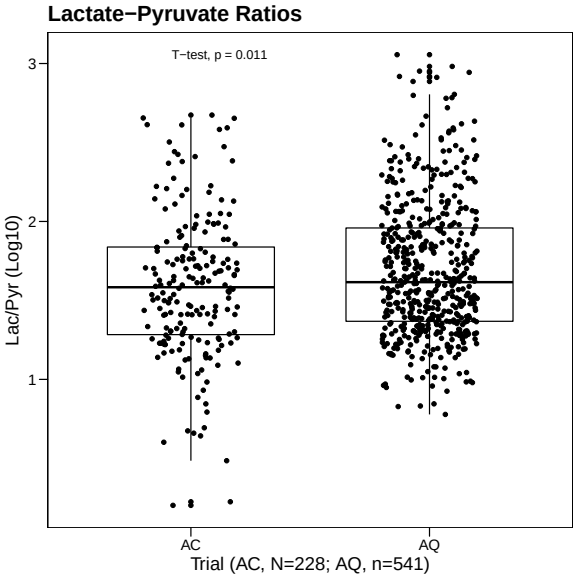


Fig. 1.9 **Lactate-to-pyruvate ratios in patients with melioidosis and severe falciparum malaria.** Patients with melioidosis were from Thailand (AC study, 1989-1991, n=228) and patients with severe falciparum malaria from Vietnam (AQ study, 1991-1995, n=541). (Unpublished data, Professor Sir N.J. White, Mahidol-Oxford Tropical Medicine Research Unit).

Glucose and L-lactate turnover in malaria

Patients with severe falciparum malaria rapidly use up their glycogen stores and often rely entirely on gluconeogenesis to maintain their plasma glucose (van Thien et al., 2001). Glucose production is therefore dependent on an adequate supply of gluconeogenic precursors. L-lactate, produced in peripheral tissues due to tissue dysoxia, is an important precursor and is shuttled back to the liver and utilised in gluconeogenesis (Cori cycle, Figure 1.7), while protein catabolism increases the levels of glucogenic free amino acids.

Hypoglycaemia is an important complication in severe malaria (White et al., 1983). It has been suggested that hypoglycaemia develops due to a shortage of glucogenic precursors, however, this was not found to be the case as additional infusion with alanine had no subsequent effect on glucose production (Dekker et al., 1997; Pukrittayakamee et al., 2002).

Hypoglycaemia used to be a particular problem in patients treated with quinine (White et al., 1983). Quinine stimulates the secretion of insulin and thereby induces hypoglycaemia (Davis et al., 2002, 1993b). In children with severe malaria who received quinine, the insulin-to-glucose ratios were significantly higher than in those who received artesunate (Agbenyega et al., 2000).

Impaired L-lactate utilisation in gluconeogenesis might play a role in reduced L-lactate disposal, thereby contributing to acidosis. However, although reduced liver blood flow has been observed in patients with severe malaria (Day et al., 2000b; Pukrittayakamee et al., 1994), hepatic L-lactate disposal is generally increased (Agbenyega et al., 2000).

Classification of hyperlactataemia in severe malaria

Having established that severe malaria is associated with increased concentrations of plasma L-lactate, the follow-on question is what causes increased L-lactate production in severe falciparum malaria. To classify the causes of hyperlactataemia, I have used a classification system based on the presence (Type A) or absence (Type B) of an underlying oxygen debt. The contributors to hyperlactataemia and their relative importance in severe malaria are summarised in Table 1.3. Below follows a discussion of the integrated physiology of hyperlactataemia in severe falciparum malaria.

Table 1.3 **Contributors to hyperlactataemia in severe *P. falciparum* malaria.**

Mechanism	Underlying problem	Specific cause	Contributors	Importance
Type A				
CaO_2 (↓)	Impaired oxygen carrying capacity	Anaemia	Haemolysis	(+)
			Splenic clearance	(+)
			Dyserythropoiesis	(+)
			Pre-existing	(-)
		Hb oxygen affinity	Temperature	(-)
			pH	(-)
			2,3-biphosphoglycerate	(-)
			Methaemoglobinaemia	(-)
DO_2(↓)	Macrocirculatory shock	Hypovolaemic	Severe dehydration	(+)
			Spontaneous bleeding	(-)
		Cardiogenic		(-)
		Vasodilatory	Concomitant sepsis	(+)
	Microcirculatory hypoperfusion	Obstruction	Sequestration	(+++)
			Red cell deformability	(+)
		Dysfunction	Nitric oxide depletion	(+)
			Hypoargininaemia	(+)
VO_2(↑)	Fever	Malaria infection		(-)
	Seizures	Cerebral malaria		(+)
	Concomitant sepsis	Malaria infection		(+)
Type B				
Hypermetabolic	Catecholamines (↑)	Endogenous adrenaline		(-)
		Adrenaline infusion		(-)
Exogenous	*P. falciparum* metabolism	Hyperparasitaemia		(-)
	Bacterial D-lactate	Gut translocation		(?)
	Ringers L-lactate	Infusion		(-)
Other	Thiamine deficiency	Pre-existing		(+/-)
	Salicylate toxicity	Aspirin overdose		(-)

CaO_2 = Oxygen content. DO_2 = Oxygen delivery. VO_2 = Oxygen consumption.

Type A lactic acidosis

Type A lactic acidosis is related to an underlying oxygen debt. A tissue oxygen debt can be caused by: 1) low oxygen content; 2) low oxygen delivery; 3) increased oxygen consumption.

Oxygen content (CaO_2) refers to the concentration of oxygen bound to haemoglobin and dissolved in plasma. The arterial oxygen content can be summarised as follows, where SaO_2 is the saturation of arterial oxyhaemoglobin and PaO_2 is the arterial oxygen tension:

$$CaO_2 \text{ (mL } O_2\text{/dL)} = (1.34 \cdot Hb \cdot SaO_2) + (0.0031 \cdot PaO_2) \tag{1.13}$$

Oxygen delivery (DO_2) is a function of oxygen content and cardiac output (Q), in other words, the volume of oxygenated arterial blood circulated by the heart to the tissues. Cardiac output is determined by the heart rate and the stroke volume. Oxygen delivery can be described as:

$$DO_2 \text{ (mL/min)} = CaO_2 \cdot Q \tag{1.14}$$

Oxygen consumption (VO_2) refers to the rate at which the perfused tissues remove oxygen from blood. Oxygen consumption is measured by respirometry where a patient breathes into an air chamber for a set duration of time to measure the depletion of oxygen and production of carbon dioxide and water.

$$VO_2 \text{ (mL } O_2\text{/min)} = Q \cdot (CaO_2 \cdot CvO_2) \tag{1.15}$$

Oxygen content

Severe anaemia

The epidemiology and pathophysiology of anaemia have already been discussed in Section 1.5.3. Anaemia can contribute to hyperlactataemia in severe malaria by decreasing the oxygen content in blood. Anaemia correlated with hyperlactataemia in studies on children with severe malaria (English et al., 1997a; Krishna et al., 1994c). Blood transfusion improved haemodynamic decompensation and in some cases also reduced the degree of hyperlactataemia (English et al., 1997a). However, a recent study of 286 adults with severe malaria found no correlation between haemoglobin and L-lactate (Ishioka et al., 2016). Only at critically low haemoglobin levels did anaemia correlate with plasma L-lactate, thus, very severe anaemia may contribute to hyperlactataemia. A recent study in Ugandan children showed that although severe malaria anaemia was positively related to plasma L-lactate, anaemia was not related to increased mortality, while other causes of a high lactate, such as parasite biomass, did show a relation to excess deaths (Brand et al., 2016).

Methaemoglobinaemia

Anaemia and hyperlactataemia may also have an indirect relationship. Obligatory intravascular haemolysis due to parasite schizogony increases the concentration of free haem and CFH and causes oxidative stress (Plewes et al., 2017). Toxic free haem can increase the concentration of methaemoglobin (MetHb), where the iron in haemoglobin is not in the normal ferrous Fe^{2+} state, such as in oxyhaemoglobin, but instead in the ferric Fe^{3+} state, which reduces the oxygen carrying capacity. Elevated MetHb to 2-15% (normally <2%) has

been described in children in the acute phase with in some cases a moderate increase after 10 days follow up (Anstey et al., 1996; Behera et al., 2016; Hänscheid et al., 2014).

Oxygen delivery

Shock

Oxygen delivery can be impaired due to macrovascular hypoperfusion following shock. Shock may contribute to hyperlactataemia but only in those cases who have signs of macrovascular hypoperfusion, either caused by: 1) hypovolaemic shock; 2) cardiogenic shock; or 3) septic shock. However, as stated before in Section 1.5.3, signs of circulatory shock are overall uncommon in severe falciparum malaria.

Hypovolaemic shock in severe falciparum malaria is likely related to severe dehydration. Ultrasound assessment of the inferior vena cava (IVC) of 30 children with severe falciparum malaria from Ghana showed a significantly higher IVC collapsibility index on admission in patients who were acidotic compared to patients without acidosis (52.1, SD 21.9 compared to 37.7 SD 15.4; p = 0.03) (Yacoub et al., 2010). This suggests that in this small population there might be a relation between acidosis and severe dehydration. However, among 31 adults with malaria from Bangladesh, the median IVC collapsibility index was not significantly different between patients with severe malaria or uncomplicated malaria (24, SD 21 compared to 29 SD 26; p = 0.777) nor was there a relationship with L-lactate (p = 0.41) (Leopold et al, under review, PLOS One 2018). Extracellular and total body water were measured by bioimpedance in a cohort of 180 Gabonese children with malaria and no signs were found of volume depletion or a relation between low volume status and venous L-lactate (Jarvis

et al., 2006). Thus, observational studies do not show a clear relation between dehydration and acidosis.

To determine if correction of dehydration reduces the severity of acidosis, previous studies have investigated the effects of fluid resuscitation. In a phase-1 study assessing the safety of saline or albumin resuscitation in 53 Kenyan children with severe malaria, 44% of patients had a systolic blood pressure below 80 mm Hg on admission and their base deficit improved after fluid replacement (Maitland et al., 2003). In contrast, in a study of 28 Bangladeshi adults with severe malaria, liberal fluid resuscitation on admission did not have a significant relationship with the change of venous L-lactate at six hours post-enrolment (p=0.08), while it increased the risk for developing pulmonary oedema (Hanson et al., 2013a).

In a large multi-centre randomized controlled study in 3141 African children with severe febrile illness and signs of impaired perfusion the effect on outcome following saline bolus, albumin bolus, or no bolus on admission was assessed (Maitland et al., 2011). In this study, 48-hour mortality was significantly increased in cases receiving bolus (10.6% in albumin bolus, 10.5% in saline bolus) compared to controls (7.3% no bolus) (Maitland et al., 2011). Among the 1793 children who were diagnosed with malaria in this study, mortality with fluid bolus was 9.2% compared to 5.8% in patients who did not receive a fluid bolus. Overall, in a retrospective secondary analysis, increased mortality was associated with cardiovascular collapse (Maitland et al., 2013).

Cardiogenic shock in severe malaria is unlikely as the cardiac index is usually normal or increased, as was previously found in a study of 183 Ghanese children (Nguah et al., 2012). In this study, the cardiac index was increased and negatively associated with haemoglobin,

suggesting a high output anaemia, while it was unrelated to acidosis (Nguah et al., 2012). Elevated cardiac enzymes have been noted in patients with acute malaria and could signify cardiac damage resulting from parasite sequestration in cardiac muscle tissue (Ehrhardt et al., 2004; Günther et al., 2003); however, this does not seem to result in clinically significant reductions in cardiac function.

Septic shock in severe malaria may occur following concomitant bacteraemia. In sepsis, the host response is characterized by a cytokine storm with downregulation of a1-adrenergic receptor expression (Bucher et al., 2003) and vasopressin deficiency (Landry et al., 1997), with subsequent vasodilatory shock. Normally, in severe malaria peripheral vascular resistance is maintained, likely due to the vasoconstrictive effects of CFH and free haem, which are potent nitric oxide scavengers (Rohlfs et al., 1998). Peripheral vascular resistance may drop and patients might rapidly decompensate when severe malaria coincides with sepsis. Therefore, in the small number of cases who develop concomitant sepsis, there may be vasodilatory shock with macrovascular hypoperfusion and subsequent hyperlactataemia.

Thus, there is only limited observational evidence that suggests that hypovolaemic shock is related to hyperlactataemia, however, there is evidence suggesting that liberal fluid resuscitation in severe malaria might be harmful. Cardiac muscle damage may occur due to cardiac sequestration by parasitised red blood cells but does not significantly impair cardiac function. Septic shock, although uncommon, may increase blood lactate in patients who develop fulminant concomitant sepsis.

Microvascular hypoperfusion

Oxygen delivery in severe falciparum malaria is impaired due to microvascular obstruction. Two major pathogenic mechanisms contribute to microvascular hypoperfusion: parasite sequestration and endothelial dysfunction.

Parasite sequestration occurs across vital tissues in the body and is central to the pathogenesis of severe malaria (White et al., 2013). Parasitised red blood cells adhere to the vascular endothelium of capillaries and post-capillary venules. Sequestration intensifies as the total body parasite biomass expands (Cunnington et al., 2013; Dondorp et al., 2005). Histopathology studies showed a correlation between pre-mortem organ failure and post-mortem organ sequestration (Nguansangiam et al., 2007; Pongponratn et al., 2003; Ponsford et al., 2012; Taylor et al., 2004). For example, in patients who developed severe malaria with renal failure, post-mortem examination showed relatively high sequestration in the kidneys (Nguansangiam et al., 2007). Similarly, children who had died from cerebral malaria had higher numbers of parasitised red cells in their brain compared to children who died from other causes (Pongponratn et al., 2003; Ponsford et al., 2012). Important L-lactate producing tissues include skeletal muscle (Davis et al., 1999) and the intestines (Seydel et al., 2006).

Evidence for a relationship between sequestration and microvascular hypoperfusion in patients with severe malaria comes from studies where the microcirculation was directly observed. Malaria-specific retinopathy observed *in vivo* was associated with post-mortem parasite sequestration in retinal blood vessels (Lewallen et al., 2000). In a cohort of 326 Malawian children with complicated malaria, retinopathy was related to cerebral malaria (CM) where 104 out of 170 (61%) patients with CM had retinal changes including haemor-

rhages and retinal whitening (Beare et al., 2004). Among 210 Bangladeshi adults with severe falciparum malaria, similar patterns of retinopathy were observed by direct ophthalmoscopy in 31 out of 75 (41%) patients with CM (Abu Sayeed et al., 2011). Impaired retinal perfusion was described by performing fluorescein angiography in 28 out of 34 (82%) children with cerebral malaria enrolled in Malawi (Beare et al., 2009). Cerebrospinal fluid studies show elevated levels of L-lactate suggesting anaerobic glycolysis in the brain (Medana et al., 2002; Molyneux et al., 1989b; Warrell et al., 1988). This is underlined by observations that the brain is a net producer of L-lactate in patients with cerebral malaria (White et al., 1985).

Microvascular perfusion abnormalities have been directly observed through *in vivo* video microscopy of the rectal microcirculation of 43 adult Bangladeshi patients with severe malaria; here, the proportion of blocked capillaries correlated with base deficit ($r = 0.66$ and $p = 0.001$) and L-lactate (rho = 0.54 and $p = 0.001$) (Dondorp et al., 2008a). In a separate study of 28 adults with severe malaria admitted to intensive care in Bangladesh and India, blocked capillaries correlated with L-lactate (rho = 0.55, $p = 0.003$) (Hanson et al., 2012). A combined analysis of adult patients with severe malaria who underwent video microscopy, including the aforementioned patient cohorts confirmed a correlation between the proportion of blocked capillaries and venous L-lactate (rho = 0.38, $p < 0.0001$) (Hanson et al., 2015a).

Animal models show lactic acidosis also develops in *P. berghei* ANKA infected mice (Holloway et al., 1991). In *P. coatneyi* infected rhesus monkeys post-mortem evidence for parasite sequestration was related to biochemical dysregulation observed *in vivo* (Davis et al., 1993a).

In humans, infected as well as uninfected red cells lose their natural deformability during falciparum malaria infection (Dondorp et al., 1999, 1997). As red cells become less deformable, they are more likely to be become sequestered and cause microvascular obstruction. Reduced red cell deformability in patients with malaria was associated with hyperlactataemia (Ishioka et al., 2016).

Endothelial cell activation has been associated with tissue sequestration in histopathological studies (Garcia et al., 1999; Turner et al., 1994, 2009), is associated with low nitric oxide bioavailability (Lopansri et al., 2003; Yeo et al., 2007, 2014), and is characterised by increased plasma markers, including Angiopoietin-2 and VEGF (Medana et al., 2010; Petersen et al., 2016; Yeo et al., 2008c). Nitric oxide regulates capillary blood flow (Yeo et al., 2013a) and reduces the expression of endothelial host receptors involved in parasite adhesion (De Caterina et al., 1995). Nitric oxide is depleted in severe falciparum malaria by increased breakdown and reduced production. Loss of NO is likely due to scavenging by CFH or the presence of nitric oxide synthase inhibitors such as asymmetrical dimethylarginine (ADMA) (Yeo et al., 2010). Reduced production of nitric oxide results from depletion of its precursor L-arginine.

L-arginine deficiency is reported in severe and uncomplicated malaria (Yeo et al., 2008a, 2007, 2008b, 2013b). Metabolism of L-arginine occurs through nitric oxide synthase (NOS), arginases can also metabolise L-arginine to produce urea and ornithine. Arginases that speed up the breakdown of L-arginine may be related to the parasite (Cobbold et al., 2016; Olszewski et al., 2009) or endogenous host erythrocyte arginases. However, a recent study did not suggest a role for parasite derived arginases in the pathogenesis of L-arginine deficiency

in vivo (Alkaitis et al., 2016). Instead, reduced production of L-arginine from L-citrulline may contribute to a L-arginine deficiency (Alkaitis et al., 2016). In a longitudinal study of 49 patients with severe malaria and 48 patients with moderate severe malaria, endothelial dysfunction was related to low bioavailability of plasma L-arginine and associated with plasma L-lactate (Yeo et al., 2008c). This was consistently found in another study in 51 patients with severe malaria and 48 moderate adults, where reduced exhaled NO correlated with measures of intravascular haemolysis, reduced vascular responsiveness and L-lactate (Yeo et al., 2007). Potentially, dysregulation of the L-arginine/NO pathway may contribute as a factor aggravating microvascular perfusion, and as such, this could worsen an existing hyperlactataemia.

Adjunctive therapies that increase nitric oxide bio-availability may improve microvascular blood flow and could thereby improve outcome. Since nitric oxide itself is unstable, therapies to replace it directly have generally been less successful (Hawkes et al., 2015). Studies that aimed to replace the L-arginine precursor have shown promising effects (Yeo et al., 2008a, 2007, 2008b, 2013b) but there are worries that L-arginine hydrochloric acid may induce acidosis. Possibly, replacement of L-citrulline may improve L-arginine and nitric oxide bioavailability (Alkaitis et al., 2016).

Thus, measures of tissue sequestration, endothelial dysfunction and microcirculatory obstruction strongly correlated with L-lactate. Therefore, microcirculatory hypoperfusion is a major cause of lactic acidosis in severe falciparum malaria.

Oxygen consumption

Basal metabolic rates are increased in acute malaria infection and as in other severe infection, the catabolic state results in accelerated whole-body protein turnover (Berclaz et al., 1996). During the acute phase of septicaemia, hepatic extraction of plasma amino acids is commonly increased, probably to due to upregulated hepatic gluconeogenesis from glucogenic amino acids (Druml et al., 2001; Fischer et al., 1995; Sprung et al., 1991). As a consequence, patients with malaria develop a negative nitrogen balance when free amino acids are utilised as energy supply. These observations indicate increased metabolic activity during infection. High fever and an inflammatory state further increase basal oxygen consumption. In patients who have seizures, generalized tonic-clonic convulsions can increase oxygen consumption in skeletal muscle.

Type B lactic acidosis

Stress induced accelerated glycolysis

Endogenous or exogenous catecholamines, such as (infused) adrenaline, accelerate (aerobic) glycolysis. Accelerated glycolysis plays a role in the pathogenesis of lactic acidosis in sepsis. In a study of 14 adults with septic shock, bilateral femoral microdialysis catheters were inserted and repeated L-lactate and pyruvate measurements were done over time (Levy et al., 2005). In one of both microdialysis catheters, ouabain was infused, a specific inhibitor of Na+ K+ ATPase. The Na+ K+ ATPase generates cytosolic ADP which activates the rate limiting glycolytic enzyme phosphofructokinase. In the study by Levy et al, ouabain slowed down glycolysis by inhibiting the catecholamine inducible Na+ K+ ATPase, and thereby

reduced the overproduction of pyruvate and L-lactate around the microdialysis catheter in the ouabain infused leg (p = 0.0001) (Levy et al., 2005). These data show that L-lactate production in patients with sepsis could be lowered by inhibiting a catecholamine mediated pathway, indicating hyperlactataemia resulted from aerobic acceleration of glycolysis rather than anaerobic glycolysis due to hypoxia. Vice versa, infusion of adrenaline worsens hyperlactataemia in sepsis (Levy et al., 1997) and in severe falciparum malaria (Day et al., 1996).

Thiamine deficiency

Thiamine deficiency precipitates hyperlactataemia in the presence of normal tissue oxygenation. Thiamine is a necessary co-factor of pyruvate dehydrogenase. Thiamine deficiency reduces PDC activity leading to an accumulation of pyruvate in the cytosol. To balance the redox state of the cytosol, pyruvate is instead converted to L-lactate. Thiamine deficiency can be quantified by direct measurement of thiamine levels using high performance liquid chromatography (HPLC) or by measuring erythrocyte transketolase activity. In areas where malaria is endemic, patients may develop thiamine deficiency due to poor nutrition, often because of a monotonous diet of polished rice. The link between clinical symptoms and thiamine deficiency was first made by Christiaan Eijkman, working in Indonesia, who received the Nobel prize for his observations. Thiamine deficiency has been reported in patients with severe malaria from Thailand (Krishna et al., 1999) and patients with uncomplicated malaria from Laos (Mayxay et al., 2007). Thiamine deficiency could therefore aggravate lactic acidosis in severe malaria.

Exogenous L-lactate

P. falciparum parasites produce L-lactate when they are kept in continuous culture (Cranmer et al., 1995; Kanaani and Ginsburg, 1991; Pfaller et al., 1982; Senczuk et al., 2001). *P. falciparum* relies entirely on glycolysis for the supply of energy. *In vitro*, the utilization of glucose of infected red cells increases up to a 100-fold when compared to uninfected red cells (Wu et al. 2015). L-lactate is exported by a formate L-lactate transporter (Marchetti et al., 2015) and contributes to the acidification of the surrounding culture medium (Jensen et al., 1983). The parasite relies on host glucose for glycolysis and on host amino acids for its metabolism since *de-novo* amino acid production is nearly absent in *P. falciparum* parasites (Liu et al., 2006). Modelling of the parasite-derived proportion of L-lactate to host cell L-lactate metabolism shows that only at (very) high parasite biomass the contribution of parasite derived L-lactate may contribute to acidosis (Leopold et al., 64th Meeting of the American Society of Tropical Medicine and Hygiene, 2015). It is estimated that 5-6% of the total L-lactate produced by *P. falciparum* is of the dextro-isomer form (Vander Jagt et al., 1990). As overall parasite derived L-lactate is thought to contribute little until high parasite biomass is reached, parasite (DL)-lactate is also unlikely to contribute to acidosis.

Treatment of lactic acidosis

Effective treatment of metabolic acidosis is achieved when the underlying cause of acidosis is resolved. L-lactate is rapidly metabolised, therefore, once the underlying cause of lactic acidosis is treated, acidosis is expected to resolve over time. In severe falciparum malaria, the major cause of hyperlactataemia is microvascular obstruction following sequestration

of mature stage *P. falciparum* parasites. Fluid resuscitation has limited effect on lactic acidosis and acid-base status (Hanson et al., 2013b). Optimal therapies for lactic acidosis in severe falciparum malaria should thus aim to provoke de-sequestration and prevent further cytoadhesion by *P. falciparum* infected red blood cells through antimalarial action on the early ring stages. Recently proposed low anticoagulant heparins, such as sevuparin, might be beneficial by inducing de-sequestration (Leitgeb et al., 2011, 2017).

Symptomatic treatment of lactic acidosis by intravenous sodium bicarbonate infusion is not generally recommended as it may lead to intracellular acidosis (WHO, 2014). The Surviving Sepsis Campaign issued a recommendation against the use of sodium bicarbonate for hypoperfusion-induced lactic acidosis in patients with sepsis and a pH$\geq$7.15 (Rhodes et al., 2017), and whether sodium bicarbonate infusion is effective in reducing mortality below the given threshold is unknown. Intravenous sodium dichloroacetate may improve L-lactate clearance by stimulating the pyruvate dehydrogenase complex (Agbenyega et al., 2003; Krishna et al., 1995, 1994a, 1996), but did not improve haemodynamics or mortality in a randomised controlled trial in 252 patients with lactic acidosis (Stacpoole et al., 1992). Similarly, hepatic prolyl hydroxylase 2 (PHD2) inhibition in the hypoxia inducible pathway might improve the clearance of L-lactate by stimulating hepatic extraction of L-lactate through the Cori cycle in mice (Suhara et al., 2015), but whether this has an effect on patient outcomes is unknown. To my knowledge, no previous studies have evaluated the effect of dialysis on acidosis in patients with severe falciparum malaria without acute kidney injury.

In conclusion, metabolic acidosis may be effectively treated by removing the underlying cause. In severe malaria, L-lactic acidosis is likely caused by microvascular obstruction

and therapies may therefore aim to prevent and reduce tissue sequestration. The causes of non-lactate acidosis in severe falciparum malaria remain unknown and require further study to identify new targets for adjunctive therapy.

1.8.2 Unmeasured acids

Evidence for an unidentified circulating acid load in patients with severe falciparum malaria comes from studies on the pathogenesis of acidosis in adults from South-East Asia and African children (Day et al., 2000b; Dondorp et al., 2004a; Herdman et al., 2015; Sasi et al., 2009). In a prospective study in adult patients with severe malaria admitted in an intensive care unit in Vietnam it was found that 198 of 296 (67%) patients were acidotic with a standard base deficit >3.3mol/L (Day et al., 2000b). Of these, 195 (99%) had a metabolic acidosis while only three had signs of a respiratory acidosis. The standard base deficit was 11.3 mmol/L (9.8 to 13.2) in patients who died and it was 4.41 mmol/L (3.92 to 5.02) in patients who survived (Day et al., 2000b). It was found that SBD was the strongest predictor of mortality with an odds ratio of 1.20 (1.12 to 1.29) per mmol/L increase ($p < 0.001$). The proportion of SBD explained by L-lactate and creatinine was only 63%, implying at least one third of the SBD was unaccounted for.

Further characterisation of metabolic acidosis using the Stewart approach in a separate follow-up study in 268 consecutive adults with severe falciparum malaria, also from Vietnam, showed a marked elevation in unidentified acids (Dondorp et al., 2004a). The strong ion gap was raised to 15.9 (1.1 to 30.7) mmol/L in patients who died compared to 9.7 (-0.7 to 20.1) in patients who survived. To determine predictors of death, a multivariate regression model was

built using SIG, L-lactate, creatinine, and haemodynamic shock. This model showed SIG was an independent predictor of death (OR 1.10 (1.02 to 1.19), p=0.016). A correlation was found between SIG and creatinine (rho = 0.37, p<0.001), however, in a subgroup of patients without renal failure the SIG on admission remained an independent predictor of mortality.

The determinants of acid-base disorders were characterised in Kenyan children with acute non-surgical conditions (Sasi et al., 2009). Using the Stewart approach, it was found that 51 out of 81 (63%) acidotic children with malaria had evidence of unidentified acids (SIG >5 mmol/L) (Sasi et al., 2009). Among these children, 46% had elevated plasma beta-hydroxybutyric acid ($\leq$ 4 mmol/L). Ketones were similarly elevated in control groups of children with lower respiratory tract infection, acute gastroenteritis and severe malnutrition. Ketones also correlated with renal function and predicted standard base deficit.

To establish the identity of acids in acidotic patients with severe falciparum malaria, recent studies have measured a selection of ketones and organic acids in the plasma (Herdman et al., 2015; Sriboonvorakul et al., 2013). Relevant ketones included alpha-hydroxybutyric acid and beta-hydroxybutyric acid, and although these were elevated in patients with severe malaria they only slightly contributed to acidosis in severe malaria. Interestingly, a phenylalanine/tyrosine metabolite, hydroxyphenyllactic acid (HPLA), was increased in severe malaria and independently predicted mortality from severe malaria (OR 3.5 (1.6 to 7.5), p<0.001) with a comparable predictive power as L-lactate. The source of HPLA was not clear, and it was postulated to derive from parasite or bacterial metabolism. Since HPLA was strongly associated with mortality, further characterisation of its metabolism might reveal a pathogenic pathway.

There have been concerns that specific metabolites related to the use of aspirin and paracetamol may cause toxicity. Salicylate poisoning has been described in children with severe malaria (English et al., 1996a). Incorrect home treatment with aspirin (salicylic acid) can cause toxicity with initially an activation of the respiratory centre resulting in hyperventilation and respiratory alkalosis, and subsequent dehydration, hypokalaemia, and metabolic acidosis. However, data from 475 children with an acute non-surgical condition in Kenya (including malaria), showed that 11% had quantifiable evidence of prior aspirin use but there was significant difference in the use of aspirin between patients with or without acidosis (Sasi et al., 2009). Possibly, acidosis could be related to paracetamol poisoning. In this situation pyroglutamic acid, also referred to as 5-oxoproline, is the responsible metabolite acidosis coming from the gamma-glutamyl cycle (Liss et al., 2013; Luyasu et al., 2014; Myall et al., 2011).

Studies in other disease-states have evaluated the contribution and identity of unidentified anions in critically ill patients (Gunnerson et al., 2010; Moviat et al., 2008a, 2013, 2008b), sepsis (Noritomi et al., 2009), acute kidney injury (Maciel et al., 2013; Moviat et al., 2012), diabetic ketoacidosis (Forni et al., 2005), and liver impairment (Funk et al., 2007; Kneidinger et al., 2013). The approaches have been to enzymatically quantify organic acids, for example from the tricarboxylic acid cycle (Forni et al., 2005). Alternatively, selected organic acids have been measured by mass spectrometry (Herdman et al., 2015; Moviat et al., 2013, 2008b). These studies were limited by their technical methods as they had to reduce their selection of organic acids to a small number of metabolites.

Metabolomics

Metabolomics refers to the study of metabolism by measuring changes in the composition of low-molecular weight molecules, called metabolites, in a certain tissue or biological fluid. For example, blood contains at least 3189 detected metabolites involved in amino acid, energy, or lipid metabolism, which together constitute the blood metabolome (Human Metabolome Database, HMDB V4.0, 2018). With new technological advances in mass spectrometry it has become feasible to quantitatively screen metabolites from a wide range of metabolic pathways in a single sample. Together with the improvement of analytical hardware and chemical methods there is a growth in the number of publicly available online reference databases for the study of metabolites and their pathways, such as the Human Metabolome Database (HMDB) (curated by the University of Alberta, Canada), METLIN (Scripps Institute, California, USA), KEGG (University of Kyoto, Japan), PubChem (NCBI, USA) and others. With regard to bioinformatics, new software has been developed for the analysis of raw data from mass spectrometry experiments, including software for the optimisation of mass spectrometry peak picking, and statistical tools for robust regression and machine learning, making metabolomics a rapidly evolving and promising research area.

A broad-spectrum analysis of organic acids in plasma of patients with severe falciparum malaria has not been done before. Prior metabolomic analyses in malaria have been performed in *P. falciparum* cultures (Olszewski et al., 2009; Park et al., 2015; Sana et al., 2013; Srivastava et al., 2015), and in *P. berghei* ANKA infected mice (Basant et al., 2010; Ghosh et al., 2012, 2013; Li et al., 2008; Sengupta et al., 2013; Tritten et al., 2013). One recent study investigated the metabolomic perturbations in a controlled human malaria infection with *Plasmodium*

vivax (Sengupta et al., 2015). So far, no metabolomics studies have been performed in controlled human malaria infections. Two metabolomic analysis have been done in patients with non-severe malaria (Abdelrazig et al., 2017; Lakshmanan et al., 2012), but these have generally included few patients (n<25). No known previous metabolomic analyses have been done in severe falciparum malaria, in particular there has been no previous metabolomic analysis of acidosis in malaria.

The study of organic acids and primary and secondary amino acids by mass spectrometry based metabolomics in patients with severe falciparum malaria may reveal patterns of metabolic dysregulation related to acidosis that would not be possible to determine using classical biochemical techniques. The application of a metabolomics approach to study the composition of the plasma acid metabolome may be helpful in establishing the identify of unknown acids.

Table 1.4 **Previously published metabolomic analyses in malaria.**

Untargeted metabolomic analyses in malaria				
Author (Year)	**Organism**	**Sample**	**Methods**	**Main findings**
Olszewski et al. (2009)	*Pf*3D7	pRBC media	LCMS	Depletion of L-arginine from culture media related to parasite arginases
Sana et al. (2013)	*Pf*3D7	pRBC	LCMS	Intense glycolysis and signs of a branched TCA metabolism
Park et al. (2015)	*Pf*3D7	pRBC media	LCMS	Several previously unknown biomarkers of infection
Li et al. (2008)	*P. berghei* ANKA	Plasma, urine	^{1}H−NMR	Elevated pipecolic acid related to a disturbance of the gut microbiota.
Basant et al. (2010)	*P. berghei* ANKA	Serum, urine, brain	^{1}H−NMR	Simultaneous increases in L-lactate, alanine, lysine, kynurenic acid, quinolinic acid.
Ghosh et al. (2012)	*P. berghei* ANKA	Serum, brain liver	^{1}H−NMR	CM mice have a differential metabolic profile from non-CM mice characterized by perturbations the ammonia detoxification pathways.
Ghosh et al. (2013)	*P. berghei* ANKA	Kidney, spleen	^{1}H−NMR	In CM mice the kidney and spleen glutamine levels were decreased.
Tritten et al. (2013)	*P. berghei* ANKA	Urine	^{1}H−NMR	Elevated urinary pipecolic acid and other previously unknown parasite related metabolites.
Lakshmanan et al. (2012)	Humans, *Pf*	Plasma	LCMS	Identification of the alpha linolenic acid pathway in *P. falciparum*
Abdelrazig et al. (2017)	Human, *Pf*	Urine	LCMS	Elevated urinary pipecolic acid, taurine,1,3-diacetylpropane, N-acetyl- spermidine, N-acetylputrescine.

Pf3D7 = Plasmodium falciparum 3D7 strain. pRBC = parasitised red blood cell. LCMS = liquid-chromatography mass-spectrometry. ^{1}H−NMR = proton-nuclear magnetic resonance spectroscopy. CM = cerebral malaria.

1.8.3 Summary of conclusions

Severe falciparum malaria is associated with unacceptably high case fatality rates between 10-30%. The pathogenesis of severe malaria is related to a rapidly expanding *P. falciparum* biomass inside the bloodstream where circulating parasites sequester in the microcirculation causing widespread microvascular obstruction and organ damage. Major clinical complications that follow from sequestration include acidosis, coma, acute kidney injury, severe anaemia, and concomitant sepsis. Of these complications, acidosis is the strongest predictor of death. However, the pathogenesis of acidosis is incompletely understood. To investigate the pathogenesis of acidosis a good outcome parameter is the standard base deficit corrected for plasma albumin and phosphate. The standard base deficit is elevated in severe falciparum principally due to L-lactate. Hyperlactataemia coincides with acidosis and arises from anaerobically produced L-lactate from host-tissues. However, besides L-lactate other still unidentified metabolic acids contribute to acidosis. The unmeasured acid load in severe malaria is independently prognostic of outcome. Classical biochemical methods have so far not been able to determine the constituents of this unmeasured acid load.

The aim was to determine the identity and source of previously unmeasured acids elevated in patients with acidosis and severe falciparum malaria. In the following Chapters, I will describe the methods (Chapter 2) and results from metabolomic studies of plasma from acidotic patients with severe malaria from Bangladesh (Chapter 3), and of culture medium from *Pf*3D7 infected red cell cultures (Chapter 4). Follow-on studies were conducted to further characterise the source of biochemical derangements observed in patients with severe malaria (Chapters 5 and 6).

Chapter 2

Methods

2.1 Clinical studies

2.1.1 Ethics

The observational study protocol and its associated documents were submitted and approved by the Chittagong Medical College Ethics Committee, Bangladesh (Reference: CMC/PG/2015/26), and the Oxford Tropical Research Ethics Committee (OxTREC), in the United Kingdom (Reference: 08-15). The trial was registered at *ClinicalTrials.gov* (Trial number: NCT02451904).

2.1.2 Study site

The observational studies on severe falciparum malaria were executed at a study site in Bangladesh where the regional care for patients with severe malaria was centralised. Chit-

tagong Medical College Hospital is a large government hospital and referral centre for malaria in South-East Bangladesh. Here, I planned, coordinated, and carried out the clinical studies described in this thesis, and provided patient care to all included cases. The hospital is located in a low-transmission coastal region with seasonal malaria, with peaks in malaria transmission during the wet-season between June and August (Maude et al., 2012). Approximately 30 km east of the port-city of Chittagong are the Chittagong Hill Tracts where there are high-transmission malaria hotspots. Further land-inwards, approximately 75 km east of Chittagong, is the Myanmar border, Figure 2.1.

Chittagong Medical College Hospital is a large government hospital with approximately 1300 beds, and it is the only public hospital of this size in a city of approximately five million people. There are two other public hospitals but they have a limited bed capacity (<200 beds). CMCH charges a patient fee of around 30 Bangladeshi Thaka (equivalent to 0.35 USD) for hospital admission, which is more affordable than the surrounding private clinics, and therefore the hospital patient population is skewed towards the poorer segment of the population. At the time of the study, the hospital had three internal medicine wards with 66 beds each, which were partitioned between a male (33 beds) and a female side (33 beds). On an average weekday, a single ward could be occupied by as many as 100-120 patients. Oxygen was available on all of the study wards and haemodialysis was available in a separate renal unit operating eight haemofiltration machines. The hospital has an intensive care unit with 12 beds of which seven were equipped with mechanical ventilators at the time of the study.

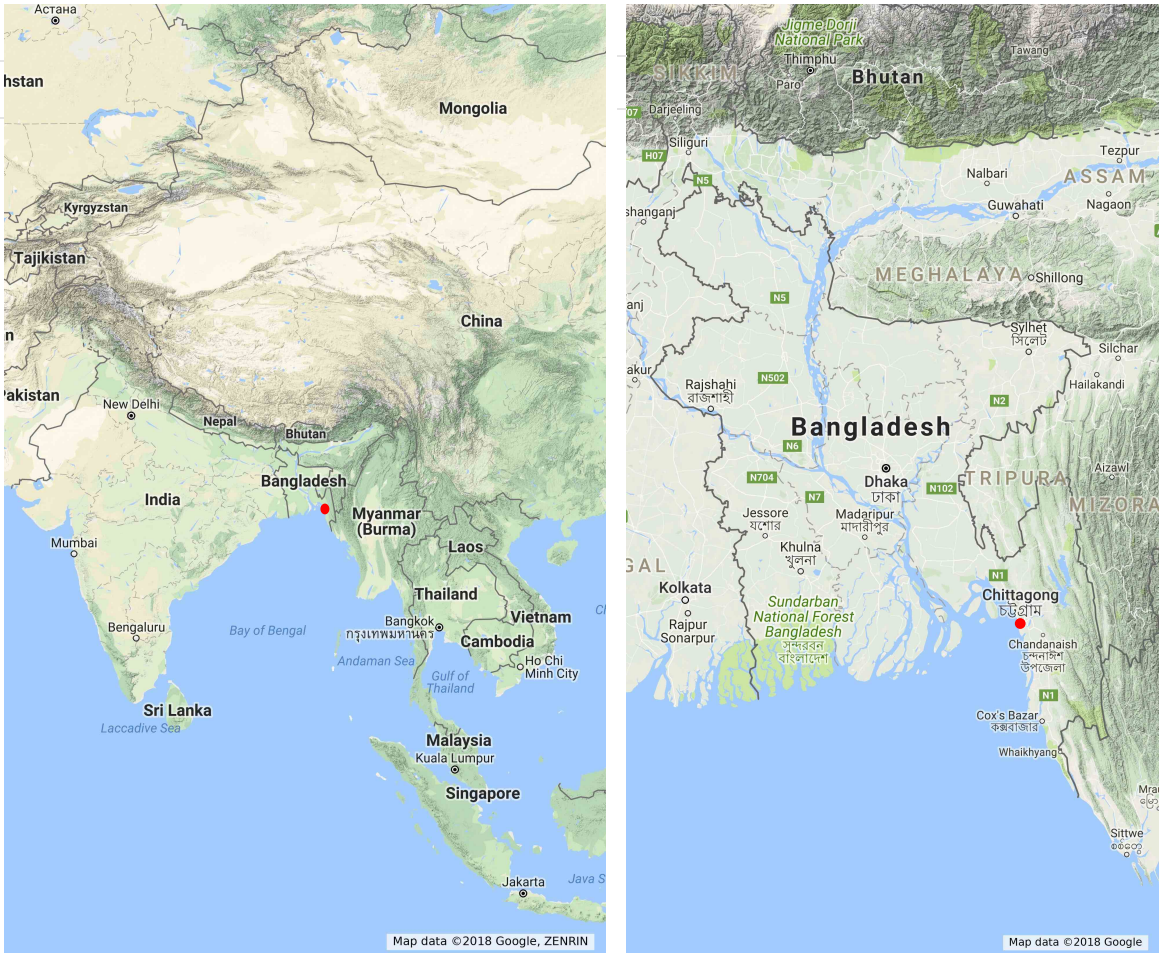


Fig. 2.1 **Location of the study site: Chittagong Medical College Hospital, Chittagong, Bangladesh.** (Map data © 2018 Google).

2.1.3 Design

A prospective observational cohort of patients with severe falciparum malaria, uncomplicated falciparum malaria, SIRS/sepsis, and healthy controls in Chittagong Medical College Hospital (CMCH), Chittagong, Bangladesh.

2.1.4 Participants

Eligibility criteria

We consecutively enrolled participants into four study groups: severe falciparum malaria, uncomplicated falciparum malaria, SIRS/sepsis, or healthy controls.

Severe Malaria

Male or female adults (>12 years) were classified as having severe malaria using modified WHO criteria for severe malaria (World Health Organization, 2015), as described previously (Hien et al., 1996). Severe malaria was defined as any *P. falciparum* parasitaemia in adults, confirmed by asexual blood stages on a peripheral thick or thin blood smear (including mixed infections with non-falciparum parasites), in combination with one or more of the following criteria for severe malaria:

1. Glasgow Coma Scale <11 out 15;
2. Haematocrit <20 % with a peripheral parasite count >100,000 per µL;
3. Jaundice with bilirubin >2.5 mg/dl with parasite count >100,000 per µL;
4. Serum creatinine >265 µmol/L (>3 mg/dL);

5. Hypoglycaemia with venous glucose <40 mg/dL;
6. Systolic blood pressure < 80 mm Hg with cool extremities;
7. Peripheral asexual stage parasitaemia >10 %;
8. Peripheral venous L-lactate > 4 mmol/L;
9. Peripheral venous HCO_3^- <15 mmol/L;
10. Respiratory insufficiency.

Patients were excluded if they received treatment for >24 hours with any antimalarial.

Uncomplicated Malaria

Male or female adults (>12 years) were classified as having uncomplicated malaria if they had *P. falciparum* parasitaemia, confirmed by asexual forms on a peripheral thick or thin blood smear (including mixed infections with non-falciparum parasites), in the absence of any of the complications of severe malaria. Patients were excluded if they received treatment for >24 hours with an antimalarial.

SIRS / Sepsis

Sepsis was defined using criteria for systemic inflammatory reaction syndrome (SIRS) in combination with a suspected focus of infection, as described previously (Dünser et al., 2012). We applied the SIRS criteria as these provided practical disease definitions in resource-constrained study settings where arterial blood gas analysis may not be available for each patient. Male or female adults (>12 years) were classified as having sepsis if they had suspected bacterial infection with any two or more of the following SIRS criteria:

1. Heart rate >90 beats per minute;
2. Respiratory rate >20 breaths per minute OR a $PaCO_2$ <32 mm Hg OR the use of mechanical ventilation for an acute respiratory process;
3. Tympanic temperature >38 °C OR <36 °C;
4. White blood cell count of >12 x 10^9/L OR <4 x 10^9 /L OR a differential count showing 10% immature neutrophils.

Malaria had to be excluded based peripheral microscopy for malaria parasites of thick and thin blood smear and a negative rapid diagnostic test for *Pf*HRP2 (Paracheck-Pf, Orchid, India). Bacterial cultures were not routinely available at the study site because quality controlled blood culture facilities were unavailable at the study site. Therefore, a positive blood culture was not a requirement for the current sepsis classification. Patients were excluded if they had a known malignancy or a known pre-existing chronic lung disease.

Healthy controls

Healthy controls were recruited inside the study hospital and usually included family members of hospitalised individuals who were not enrolled in the study. Healthy controls were excluded if they had any chronic health conditions, if they were taking medication, pregnant, or if they had abnormal vital signs (including hypertension). All healthy controls were tested negative for malaria based on peripheral microscopy for malaria parasites of thick and thin blood smear and a negative RDT (Paracheck-Pf, Orchid, India). We offered a small fee to reimburse for their loss of time.

2.1.5 Clinical procedures

Screening

We screened consecutive patients admitted to the department of internal medicine who were suspected clinical cases of malaria. Active screening for malaria was done by daily visits to the wards, passive screening took place by receiving requests from the attending ward physicians to test patients for malaria. As part of standard of care, a screening blood sample was taken from the antecubital fossa and collected in a 1mL Ethylenediaminetetraacetic acid (EDTA) blood tube. Venous blood samples were immediately tested for malaria using by light microscopy of a thin and thick blood smear and an RDT for malaria.

Thick and thin Giemsa-stained blood films were prepared on the same glass slide by a trained laboratory technician. Patient identifiers were written on the side of the slide and slides were cleaned using an alcohol pad and dried by air before two or three drops of blood were added for thick smear and a single drop of blood for a thin smear. Following, slides were placed on a flat surface to dry, and once they were dry the slides were immersed in methanol. Slides were stained with Giemsa for 15-20 minutes, after which they were dried and read under the microscope. Parasite counts were calculated per µL of blood as follows:

$$\text{Parasitaemia per µL (/1000 RBC)} = \text{parasite count} \cdot \text{Hct} \cdot 125.6 \tag{2.1}$$

$$\text{Parasitaemia per µL (/200 WBC)} = \text{parasite count} \cdot 40 \tag{2.2}$$

$$\text{Parasitaemia per µL (/500 WBC)} = \text{parasite count} \cdot 16 \tag{2.3}$$

Rapid diagnostic tests were based on the detection of *Pf* HRP2 in whole blood (Paracheck-Pf, Orchid, India), which was carried out according to the manufacturers' instructions. Patient identifiers were written on the side of the RDT. The RDTs were loaded with EDTA whole blood using a micropipette, diluted using the suppliers' buffer, and read after 20 minutes.

If an RDT and thick or thin blood smear were positive for malaria, patients were assessed using the criteria for severe and uncomplicated malaria. All blood smears were checked by an experienced microscopist from the Mahidol-Oxford Tropical Medicine Research Unit (MORU). If all malaria tests were negative, patients were assessed for eligibility in the SIRS/sepsis study group.

Informed consent and the Declaration of Helsinki

Participants who met the inclusion criteria and did not meet any of the exclusion criteria were informed about the study and were asked to read the patient information sheet (PIS) and informed consent form (ICF). The PIS/ICF were translated into Bengali and back-translated in English to verify their content. They included information about the nature of the study and the implications of the protocol, and the potential benefits and risks for the patients to take part in the study. There was a clear statement that participation is voluntary. Patients or their relatives were free to withdraw from the study at any time without giving any particular reason and without any consequences for future care. If patients or their relatives could not read or write, one of the Bengali speaking research assistants would read out the PIS/ICF and explain any of the details where necessary. Patients or their relatives were provided time to discuss the study with the investigator and amongst themselves. Patients were asked to

provide written informed consent (or a thumbprint if illiterate) or if they were unable to give consent themselves, an attending first degree family member was asked to provide written informed consent. A copy of the consent form was given to the participants.

All members of the study team who were involved with patient care or laboratory work had completed a recent course on Good Clinical Practice from the Mahidol-Oxford Tropical Medicine Research Unit / University of Oxford. I ensured that the study was carried out according to the ICH Guidelines for Good Clinical Practice and in compliance with the Declaration of Helsinki.

Clinical procedures

Initial clinical assessments aimed to determine if patients were haemodynamically stable and to ensure they had received antimalarial treatment. Vital signs were collected on enrolment and any life-threatening conditions were treated without delay by the study team together with the attending ward physicians. All patients underwent a full physical examination and neurological assessment. A clinical history was taken at baseline by one of the Bengali speaking study physicians, and included a premedical history, travel history and treatment history. All collected data were systematically recorded on a paper case record form (CRF).

A new venous cannula (18G or 20G) was inserted in the forearm, which was exclusively used for repeated venous blood collection. In patients with severe malaria, an additional venous cannula was inserted in the other forearm for the administration of injectable artesunate and antibiotics.

Follow-up procedures included six-hourly visits for the monitoring of vital signs and parasitaemia until fever, coma, and parasite clearance were achieved. Serial blood sample collection was done more extensively 24 hourly, up to (and including) 72 hours. Patients were followed until discharge or in-hospital death.

Antimalarial treatment

All patients with malaria were treated according to WHO guidelines (World Health Organization, 2015). Patients with severe malaria received parenteral artesunate dosed as outlined in Table 2.1. At least three doses of intravenous artesunate were administered before a patient was switched to oral medication, which included a full course of artemether/lumefantrine for a duration of three days taken together with food or drinks rich in fat (White et al., 1999).

Table 2.1 **Antimalarial treatment regimens for patients included in observational studies in Chittagong, Bangladesh.**

Intravenous antimalarial treatment	
Formulation	**Manufacturer**
Artesunate 60 mg powder for injection	Guilin Pharmaceutical Co., Ltd. (China)
Dosing Category	**Dose**
Children <20 kg:	3 mg/kg body weight per dose
Children and adults >20 kg:	2.4 mg/kg body weight per dose
Regimen	
Day 1 Dose 1:	On admission (0 hr)
Day 1 Dose 2:	12 hr later
Day 2 Dose 3:	24 hr after first dose
Continued daily until taking orally	
Oral antimalarial treatment	
Formulation	**Manufacturer**
Artemether 20 mg / lumefantrine 120 mg	Guilin Pharmaceutical Co., Ltd. (China)
Dosing Category	**Dose**
5 to <15 kg:	1 Tablet
15 to <25 kg:	2 Tablets
25 to <35 kg:	3 Tablets
35 kg and above:	4 Tablets
Regimen	
Day 1 Dose 1:	On admission or switch to oral (0 hr)
Day 1 Dose 2:	8 hr after first dose
Day 2 Dose 1:	Morning
Day 2 Dose 2:	Evening
Day 3 Dose 1:	Morning
Day 3 Dose 2:	Evening

Adapted from WHO (2014) and from Severe Malaria Observatory (2018).

Sample collection

Serial samples for haematology, biochemistry and metabolomics were collected after discarding of the first mL of blood from a saline-locked venous cannula. All blood samples were slowly mixed inside the collection tubes and immediately processed in the laboratory.

Whole blood was collected in EDTA collection tubes for haematology from an external quality controlled laboratory at the study site (Chevron, Chittagong, Bangladesh).

Venous and arterial blood gas samples were collected in blood gas syringes coated with lithium-heparin (Terumo, Tokyo, Japan). Samples were gently mixed for 20 seconds and immediately analysed (<15 minutes following blood collection) using $CG4^+$ and $Chem8^+$ cartridges on an iStat blood gas analyser (Abbott, Chicago, Illinois). We recorded pH, bicarbonate, sodium, potassium, chloride, and ionised calcium from the same blood sample.

Prior to each of the study seasons, the iStat machines received servicing and software updates from the company. The iStat machines were re-calibrated and their accuracy was confirmed using a three-point concentration scale (i-STAT TriControls Level 1, 2 and 3, Abbott, Chicago, Illinois). All newly acquired iStat cartridges were validated per batch prior to their use in the field. iStat cartridges were transported and stored between 2-8 °C, and they were left to adjust to room temperature at least 5 minutes before clinical measurements. All iStat measurements were performed in an air-conditioned room at room temperature. At the study site, the iStat machines underwent weekly quality control using an iStat Electronic Simulator (Abbott, Chicago, Illinois).

Serum samples were collected for biochemistry, albumin, phosphate, and magnesium, analysed at clinical laboratory of the Hospital for Tropical Diseases in Bangkok, Thailand.

Plasma samples were collected in lithium heparin collection tubes and immediately processed by separating plasma through centrifugation of the blood tubes for 5 minutes at 1500 rpm and 4 °C. Plasma was immediately aliquoted in cryovials (Corning, MA, USA) and flash-frozen in liquid nitrogen. Multiple heparinised plasma aliquots were stored for metabolomics, amino acid quantification, and quantification of soluble markers of gut integrity.

Stool samples for 16S rRNA microbiota analysis were initially collected in stool containers. An amendment was made in May 2016 to allow for stool sample collection using a rectal swab (FLOQ swabs, COPAN, USA).

Estimation of unmeasured acids

To estimate the concentration of unmeasured circulating acids, we calculated the standard base deficit, the anion gap and the strong ion gap as described previously (Story et al., 2002).

Standard base deficit (SBDc) was calculated using the modified Van Slyke equation (Siggaard-Andersen, 2005) and corrected for differences in plasma albumin and phosphate using correction factors developed by Wooten (Wooten, 2003). Here, albumin is expressed in g/dL and phosphate in mg/dL.

$$\text{SBDc}=([HCO_3^-] - 24.4) + (8.3 \cdot [\text{Albumin}] \cdot 0.15 + 0.29 \cdot [\text{Phosphate}] \cdot 0.32) \cdot (\text{pH} - 7.4) \tag{2.4}$$

The anion gap (AGc) was estimated and corrected for the negative charge of albumin (2.8 mEq/L per g/dL at a pH of 7.4) and phosphate (0.59 mEq/L per mg/dL at a pH of 7.4). Here, albumin is expressed in g/dL and phosphate in mg/dL.

$$AGc=([Na^+] + [K^+]) - ([Cl-] + [HCO_3-]) - (2 \cdot [Albumin] + 0.5 \cdot [Phosphate] \quad (2.5)$$

For calculation of the strong ion gap we used Stewart's approach (Stewart, 1983) modified by Figge (Figge et al., 1992) and determined the apparent strong ion difference (SIDa) and the effective strong ion difference (SIDe), as follows:

$$SIDa=([Na^+] + [K^+] + [Mg^{2+}] + [Ca^{2+}]) - ([Cl-] + [L-lactate]) \quad (2.6)$$

$$SIDe=(2.46 \cdot 10^{-8} \cdot PCO_2/_{10}{}^{-pH}) + ([Albumin] \cdot (0.123 \cdot pH–0.631)) + ([PO_4^-] \cdot (0.309 \cdot pH–0.469))) \quad (2.7)$$

Finally, the strong ion gap (SIG) was calculated by subtracting the apparent and effective strong ion differences:

$$SIG=SIDa - SIDe \quad (2.8)$$

Sample storage and transport

At the study site, all plasma and stool samples were stored in liquid-nitrogen (LN) with regular monitoring and replacement of the LN levels throughout the study season. Serum samples for regular biochemistry were stored at -20 °C.

Samples were shipped on dry ice to the Mahidol-Oxford Tropical Medicine Research Unit in Bangkok by the commercial company World Courier. Packing and transportation was carried out following the IATA infectious substances and diagnostic specimens shipping guidelines and samples were shipped in four tranches classified as biological substances, Division 6.2, Category B (United Nation committee of Experts/Technical instruction).

Once samples arrived at the Mahidol-Oxford Tropical Medicine Research Unit in Bangkok they were verified on dry ice and stored until analysis at -80 °C.

Source data

All the source data and case record forms were scanned and sent back to the Clinical Trials Support Group at the Mahidol-Oxford Tropical Medicine Research Unit in Bangkok. Data were entered into a MACRO database (MACRO Electronic Data Capture, Elsevier, the Netherlands) and underwent extensive cleaning through range checks and logical cross-checks. Final clinical datasets were verified for inconsistencies in Stata software (V12, StatCorp, Texas, USA).

2.2 Laboratory investigations

2.2.1 Metabolomics

Metabolomics provides a novel approach to study a broad range of metabolites in the same sample. However, metabolomic data analysis is currently in a relatively early stage with regard to method development, when compared to for example genetics. Prior methods have

been described for metabolite extraction, data acquisition, peak extraction and normalisation, as well as quality control measures, Supplementary Figure 1. However, there is a paucity of existing approaches available that combines these techniques for the complete analysis of metabolomics data, which necessitated for me the need to design a customised bioinformatics pipeline. The result of the current analytical approach can be summarised as follows, and is described in a flow chart in Supplementary Figure 1:

1. Metabolite extraction (Wet lab)
2. Data acquisition (Analytical chemistry)
3. Metabolomic data analysis (Dry lab)
    (a) Metabolic peak extraction
    (b) Data normalisation
    (c) Quality control
    (d) Metabolite identification
    (e) Feature selection
4. Statistical analysis (Dry lab)

Each stage in this workflow has been previously published and validated elsewhere. To establish this analytical workflow, I have spent considerable time developing a customised bioinformatics pipeline from the ground up to perform data normalisation, quality control, feature identification and feature selection. In the following Sections, I describe in more

detail the methods that were applied and data optimisation steps that were considered. I will start by describing the metabolite extraction techniques used for patient plasma and *Pf*3D7 cultures. After this, I describe the mass spectrometry analysis. Finally, I describe the metabolomic data analysis, including peak extraction, normalisation, quality control and identification.

As mentioned before in Section 1.8.2 (Metabolomics), few previous studies have reported metabolomic analyses of samples collected from patients with uncomplicated malaria, and no prior metabolomic studies have been conducted among patients with severe malaria. Because there were no prior metabolomic data analysis workflows available, specific to malaria, it was in some cases necessary to optimise the parameters of existing methods in order to adjust them to our specific needs. In specific, although the dynamic range of UHPLC-Orbitrap-MS is high – allowing quantification from nanomolar to millimolar concentrations – optimisation was required to ascertain that metabolite peaks would fall on the linear portions of the predicted curves. To ascertain that metabolites did not fall below the limit of detection, we performed pilot studies using multiple dilutions of pooled QC samples (representing the full spectrum of metabolites in the experiment) to determine optimal reconstitution ratios. The final reconstitution ratios are stated below in Section 2.2.1.

Metabolite extraction from patient plasma (Chapter 3)

Plasma samples were extracted for metabolomics analysis at the Department of Pharmacology of the Mahidol-Oxford Tropical Medicine Research Unit in Bangkok. I planned and carried out the extraction of metabolites using two extraction methods and designed a quality control

procedure for post-hoc signal correction using integrated QC samples. Before the extraction was carried out, all samples were randomised and blinded. A total of 381 samples (including serial samples from patients with severe malaria) were extracted in duplicate using two different extraction methods: 1) solid phase extraction (SPE) and 2) protein precipitation using methanol (MeOH).

Solid phase extraction

Solid phase extraction was performed as described previously (Sriboonvorakul et al., 2013). In summary, an initial plasma volume of 100 µL was transferred onto a 96-well plate on ice and mixed with ammonium acetate buffer (pH 8). Using a multi-stepper autopipette all samples were spiked with an internal standard solution of stable isotopes. We mixed the 96-well plates for 10 minutes at 600 rpm using a MixMate system (ThermoFisher, Waltham, Massachusetts). Following, the samples underwent solid phase extraction through an Isolute PE-AX 96-well cartridge (Biotage Uppsala, Sweden) according to the manufacturer's instructions. Extracted metabolites were collected on a fresh 96-well plate and evaporated to dryness with nitrogen using TurboVap (Biotage Uppsala, Sweden) for 1 hour at 37 °C before they were sealed for dry storage at -80 °C.

Methanol extraction

A second aliquot of the same plasma samples was extracted using protein precipitation with methanol (Roberts et al., 2012). An initial plasma volume of 100 µL was transferred to Eppendorf tubes on ice and 900 µL of LCMS grade methanol was added (FLUKE) (Sigma, St

Louis, Missouri) using a multi-stepper pipette (Roberts et al., 2012). Samples were vortexed individually for 30 seconds at full speed. Following, samples were centrifuged for 15 minutes at 15.800 rpm and 4 °C. An aliquot of 100 µL from the upper organic layer was transferred onto a 96-well plate and evaporated to dryness using a TurboVap for 3 to 4 hours at 37 °C before they were sealed for dry storage at -80 °C.

SPE metabolite reconstitution

All sealed plates containing the pellets of extracted plasma metabolites were shipped by World Courier to the Huck Institute of Life Sciences, PA, USA (Manuel Llinás laboratory). There, I reconstituted the metabolites in preparation for their analysis by ultra-high-performance liquid-chromatography Orbitrap mass-spectrometry (UHPLC-Orbitrap-MS). The SPE extracted samples were reconstituted by adding HPLC-grade water (Chromasolv) (Sigma, St Louis, Missouri) in a 1:1.6 ratio. The optimal reconstitution ratio was determined beforehand by assessing the linearity of the MS signal of multiple dilutions and monitoring the peaks of L-lactate (m/z 89.0244, M-H), we optimised for a dilution within the limits of detection. Following reconstitution in water, the SPE samples were mixed on the 96-well plates for 10 minutes at 600 rpm and room temperature, and centrifuged for 15 minutes at 3200 rpm and 4 °C. Samples were transferred to glass snap-cap low volume MS microvials (ThermoFisher, Waltham, Massachusetts) and were kept for 1-3 days at -80 °C before they were analysed. During LCMS, the samples were kept at 4 °C in the sample loading deck.

MeOH metabolite reconstitution

Protein precipitated samples were reconstituted by adding HPLC-grade water (Chromasolv) but in a 1:1.4 ratio. The 96-well plates were mixed for 10 minutes at 600 rpm and room temperature and reconstituted extracts were transferred into Eppendorf tubes for a final round of centrifugation for 15 minutes at 15.000 rpm and 4 °C. Supernatant was transferred to glass snap-cap low volume MS microvials (ThermoFisher, Waltham, Massachusetts) and these were also kept for 1-3 days at -80° before they were analysed.

Quality Control

Prior to the extraction process, I created a pool of all patient samples by adding 10 µL of each sample in a single vial. The QC pool was then extracted along with the individual plasma samples. The advantage of this QC pool was that it represented a mixture of all metabolites from all patients which allowed for QC-based signal-correction as described previously (Dunn et al., 2011). QC samples were regularly interspersed in the final sample analysis sequence in a 1:10 ratio, Figure 2.2. All samples including QC and water blanks underwent high-resolution mass spectrometry as outlined below in Section 2.2.1. The full mass spectrometry experiments for the analysis of all plasma samples took a total of 9 consecutive days. QC samples allowed for the inspection of retention time shifts and changes in mass accuracy throughout the continuous run-time of the experiment.

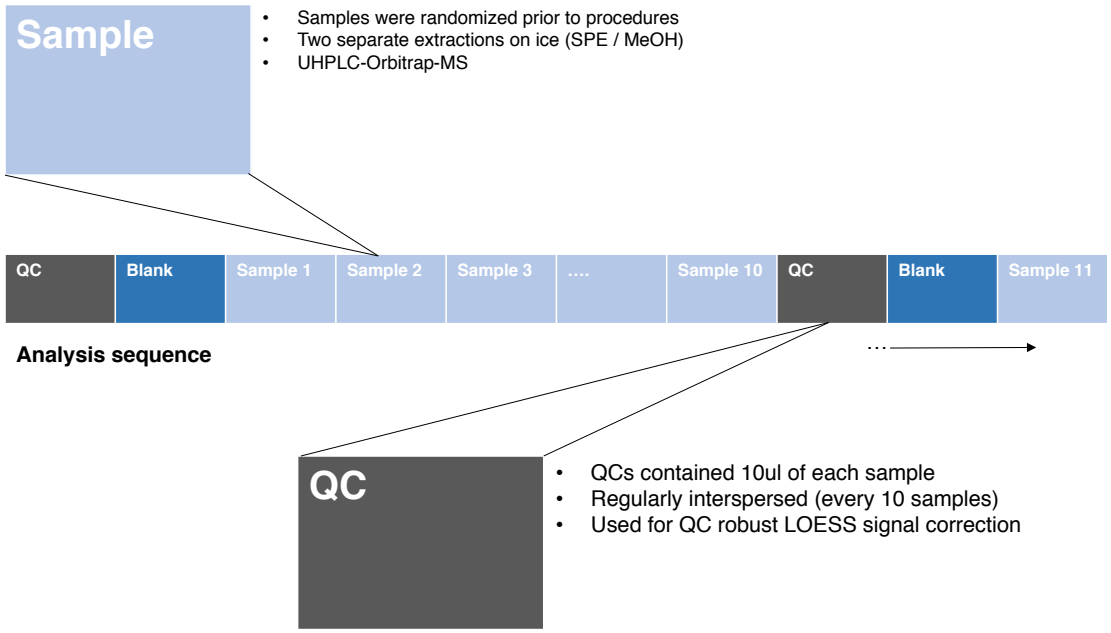


Fig. 2.2 **UHPLC-Orbitrap-MS analysis sequence of patient plasma.**

Metabolite extraction from *Pf* 3D7 culture media (Chapter 4)

In addition to a metabolomic analysis of patient plasma, a study was conducted on the temporal fluctuations of metabolites in the culture media of *P. falciparum* infected red blood cells. The following section describes the sample extraction techniques for these culture studies. All *Plasmodium falciparum* assays were done at the laboratory of Manuel Llinás at the Huck Institute of Life Sciences, Pennsylvania, USA, where I planned and carried out the experiments.

P. falciparum cultures

P. falciparum (3D7 strain; MR4, cat. no. MRA-102) was cultured and maintained using standard methods in a mycoplasma-free hood (Trager and Jensen, 1976). The *P. falciparum*

clone 3D7 was selected because it is a common parasite clone that grows efficiently in vitro of which the entire genonomic sequence has been characterised (PlasmoDB), as well as the transcriptome of the intraerythrocytic developmental cycle (Llinás et al., 2006).

Culture flasks were kept, with the lid half-closed, in a humidified incubator at 37 °C and 5% CO_2 and maintained in RPMI-1640 culture medium (Thermo Fisher Scientific) containing 15 mmol/L HEPES (Sigma-Aldrich), 2.5 g/L Albumax (Thermo Fisher Scientific), 50 mg/L gentamycin (Sigma-Aldrich), 2 mg/mL sodium-bicarbonate (Sigma Aldrich) and 10 mg/L hypoxanthine (Sigma-Aldrich). Cultures were confirmed Mycoplasma-free by PCR using an IntronBio (eMyco) Mycoplasma detection kit (Boca Scientific, No. 25235).

A *P. falciparum* culture was expanded (10% parasitaemia) and synchronised twice using 5% sorbitol (Sigma-Aldrich) over two subsequent developmental cycles. The highly synchronised *P. falciparum* culture (in-sync between 4 to 6 hours) was split into three flasks and diluted to 3%, 2%, and 1% parasitaemia all kept at 2% haematocrit. Alongside of the parasitised red cell cultures, an uninfected red blood cell control culture was prepared at 2% haematocrit with blood from the same donor. All flasks were given fresh pre-warmed media (37 °C) 1 hour before the assays started and were not refreshed thereafter.

The culture media were assayed at multiple time points during the intraerythrocytic developmental cycle. Sampling time points corresponded to the parasite stage: small ring stage (6-12 hours post invasion (hpi)), large ring stage (18-24 hpi), trophozoite stage (24-30 hpi), schizont stage (20-36 hpi and 42-48 hpi), re-invasion (48-52 hpi). Parasite viability was assessed through parasite counts during the intra-erythrocytic life-cycle and after re-invasion. Spent media were sampled in duplicate at each time point (technical duplicates, n=2),

parasitaemia was assessed by microscopy and cell counts were done using a haemocytometer. The metabolomics studies were carried out in duplicate.

Sample preparation for *Pf*3D7 media metabolite profiling

Metabolite extractions were performed on 100 µL of the spent media by immediately adding 900 µL pre-chilled methanol at (4 °C). The methanol extraction solution was spiked with 5 µM of internal standard $^{13}C_4$ - $^{15}N_1$ -aspartate to enable post-hoc corrections for variations in sample handling. Precipitated samples were vortexed individually for 30 seconds at full speed and then resuspended. Following, the samples were centrifuged for 15 minutes at 15.000 rpm and 4 °C. A total of 50 µL was transferred to an Eppendorf tube and was evaporated to dryness using nitrogen.

In a cold room (4 °C), the metabolite pellets were re-suspended by adding 67 µL of HPLC-grade water (Chromasolv, Fisher-Scientific) (33% signal intensification) and vortexing the individual samples for 30 seconds at full speed, followed by centrifugation for 15 minutes at 15.000 rpm and 4 °C. A final 50 µL of the upper layer was transferred into glass snap-cap low volume MS microvials (ThermoFisher, Waltham, Massachusetts).

Quality control

For this experiment, fresh RPMI 1640 media (the same as used for the cultures) was used to prepare quality control samples that were regularly interspersed along with water blanks between the media samples. We chose not to use a pooled sample but rather to use fresh

RMPI 1640 media which was considered a stable QC and reflected many of the standard constituents that could be found in all media.

UHPLC-Orbitrap-MS

Metabolites extracted from patient samples and from *Pf* 3D7 culture media were analysed on the same analytical platform and using the same LC-MS parameters. We performed ultra-high-performance liquid-chromatography mass-spectrometry using a Thermo Exactive Plus Orbitrap™ (ThermoFisher, Waltham, Massachusetts). Reversed-phase liquid-chromatography was done using a new pre-primed Synergi™ Hydro-RP C18 column (100 x 2 mm, particle size 2.5 µm, No. 00D-4387-B0, Phenomenex, Torrance, California). The column was fitted with a pre-column filter (Restek, Bellefonte, Pennsylvania). The UHPLC-parameters have been described previously (Lu et al., 2010) and are summarised in Table 2.2.

Each experiment was done in negative ion mode with electrospray ionisation and was preceded by calibration prior to data acquisition using Pierce Negative ESI calibration solution (ThermoFisher, Waltham, Massachussets). The MS parameters were adapted from previous studies (Lu et al., 2010). The scan range covered between an *m/z* 70-1000 with a resolution of 140,000 at *m/z* 200, Table 2.2.

Table 2.2 **Instrument settings used for UHPLC-Orbitrap-MS at the Huck Institute of Life Sciences, Pennsylvania, USA.**

Instrument Settings (Metabolomic analysis)	
UHPLC	
Column:	Synergi™ Hydro-RP C18 (Phenomenex)
Mobile Phase:	A: 97:3 water/methanol + 15 mM acetic acid
	+ 100 mM tributylamine (ion pairing agent)
	B: Methanol
LC Method:	0.0 min 0% B
	2.5 min 0% B
	5.0 min 20% B
	7.5 min 20% B
	13.0 min 55% B
	15.5 min 95% B
	18.5 min 95% B
	19.0 min 0% B
	25.0 min 0% B
Flow rate:	200 µL/min
Auto-sampler temperature:	4 °C
Column temperature:	25 °C
Injection volume:	10 µL
MS	
Scan type:	MS
Mass spectrometer:	Thermo Exactive Plus Orbitrap™
Mode:	Negative Ion
Scan range:	70-1000 m/z

Adapted from Lu et al. (2010).

Metabolomic data analysis

For mass spectral analysis, quality control, metabolite annotation, and variable selection of plasma metabolites, I developed an analytical pipeline by combining different methodologies from previously described analysis techniques, as depicted in a flowchart in Supplementary Figure 1. This pipeline was used for metabolomic analysis of plasma and of *Pf* 3D7 culture media. Specific details included in this analysis are described in the following sections.

Deconvolution of mass spectrometry data

We converted the raw mass spectrometry data files using proprietary software Xcalibur (ThermoFisher, Waltham, Massachusetts) to .raw and .mzXML files, and used a combination of R software (R version 3.3.3, 'Another Canoe', www.r-project.org) and the MZmine interface (V2.0) (http://mzmine.github.io/) for mass spectral analysis.

An IPO algorithm was used to aid in determining optimal peak picking parameters (Libiseller et al., 2015). I loaded the mass spectrometry data per batch for each of the separate extraction procedures (Set 1: plasma SPE; Set 2: plasma MeOH; Set 3: *Pf* 3D7 media MeOH) and performed mass spectral analysis for each individual dataset.

Mass spectral deconvolution was performed in MZmine 2 software (Pluskal et al., 2010) in the following order: 1) baseline correction using a RollingBall algorithm; 2) mass detection; 3) chromatogram deconvolution; 4) peak smoothing; 5) isotope and duplicates filtering; and finally, 6) peak identification and annotation.

The base peak chromatograms for the two extractions of patient plasma metabolites are provided in Supplementary Figure 2. The base peak chromatograms for the *Pf* 3D7 culture media metabolites are provided in Supplementary Figure 3.

Metabolite annotation and source analysis

Peak annotation was done using an in-house list of 292 'known' metabolites which had been previously individually chemically confirmed on the same analytical platform using the same LCMS parameters. This 'knowns list' contained primary and secondary amino acids, glycolytic intermediates, and other metabolites from a wide range of malaria parasite metabolic pathways, as described previously (Olszewski et al., 2009).

We had expanded the knowns list by adding a range of organic acids that were previously identified in patients with severe malaria acidosis (Herdman et al., 2016), such as beta-hydroxybutyric acid, hydroxyphenyllactic acid, gamma-amino butyric acid, 5-aminopentanoic acid.

For new metabolic features that were not identified by this list of known metabolites, I manually inspected the peaks and used the publicly available Human Metabolome Database (V3.0 and V4.0; www.hmdb.ca) to perform a putative annotation (Wishart et al., 2018). The mass accuracy of UHPLC-Orbitrap-MS was considered to be sufficiently high that these peaks could be accurately putatively annotated using an *m/z* tolerance of 10 ppm (Negative ion mode, M-H).

All metabolites were mapped to their metabolic origin using the publicly accessible HMDB datasets. I filtered the HMDB V4.0 dataset for metabolites that were detected in

blood (total number of 3028 metabolites) and extracted the XML data files into R. Those metabolites that were reported as having a microbial origin by HMDB were called microbial in the current human plasma datasets (Chapter 3), as described in Supplementary Table 1.

Signal correction for plasma samples

For all the clinical samples, I performed a previously described QC robust LOESS signal correction (QC-RLSC) (Dunn et al., 2011) using the MetMSLine R package (Edmands et al., 2015), Supplementary Figure 4. Correlations of UHPLC-Orbitrap-MS data were assessed through comparison of L-lactate with bedside L-lactate measurements from the iStat system, Supplementary Figure 5. Metabolic features were discarded if median mass intensity was higher in blanks than in QC samples. Duplicates were removed by selection of the metabolite with the highest median relative intensities in the QC samples. For quality assurance, we removed features if they were found in <50% of all QC samples, or if the RSD of the metabolites was >20% among the QC samples (Dunn et al., 2011).

Signal correction for *P. falciparum* media samples

P. falciparum metabolite data were normalised by dividing the peak abundance with the internal standard peak abundance. Samples were normalized for haemocytometer and parasite cell counts. The mean of the blanks was calculated for a blank subtraction for each of the compound blank subtraction. Finally, mean, standard deviation, residual standard deviation and fold changes were calculated for the technical duplicates.

2.2.2 Amino acid quantification

Amino acid extraction from patient plasma (Chapter 5)

Amino acids were extracted from patient plasma samples for amino acid analysis at the Department of Pharmacology of the Mahidol-Oxford Tropical Medicine Research Unit in Bangkok. I planned and carried out the extraction of metabolites using a commercially available amino acid analysis kit (Phenomenex EZ:faast™), as described previously (Badawy et al., 2008).

Amino acids were extracted using an SPE procedure based on sorbent tips. In brief, plasma samples were thawed on ice, and a volume of 100 µL was mixed with 100 µL of a solution containing three internal standards (Homoarginine, Methionine-d3, homophenylalanine). The mixture was then passed through a sorbent tip and washed with 200 µL N-propanol. Following, the sorbent was eluted by adding a 3:2 mixture of sodium Hydroxide and N-propanol. An organic emulsification was attained by adding chloroform and propyl chloroformate and vortexing the emulsified sorbent for 30 seconds at full speed. Following, iso-octane was added to create an organic solution and the sample was vortexed for 5 seconds at full speed. The upper organic layer containing the extracted metabolites was transferred onto a fresh glass 96 well and evaporated to dryness with nitrogen using TurboVap (Biotage Uppsala, Sweden) for 1 hour at 37 °C before the plates were sealed for dry storage at -80 °C.

Reconstitution of SPE extracted samples was done by adding 100 µL of phase A, Table 2.3, into the dry wells and mix for 10 minutes at 600 rpm and room temperature using a MixMate system (ThermoFisher, Waltham, Massachusetts). Reconstituted extracts were transferred

into glass snap-cap low volume MS microvials (ThermoFisher, Waltham, Massachusetts) and these were also kept for 1-3 days at -80 °C before they were analysed.

HPLC-MS/MS-MRM

Plasma free amino acids (PFAA) were quantified through LCMS using an ABSciex API 5000 Triple Quadrupole LC/MS/MS Mass Spectrometer. We performed HPLC using an AAA-MS™ column (250 x 3.0 mm, Phenomenex, Torrance, California). The column was placed into a Thermo LC system and the LC-parameters were followed as described in the Phenomenex EZ:faast™ amino acid analysis kit, Table 2.3.

Mass spectrometry with multiple reaction monitoring was done in positive ion mode with electro-spray ionisation. Prior to data acquisition we optimized the LC system to detect the internal standards. The scan range covered between an *m/z* 100-600 Table 2.3. Inter-assay variation scores were determined for three amino acid standards. Concentrations were based on interpolation on a linear standard curve.

Table 2.3 **Instrument settings used for HPLC-MS/MS-MRM at the Department of Pharmacology, Mahidol-Oxford Tropical Medicine Research Unit, Bangkok, Thailand.**

Instrument Settings (Amino acid analysis)	
HPLC	
Column:	AAA-MS™ (Phenomenex)
Mobile Phase	A: 10 mM Ammonium formate in water
	B: 10 mM Ammonium formate in methanol
LC Methods:	0.0 min 68% B
	13.0 min 83% B
	13.1 min 68% B
	17.0 min 68% B
Flow rate:	250 µL/min
Auto-sampler temperature:	4 °C
Column temperature:	35 °C
Injection volume:	1 µL
MS	
Scan type:	MRM*
Mass spectrometer:	ABSciex API 5000 Triple Quadrupole
	LC/MS/MS Mass Spectrometer
Mode:	Positive Ion
Scan range:	100-600 m/z

Adapted from Badawy et al. (2008).

*MRM = Multiple reaction monitoring.

2.2.3 Plasma proteins

*Pf*HRP2

Plasma *Pf*HRP2 was quantified using a commercially available sandwich ELISA assay (Cellabs, Sydney, Australia), as described previously (Dondorp et al., 2005; Hendriksen et al., 2012). Standard curves were constructed by using calibrated pooled plasma reference samples with recombinant *Pf*HRP2 (provided by D. Sullivan, John Hopkins School of Public Health, Baltimore, Maryland, US). All samples were diluted and analysed in duplicate. Plasma *Pf*HRP2 concentrations were determined on the linear segment of the standard curve.

D-lactate

Plasma D-lactate concentrations were determined by colorimetric ELISA kits, purchased from Sigma-Aldrich (MAK058). Assays were performed according to the vendors instructions. In brief, plasma samples stored at -80 ºC, were thawed and pipetted on to 96-well plates. The samples were then diluted 7.5x by adding D-lactate sample buffer. In each well, a reaction mixture of D-lactate enzyme mix, buffer and substrate were added and a reaction was incubated up to 30 minutes. A standard curve was established by five dilutions of the D-lactate standard. All samples and standards were analysed in duplicate, without a sample blank. Readings were done on a plate reader at 450nm, and each sample was corrected for background intensity. Final concentrations were established from the standard curve and multiplied by initial sample dilution.

2.2.4 16S rRNA sequencing

Gut microbiota profiling

We profiled the human gut microbiota composition of patients and healthy individuals by sequencing the V4 region of the 16S rRNA gene in DNA extracted from stool samples. In summary, DNA was extracted using a bead-beating method and a Maxwell 16 Tissue LEV Total RNA purification kit (Promega, Leiden, The Netherlands). A 2-step PCR strategy was used to generate barcoded amplicons from the V4 region of 16S rRNA genes which were sequenced on an Illumina Miseq platform, as described previously (Fransen et al., 2017). I extracted the DNA from the stool samples at the Department of Microbiology of the University of Wageningen, the Netherlands. DNA sequencing was done commercially in Germany (GATCBiotech, Konstanz, Germany). Sequence processing was done by dr. Floor Hugenholtz in a collaboration with the Academic Medical Center, University of Amsterdam, the Netherlands.

DNA extraction

Faecal DNA was extracted by adding beads and STAR buffer (Stool Transport and Recovery) (Roche, Basel Switzerland) to the faeces samples and carrying out bacterial cell disruption using a bead-beater, as described previously (Yu and Morrison, 2004). The faecal pellets were centrifuged and 250 µL of the supernatant was diluted in 50 µl DNase free water followed by purification using a Maxwell 16 Tissue LEV Total RNA Purification Kit.

Barcoding

We amplified the 16S rRNA gene using 20 ng of DNA through 25 cycles with V4 515FB forward and 806RB reverse (Ramiro-Garcia et al., 2016). Universal Tag (UniTag) linkers were attached to the primers. UniTag sequences were selected based on their GC-content and checked against unforeseen matches in any of the major gene databases. The GC-content was kept around 66% to reduce the tendency to form secondary structures such as heterodimers, hairpin loops, and homodimers, which were assessed using an IDTDNA Oligoanalyzer 3.1 (Integrated DNA Technologies). We excluded matches in the 16S rRNA gene databases by using the "TestProbe" tool from the SILVA rRNA database project (Quast et al., 2013) (using the SSU r117 database). Furthermore, we excluded matches in genome databases using the Primer-BLAST tool (http://www.ncbi.nlm.nih.gov/ tools/primer-blast/). The final UniTags were: UniTag I (forward) GAGCCGTAGCCAGTCTGC; and UniTag II (reversed) GCCGTGACCGTGACATCG.

Two step PCR

The first PCR was performed using a total volume of 50 µl, which contained 1× HF buffer (Finnzymes, Vantaa, Finland), 1 µl dNTP Mix (10 mM; Promega, Leiden, the Netherlands), 1 U of Phusion® Hot Start II High-Fidelity DNA polymerase (Finnzymes Vantaa, Finland), 500 nM of the forward primer (515-FB: GTGYCAGCMGCCGCGGTAA) and this was appended with UniTag 1 at the 5' end, 500 nM of the reverse primer (806RB: GGAC-TACNVGGGTWTCTAAT) (http://www.earthmicrobiome.org/protocols-and-standards/16s/)

(Caporaso et al., 2011) that were 5'-extended with UniTag 2, and 0.2–0.4 ng/µl of template DNA.

We confirmed the size of the PCR products by gel electrophoresis (340 bp) for which we used 5 µl from the amplification reaction mixture on a 1% (w/v) agarose gel containing 1× SYBR® Safe (Invitrogen, ThermoFisher, Waltham, Massachusetts). We added adaptors and an 8-nt sample-specific barcode in an additional five cycle PCR amplification to 5 µl of the PCR products.

The second PCR was done using a total volume of 100 µl, which contained 1× HF buffer, dNTP Mix 2 U of Phusion® Hot Start II High-Fidelity DNA polymerase, 500 nM of a forward and reverse primer at the 5' end. We used magnetic beads (MagBio, London, UK) to purify the PCR products according to the HighPrepTM protocol of the supplier using 20 µl Nuclease Free Water (Promega Leiden, the Netherlands) and quantified using the Qubit (Life Technologies, Bleiswijk, the Netherlands). We then mixed the purified PCR products in equimolar amounts and concentrated them again by magnetic bead purification.

Sequencing and processing of sequence data

Purified amplicon pools were 250 bp paired-end sequenced using Illumina Miseq (GATCBiotech, Konstanz, Germany). We only assigned read pairs to samples if the forward and reverse barcodes matched perfectly. Following, we trimmed the initial 46-49 bases (depending on the barcode primer), with the goal to remove the barcode itself, the UniTag, and the Universal 16S V4 515F-806R (Caporaso et al., 2011) primer sequences. After samples were de-multiplexed and trimmed, we truncated the forward reads at 155 bases and the reverse

reads at 125 bases; this was done to remove poor-quality sequence proportions and to retain a margin of 20 bp between pairs for merging.

The amplicon sequence variants (ASV) were inferred using Dada2 (v1.5.2) (Callahan et al., 2017). We detected chimera sequences and removed them in case the parent variant was at least twice as abundant. The sequences were then aligned against the Silva (v128) reference database(Quast et al., 2013) using Mothur (v1.39.5) (Schloss et al., 2009). This was done with the goal of detecting and removing a-specific amplicons and to determine the orientation of the ASVs. We trimmed the edges of multiple alignments and corrected for insertions and deletions in the primer regions, followed by aggregating the counts for the new trimmed ASVs. We retained forward ASVs if the reverse complement ASV could also be inferred; we summed the counts of both ASVs.

Taxonomy was assigned using a naïve Bayesian classifier from the Ribosomal Database Project (Wang et al., 2007), species were assigned through exact database matching. We had included two positive controls to evaluate the performance of this process and the positive controls consisted of genomic material from a variety of 55 different strains (Ramiro-Garcia et al., 2016).

Population diversity indices

The population diversity of the measured gut microbiota was assessed by calculating alpha diversity metrics using phyloseq (McMurdie and Holmes, 2013). This was done by rarefying the ASV Table without replacement to the specified depths 10 times and calculating the mean and standard deviation. Population diversity indices included Shannon, Chao1 and observed

species, for which the Alpha diversity was calculated using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline (Caporaso et al., 2010a).

2.3 Statistical methods

Data analysis and statistics were performed using R software (R version 3.3.3, 'Another Canoe', www.r-project.org).

Categorical data were reported as counts and proportions. Non-normally distributed continuous variables were summarised by their median and inter quartile ranges (IQR). Normally distributed continuous variables were summarised by their mean and standard error of the mean, unless otherwise indicated. Correlations were assessed using Spearman's correlation coefficient. Wilcoxon rank sum tests were used for comparing non-normally distributed variables. Significance intervals for multiple study groups (>2) were calculated by Kruskal-Wallis tests and corrected by a Benjamini-Hochberg procedure where appropriate. Metabolites and amino acids were visualised using box plots displaying the median and interquartile ranges.

Multivariate exploratory data analysis of plasma metabolites was done through principle component analysis using the '*FactoMineR*' package in R. A differential abundance analysis of acids comparing patients with controls was done using the 'samr' package in R. Following calculation of the log2(fold change) between study groups, p-values were calculated using Welch T-tests corrected for multiple testing through a Benjamini-Hochberg procedure. Data from the differential abundance analysis were visualised in a scatter plot of the metabolites,

with the -log10(Benjamini-Hochberg adj. p-values) on the y-axis and the log2(fold change) on the x-axis.

Plasma metabolite variable selection was done through a univariate regression analysis using the '*samr*' package in R (Storey and Tibshirani, 2003; Tusher et al., 2001). This was a non-parametric univariate regression based on the calculation of local false detection rates (FDR) and q-values (Li et al., 2013; Storey and Tibshirani, 2003). In addition, Welch T-tests were done based on the assumption of unequal variance and corrected using a Benjamini-Hochberg.

Pf 3D7 metabolites measured in the technical replicates from *P. falciparum* culture experiments were first blank subtracted. We then compared change over time by calculating the log2(median fold change) of parasitised red blood cells with the non-parasitised red blood cell control for each time point.

Amino acid profiles were visualised in radar plots using the '*fmsb*' package in R. Amino acid concentrations were normalised by calculating the log2 median fold change of patients compared to healthy controls. Significance testing between two groups was done using Mann-Whitney U tests corrected by a Benjamini-Hochberg procedure. Predictors of the total concentration of plasma free amino acids were assessed using multivariate linear regression analysis ('*glm*', family = 'Gaussian', in R).

Multivariate exploratory data analysis of 16S rRNA features was done through partial redundancy analysis using Canoco software (v5.0). The 16S rRNA data were analysed on the phylum (highest order) and genus (lowest order) level. I calculated the relative abundance of

each of the levels by determining the proportion of read counts of the total number of read counts in the sample (on the same level), and reported these as the % of total 16S rRNA.

Chapter 3

Metabolomic analysis of acidosis in severe falciparum malaria

3.1 Abstract

Background

Acidosis in severe falciparum malaria is associated with high mortality but the pathogenesis remains incompletely understood. Non-lactate acids contribute to acidosis however their identity and metabolic source are unknown. Establishing the identity and metabolic source of unidentified acids may have implications for the treatment of acidosis in severe falciparum malaria.

Methods

A prospective observational cohort study was conducted in a malaria referral centre in Bangladesh with the aim of establishing the identity and source of non-lactate organic acids in patients with severe falciparum malaria and controls. Clinical and parasitological data were collected from admission daily until discharge or in-hospital death, and blood gas analysis was performed to quantify the level of unmeasured acids by calculating the standard base deficit corrected for albumin (SBDc). Organic acids were screened in plasma using high-resolution mass-spectrometry. Regression analysis was performed to determine the relation of newly identified acids with standard base deficit. Longitudinal analysis was done to determine the kinetics of newly identified markers in relation to patient outcomes.

Results

A total of 152 participants were enrolled, including patients with severe falciparum malaria (n=60), uncomplicated falciparum malaria (n=47), and local healthy controls (n=45). Among patients with severe malaria acidosis, L-lactate explained on average 47% (SD 24%) of the SBDc, indicating a presence of unmeasured metabolic acids. Among patients with severe falciparum malaria, we identified a significant association between SBDc and 36 metabolic acids (false detection rate <0.15). Among these newly detected acids there was an over-representation of metabolites from a potential microbial source (10 out 36) and amino acids. All microbial acids showed impaired clearance in patients with fatal outcome. Patients with malaria had significantly lower plasma L-citrulline levels, indicating loss of enterocyte biomass and a decreased gut barrier function.

Discussion

Acidotic patients with severe falciparum malaria had elevated plasma levels of organic acids from a potential microbial source. In addition we found acidosis was associated with widespread dysregulation in amino acid metabolism. Whether newly identified and microbial acids are produced by *P. falciparum* parasites is unclear but patients had coincidental marker of loss of intestinal barrier integrity, suggesting that a compromise in intestinal barrier function may contribute significantly to the pathogenesis of life threatening acidosis in severe falciparum malaria. Further studies are needed to assess the relationship between acidosis and amino acid derangements and to determine the metabolic origin of acids from a likely microbial source.

3.2 Background

Severe malaria remains an important cause of pre-mature death in malaria endemic countries with 445,000 deaths due to malaria infections in 2016, mostly in sub-Saharan Africa (Ashley et al., 2018; Norheim et al., 2015; World Health Organization, 2015). The vast burden of severe malaria is caused by the *Plasmodium falciparum* parasite. Early diagnosis and treatment with parenteral artesunate can reduce mortality but case fatality rates still range between 10-30% depending on the number of organs affected (von Seidlein et al., 2012). Adjunctive therapies might reduce case fatality if they target pivotal pathophysiological pathways but this requires a further understanding of the pathophysiology of severe malaria.

During the blood-stage of malaria infection, the biomass of *P. falciparum* rapidly expands through asexual replication leading to coma, renal failure, and metabolic acidosis (Dondorp et al., 2005, 2008b). The key pathogenic mechanism of severe falciparum malaria is sequestration of infected red blood cells in the microcirculation of vital tissues (Marchiafava and Bignami, 1894; White et al., 2013), mediated by parasite-derived ligands, such as *Pf*EMP1, and a range of host endothelial cell surface receptors, including CD36, EPCR, and ICAM1 (Bernabeu et al., 2016; Claessens and Rowe, 2012). Microvascular sequestration contributes to endothelial dysfunction, capillary blockage and subsequent organ failure (Hanson et al., 2012; Yeo et al., 2007). Previous histopathology studies have shown preferential sequestration of infected red cells in the brain and intestines (Pongponratn et al., 2003; Seydel et al., 2006).

Metabolic acidosis is one of the strongest predictors of mortality in both pediatric and adult cases of severe malaria (Day et al., 2000b; Dondorp et al., 2004a; English et al.,

1997a; Hanson et al., 2012; Jeeyapant et al., 2017; Krishna et al., 1994a; Marsh et al., 1995; Newton et al., 2005; Taylor et al., 1993; von Seidlein et al., 2012; White et al., 1985). The development of acidosis is harmful in itself as it causes conformational changes of intracellular and extracellular proteins and dysregulates cellular metabolism (Kraut and Madias, 2012). In severe malaria, acidosis is also a marker of localized tissue dysoxia, mainly caused by anaerobic production of endogenous L-lactate, the major contributor to the acid load (Hanson et al., 2012; Pongponratn et al., 2003). Depending on the malaria parasite biomass, parasite-derived metabolism of glucose to lactate also contributes, but is thought to be a minor proportion of the total lactic acid load (Cranmer et al., 1995; Kanaani and Ginsburg, 1991; Pfaller et al., 1982; Senczuk et al., 2001). While L-lactate is the major known acid contributing to acidosis, it does not account for the full extent of acidosis observed (Dondorp et al., 2004a; Herdman et al., 2015). The remaining unexplained non-lactate acid load in severe malaria, while of independent prognostic importance, has not been well characterised and the source of these unidentified acids is unknown.

In this study we aimed to identify the contributors to metabolic acidosis in patients with severe *P. falciparum* malaria and to map their potential sources. We used a metabolomics screening method to characterize the non-lactate acid load in blood plasma of acidotic patients with severe *P. falciparum* malaria. Our primary objective was to determine the identity and source of acids elevated in the plasma of acidotic patients.

3.3 Hypothesis

This study examined the hypothesis that metabolic acidosis in severe falciparum malaria is associated with an accumulation of non-lactate organic acids, measured by ultra-high-performance liquid-chromatography mass-spectrometry (UHPLC-Orbitrap-MS).

3.4 Objectives

The primary objective of this study was to determine the relationship between standard base deficit (corrected for albumin) and non-lactate organic acids, measured using UHPLC-Orbitrap-Mass spectrometry. Secondary objectives were:

- To establish the identity and metabolic source of newly identified organic acids.
- To determine the temporal kinetics of newly identified organic acids and the association with mortality.

3.5 Methods summary

Detailed methods are described in Chapter 2.

Ethics

The study protocol was reviewed and approved by the local ethics review board, the Chittagong Medical College Ethics Committee, Bangladesh, and the Oxford Tropical Research

Ethics Committee (OxTREC), in the United Kingdom. The trial was registered at *Clinical-Trials.gov* (Trial number: NCT02451904).

Study Design

A prospective observational case-control study in Chittagong Medical College Hospital, Bangladesh. The recruitment phase of the study covered two wet-seasons in 2014 and 2015.

Sample size justification

The effect size of individual metabolites between study groups was unknown *a-priori*, and no pilot studies had been done before. Therefore, we enrolled all consecutive patients with malaria that were willing to give consent. We aimed to include patients with different levels of disease severity to observe a wide range of variation in the degree of acidosis.

Study participants and procedures

Male and female adults (>12 years) with *Plasmodium falciparum* malaria were the target population. Patients with a blood slide positive for asexual blood stages of *P. falciparum* (including mixed infection with non-falciparum species) were eligible to participate in the study. Severe and uncomplicated falciparum malaria were strictly defined according the WHO criteria for severe malaria (World Health Organization, 2015), modified by Hien et al. (Hien et al., 1996), as detailed in Section 2.1.4. Severe acidosis was defined according to WHO criteria (World Health Organization, 2015). Acute kidney injury was defined according to the KDIGO criteria (www.kdigo.org). Written informed consent was obtained from all

participants, or from their attending relatives in case the patient was clinically too unwell to provide consent themselves. On enrolment, a medical history was obtained and physical examination and blood sampling were performed. Hospitalised patients had vital signs and serial blood samples taken daily until 48 hours post-enrolment. Severe malaria was treated as detailed in Section 2.1.5. Patients were followed until hospital discharge or death in fatal cases.

Venous blood gases and L-lactate were measured using the iStat system. The unmeasured organic acid load was estimated by the standard base deficit (corrected for albumin and phosphate) (SBDc), the anion gap, and the strong ion gap, as detailed in Section 2.1.5. We partitioned SBDc to determine the attributable-fraction of measured and unmeasured organic acids (O'Dell et al., 2005). Plasma L-citrulline as a marker of enterocyte mass was quantified using HPLC-MS/MS-MRM as described in more detail in Section 2.2.2.

UHPLC-Orbitrap-MS Metabolomics

Metabolomic analysis was done on heparinised plasma as depicted in a flow diagram in Supplementary Figure 1 and outlined in Section 2.2.1. In brief, samples were extracted in duplicate using a solid-phase-extraction technique (Sriboonvorakul et al., 2013) and by protein precipitation using methanol (Roberts et al., 2012). UHPLC-Orbitrap-MS was conducted using a C18 column (Hydro-RP, Phenomenex, Torrance, California) with tributylamine as an ion-pairing agent, analysed over a 25 minute run-time protocol in negative ion mode, covering an m/z range of 70-1000 Dalton on a Thermo Exactive Plus Orbitrap™ (ThermoFisher, Waltham, Massachusetts) (Lu et al., 2010). The total uninterrupted analysis for all serial

samples, QC samples, and water blanks, took approximately 9 consecutive days per extracted sample set. Peak annotation was carried out using an in-house library of 292 chemically confirmed metabolic intermediates from major human and *P. falciparum* metabolic pathways. Unidentified peaks were putatively annotated using HMDB (V4.0) data sets (Wishart et al., 2018) with confidence at an *m/z* tolerance of 10 ppm. We filtered the final data set down to only include acids (based on selection of pKa <7.0). The metabolomic data analysis pipeline that I developed for this study is visualised in a flow diagram in Supplementary Figure 1.

Statistical analysis

Data were explored by principle component analysis and by comparing the log2(fold change) between study groups. Welch T-tests with Benjamini-Hochberg corrections were used for calculation of p-values for individual metabolites, based on the assumption of unequal variance. Univariate regression analysis of SBDc and plasma organic acids was done using a SAM regression analysis tool ('*samr*' package, R) (Storey and Tibshirani, 2003; Tusher et al., 2001), which allowed the calculation of local False Detection Rates (FDR) and q-values (Li et al., 2013; Storey and Tibshirani, 2003); metabolites were selected if regression showed an FDR <0.15 and q-value <0.001. Log2(fold change) was calculated for individual metabolites and visualised in a heat map for patients with severe malaria, stratified by the degree of acidosis. Spearman's correlation coefficients were calculated for assessing correlations in normally distributed data. Mann-Whitney U tests were used for comparing non-normally distributed variables.

3.6 Results

3.6.1 Participants

A total of 152 participants were recruited, among them were 60 patients with severe *P. falciparum* malaria, 47 patients with uncomplicated malaria and 45 healthy local controls. Baseline characteristics are shown in Table 3.1. There were no significant differences between the study groups in age (p=0.83, Kruskal-Wallis) or sex (p=0.67, Kruskal-Wallis), the majority of the study participants were young males. Mortality from severe falciparum malaria was 20 out of 60 (33%), Table 3.1.

3.6.2 Acid-base disorders in severe malaria

Acid-base status was assessed by blood gas analysis and estimation of the unmeasured acid load in all participants on enrolment. Patients with severe falciparum malaria had significantly elevated levels of unmeasured acids compared to patients with uncomplicated malaria and healthy controls, Figure S6. We observed that the majority of patients with severe falciparum malaria had a metabolic acidosis. Severe acidosis, defined as a standard base deficit corrected for albumin (SBDc) >8mmol/L (World Health Organization, 2015), was diagnosed in 23 out of 60 (38%) severe malaria cases. Moderate acidosis (SBDc >3.3 and $\leq$8 mmol/L) was detected in 28 (47%) cases. Mild or no acidosis, defined as a standard base deficit corrected for albumin SBDc $\leq$3.3 mmol/L (Dondorp et al., 2010), was found in 9 (15%) severe malaria cases.

Table 3.1 **Baseline characteristics in patients and healthy controls (n=152) enrolled in a prospective observational study on the identity and source of non-lactate organic acids contributing to metabolic acidosis in severe falciparum malaria in Bangladesh.**

	Severe Malaria (n=60)		**Uncomplicated Malaria (n=47)**		**Healthy Controls (n=45)**		**p value**
Age (years)	28	(22 to 40)	30	(22 to 45)	29	(23 to 35)	0.831
Female sex	21	(35%)	13	(28%)	16	(36%)	0.654
BMI	21	(20 to 24)	22	(19 to 24)	24	(21 to 27)	0.001
Mortality	20	(33%)	0		0		<0.001
Clinical examination							
Temperature (°C)	38	(37 to 39)	37	(37 to 38)	36	(36 to 37)	<0.001
Coma depth*	10	(8 to 14)	15	(15 to 15)	15	(15 to 15)	<0.001
Pulse rate (per minute)	110	(99 to 130)	98	(89 to 112)	83	(78 to 91)	<0.001
Respiratory rate (per minute)	36	(28 to 44)	24	(23 to 28)	18	(16 to 20)	<0.001
Mean arterial blood pressure (mm Hg)	82	(72 to 88)	79	(73 to 87)	93	(87 to 100)	<0.001
Laboratory assessments							
Parasitaemia (parasites per µL)†	44868	(24406 to 82483)	3840	(1723 to 8558)	0	-	<0.001
*Pf*HPR2 (ng/dL)†	4356	(3218 to 5896)	647	(429 to 977)	0	-	<0.001
Glucose (mmol/L)	6.7	(5 to 8.7)	6.7	(5.8 to 7.8)	5.9	(5.2 to 6.7)	0.216
Sodium (mmol/L)	135	(131 to 138)	134	(132 to 137)	140	(139 to 141)	<0.001
Potassium (mmol/L)	4	(3.6 to 4.3)	3.4	(3.2 to 3.7)	3.6	(3.5 to 3.8)	<0.001
Chloride (mmol/L)	105	(101 to 108)	104	(99 to 107)	103	(102 to 106)	0.153
Phosphate (mEq/L)	3.2	(2.5 to 4.3)	2.7	(2.1 to 3.4)	3.7	(3.3 to 4.2)	<0.001
Magnesium (mmol/l)	2.7	(2.3 to 3.2)	2.3	(2.1 to 2.5)	2.8	(2.6 to 2.9)	<0.001
Calcium (ionised) (mmol/l)	0.9	(0.9 to 1)	1	(0.9 to 1.1)	1.1	(1 to 1.2)	<0.001
Creatinine (µmol/L)	132	(86 to 361)	92	(69 to 102)	71	(57 to 78)	<0.001
Blood urea nitrogen (mmol/L)	41	(26 to 86)	16	(11 to 25)	7	(5 to 10)	<0.001
Albumin (g/L)	27	(24 to 30)	30	(26 to 35)	45	(43 to 49)	<0.001
Haemoglobin (g/L)	90	(64 to 109)	97	(80 to 110)	135	(120 to 149)	<0.001
White blood cells ($x10^3$/µL)	9.3	(7 to 15.3)	6.1	(5.2 to 7.9)	7.9	(6.8 to 9.1)	<0.001
Platelets ($x10^3$/µL)	27	(17 to 43)	46	(37 to 95)	233.5	(178.5 to 277)	<0.001
Acid-base status							
pH	7.38	(7.33 to 7.43)	7.43	(7.4 to 7.46)	7.37	(7.35 to 7.39)	<0.001
pCO_2 (mm Hg)	29	(25 to 34)	34	(31 to 36)	46	(43 to 52)	<0.001
HCO_3^- (mmol/L)	17.8	(14.7 to 19.8)	21.3	(19.1 to 23.9)	25.6	(24.2 to 27.6)	<0.001
Plasma SBD‡ (mmol/L)	6.6	(4.5 to 9.7)	2.5	(0.2 to 5)	-1	(-2.9 to 0.3)	<0.001
SIDa (mmol/L)	41	(38.2 to 44.3)	40.2	(38.9 to 42.3)	46.1	(43 to 48.3)	<0.001
SIDe (mmol/L)	25	(22.4 to 27)	28.7	(25.8 to 30.5)	35.1	(33 to 36.5)	<0.001
Strong ion gap (mmol/L)	15.8	(13.3 to 19.5)	12.1	(10.8 to 14.3)	10.4	(8.6 to 12)	<0.001
Anion gap‡(mmol/L)	12.3	(10.3 to 14.6)	9.3	(6.9 to 11.2)	7	(5.6 to 8.4)	<0.001
L-lactate (mmol/L)	3.6	(2.2 to 4.9)	1.6	(1.2 to 1.8)	1.2	(1 to 1.4)	<0.001

Data are number (%) or median (IQR) unless otherwise indicated. BMI = body mass index. *Pf*HRP2 = Plasma *P. falciparum* Histidine Rich Protein 2. pCO_2 = partial pressure of carbon dioxide. HCO_3^- = bicarbonate. SBDc = standard base deficit. SIDa = Apparent strong ion difference. SIDe= Effective strong ion difference. SIG = Strong ion gap. AG = Anion gap.

*Depth of coma was assessed by the Glasgow Coma Scale (3-15).

†geometric mean, 95% geometric confidence interval.

‡ Corrected for plasma albumin and phosphate concentrations.

The case fatality rate among patients with severe malaria increased stepwise according to the degree of acidosis, from 10% in mild acidosis, 21% in moderate acidosis, to 56% in severe acidosis. The SBDc was significantly elevated in patients who died (10.9 mmol/L, SD 5.1) compared to patients who survived (6.0 mmol/L, SD 3.3; p=0.0004), Figure 3.1a.

The proportion of acidosis explained by L-lactate decreased as acidosis became more severe. We partitioned the SBDc and found that L-lactate explained on average 89% (SD 19%) of the SBDc in patients with mild or no acidosis, 61% (SD 28%) in moderate acidosis, and 47% (SD 24%) in severe acidosis, Figure 3.1b.

Further evidence for a prognostic unmeasured acid load was found by estimation of the anion gap (AGc) and the strong ion gap (SIG), both of which were significantly elevated in patients who died, Figure 3.1c, d.

We observed a high correlation between SBDc and other estimates of unidentified acids including AGc (Spearman's r=0.61, p<0.0001) and SIG (Spearman's r = 0.46, p <0.0001). As expected, there was also a correlation between SBDc and creatinine (Spearman's r= 0.43, p <0.0001), Supplementary Figure 6, but the attributable fraction of L-lactate to acidosis was similar in patients with and without AKI, Supplementary Figure 7. Acute kidney injury was present in 19 out of 51 (37%) patients with acidosis.

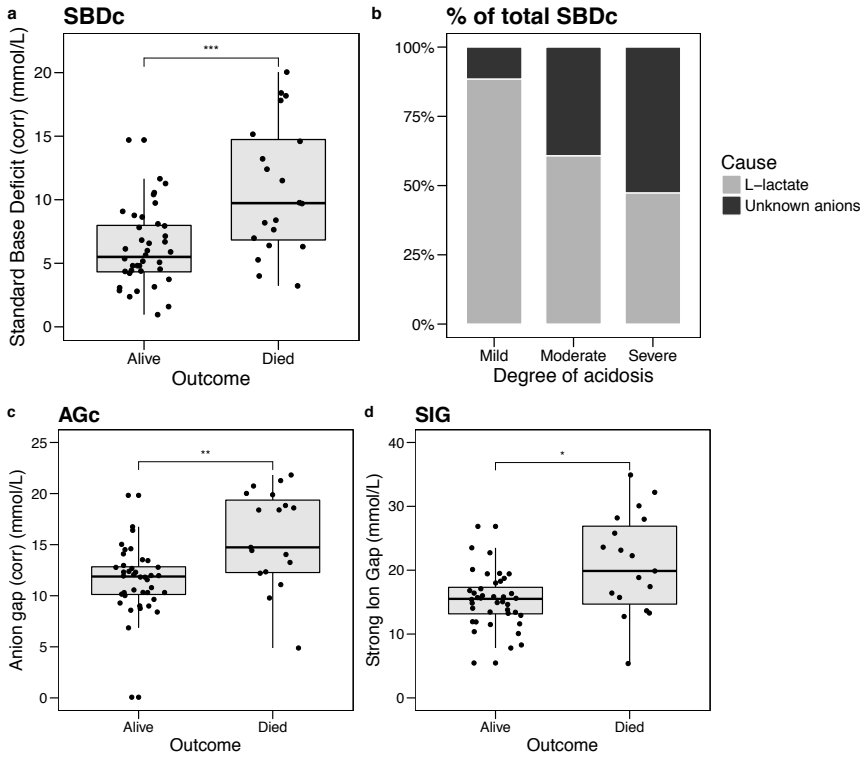


Fig. 3.1 **Elevated levels of previously unidentified organic acids in patients with severe *P. falciparum* malaria**. (**a**) Standard base deficit corrected for albumin and phosphate (SBDc) in relation to outcome in patients with severe *P. falciparum* malaria (n=60). (**b**) Mean percentage of standard base deficit attributable to L-lactate among patients with mild, moderate and severe acidosis. (**c**) Anion gap corrected for albumin and phosphate (AGc). (**d**) Strong ion gap (SIG). Box plots show median, interquartile ranges (IQR) and minimum and maximum; significance tests were done using Kruskal-Wallis tests, *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

3.6.3 Metabolomic analysis

Using high-resolution mass-spectrometry, we detected an initial 1355 molecular ion peaks in the combined extraction methods. Following rigorous quality control procedures, we selected 499 unique features, Supplementary Figure 1. Peaks were annotated and filtered to include acids with a (predicted) pKa <7. The final dataset included 86 organic acids, details are provided in Supplementary Table 1.

Variation was explored between the samples in the organic acid data set by principal component analysis (PCA), which showed separation according to study group on PC1, Figure 3.2a. Some samples from patients with uncomplicated and severe malaria overlapped but these patients also overlap clinically.

We further investigated the differences between study groups by differential abundance analysis and found 23 metabolites to be significantly elevated in patients with uncomplicated malaria when compared to healthy controls (Log2(fold change)>1, Benjamini-Hochberg adjusted p<0.01), Figure 3.2b. Furthermore, a total of 26 metabolites were significantly increased in patients with severe malaria compared to uncomplicated malaria (BH adj. p<0.01, Log2(fold change)>1), Figure 3.2c. The results from the differential abundance analyses are tabulated in Supplementary Table 2.

Major differences were observed in the levels of acetaminophen glucuronide in the plasma from patients who had malaria compared to healthy controls. Acetaminophen glucuronide is a water soluble acidic metabolite from hepatic glucuronidation of paracetamol, showing (unsurprisingly) that patients with malaria were taking antipyretics.

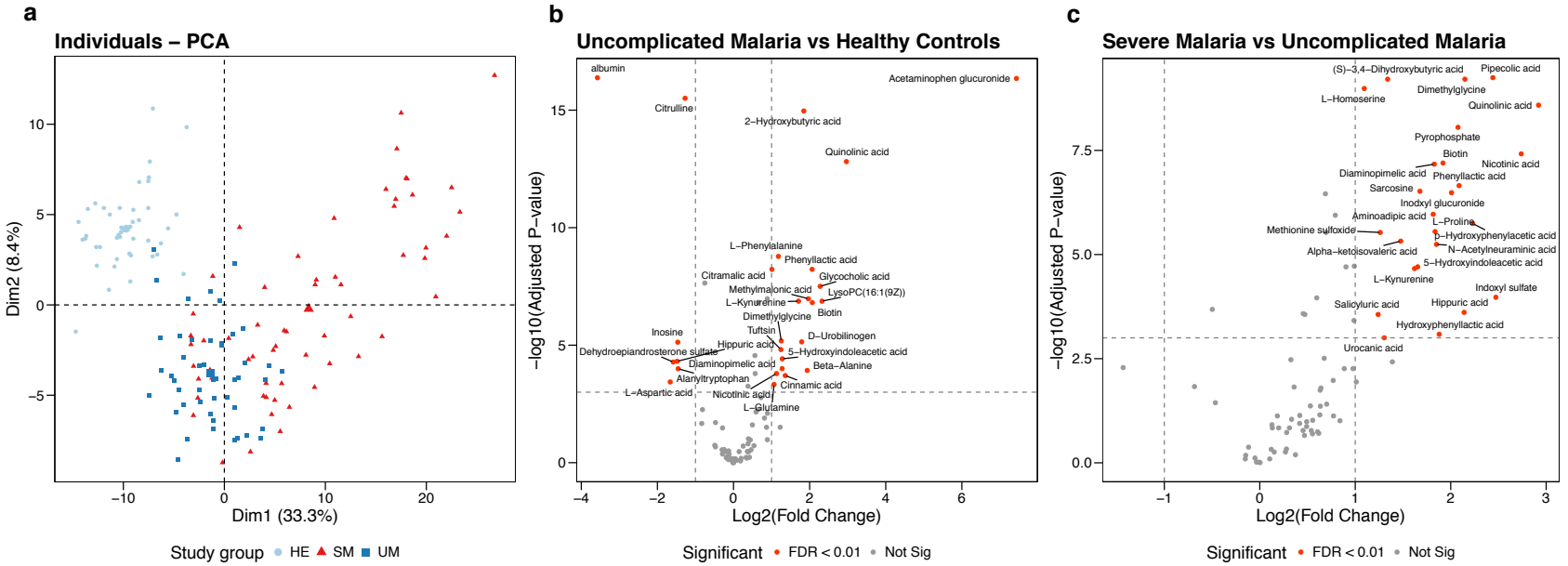


Fig. 3.2 **Metabolomic analysis of plasma from patients with *P. falciparum* malaria by UHPLC-Orbitrap-MS.** (**a**) Principal component analysis (PCA) showing separation by patient groups. (**b**) Differential abundance analysis of acids compared between uncomplicated malaria and healthy controls. (**c**) Differential abundance analysis of acids compared between patients with uncomplicated malaria and severe malaria. The Y-axis denotes -log10(Benjamini-Hochberg adj. p-values) and x-axis denotes log2(fold change).

To determine the association between acidosis and newly identified organic acids we performed a regression analysis among patients with severe malaria. A significant association between SBDc and 36 organic acids was found (FDR <0.015, q-value<0.0001), these organic acids are visualised using their log2 normalised fold change stratified according to the degree of acidosis, Figure 3.3a. Significant associations were found between SBDc and metabolites from energy metabolism such as lactate and ketones, Figure 3.3a. A widespread increase in plasma free amino acids was observed in acidotic patients with severe malaria, including proline, lysine, tyrosine, glutamine, histidine, and homoserine.

We did not observe any associations between acidosis and drug metabolites from medication previously linked to the development of acidosis, such as aspirin or paracetamol (English et al., 1996a; Myall et al., 2011). Salicyluric acid, an aspirin metabolite previously implicated in the development of acidosis in children with malaria (English et al., 1996a) was not significantly elevated in acidosis. Nor were acetaminophen glucuronide and pyroglutamic acid, which are paracetamol metabolites known to cause pyroglutamic acidosis (Myall et al., 2011).

Significantly associated organic acids were mapped to their metabolic pathways and biological origin using HMDB (V4.0) datasets. Notably, we observed an over-representation of organic acids from a microbial origin, 10 out of 36 (28%) metabolites were described as having a microbial source (HMDB, V4.0). These potential microbial acids cross-correlated significantly and were found to have a negative correlation with blood pH and bicarbonate, Figure 3.3c. We observed a gradual elevation of these potential microbial acids, starting in patients with uncomplicated malaria and increasing in severe malaria (Figure 3.3d.

The ten organic acids from a potential microbial source included bacterial cell-wall components and metabolic intermediates (Wikoff et al., 2009; Zhang and Davies, 2016), Table 3.2 and Supplementary Figure 8. We observed a significant elevation of diaminopimelic acid (DAP) in acidotic patients with severe malaria, DAP is a known component of the cell wall of Gram-negative intestinal bacteria (Goodell and Schwarz, 1985). A set of three phenyl compounds, including p-hydroxyphenylacetic acid, phenyllactic acid, and hydroxyphenyllactic acid, are known intermediates of phenylalanine metabolism in intestinal bacteria (Zheng et al., 2011). Indoxyl-sulphate has been described to derive from bacterial tryptophan metabolism and indole production by gut microbiota (Wikoff et al., 2009).

The increase in acids potentially derived from intestinal bacteria suggests a role for translocation from the gut, however *P. falciparum*-infected red blood cell may also be a source though their release of metabolites into the bloodstream during infection.

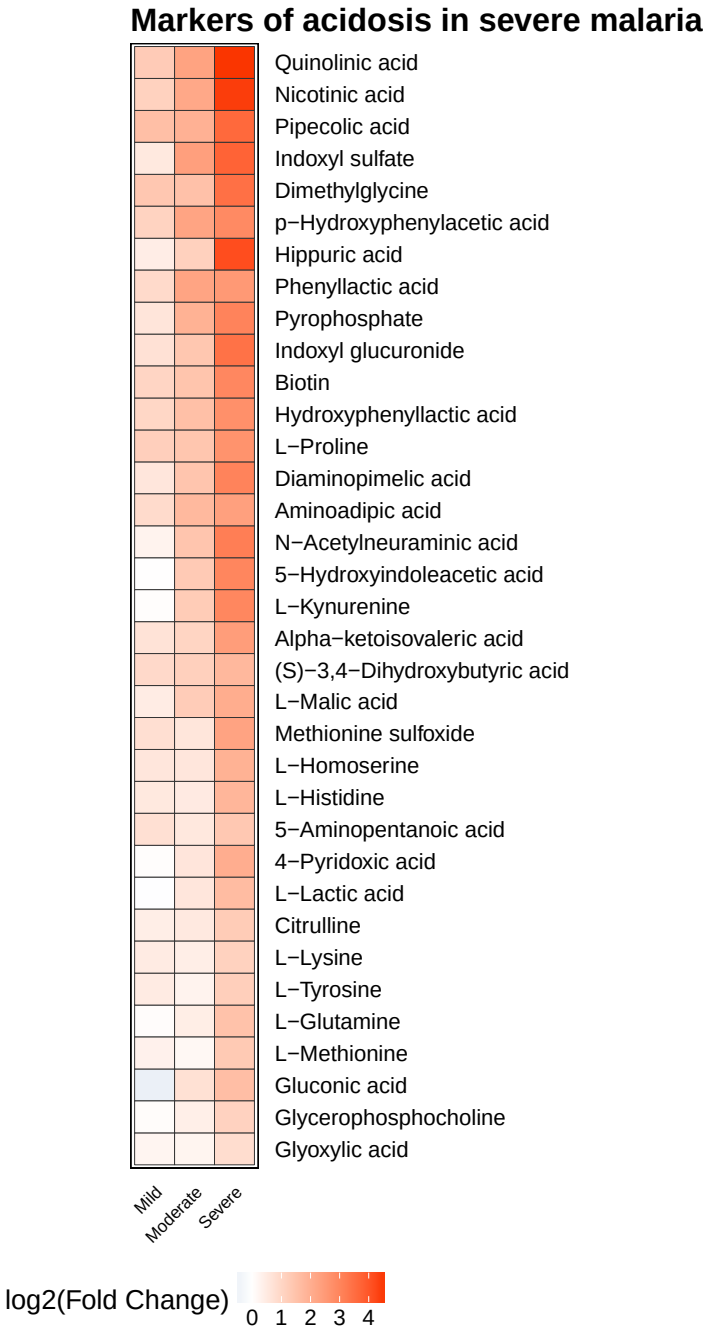


Fig. 3.3 **Metabolomic analysis of acidosis during severe *P. falciparum* malaria.** A heat map of all organic acids that were significantly associated with SBDc (FDR<0.15). Visualisation was done by calculating the log2(fold change) normalised against the levels found in patients with uncomplicated malaria, and stratified according to the degree of acidosis.

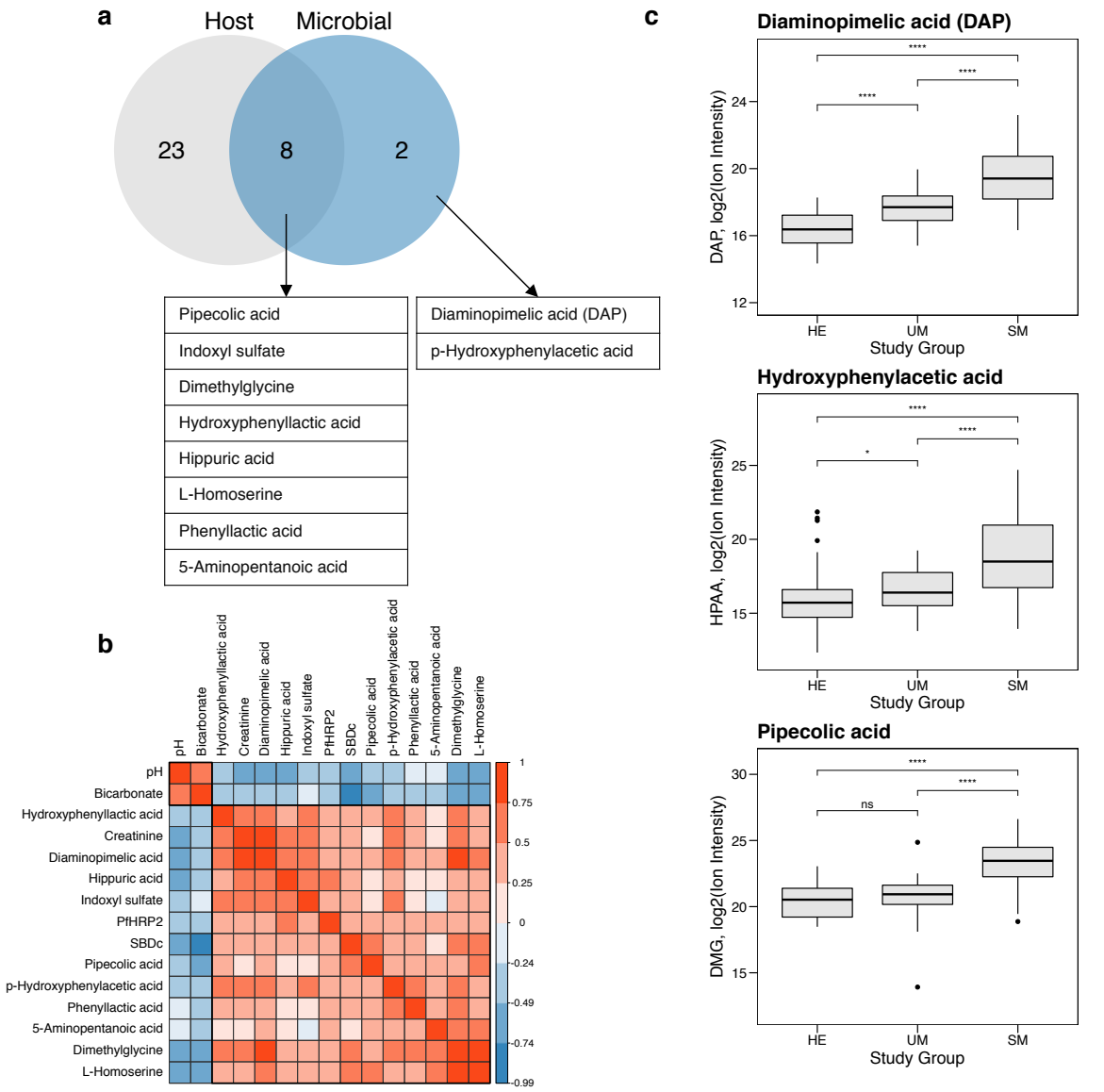


Fig. 3.4 **Metabolomic analysis of acidosis during severe *P. falciparum* malaria, continued.** (**a**) Venn-diagram of the biological origin of acids identified in acidotic patients with severe *P. falciparum* malaria. Acids were mapped to their metabolic source using the publicly available Human Metabolome Database (HMDB), version 4.0. (**b**) Correlation matrix based on Spearman's correlation coefficients of clinical variables of acid-base balance, including blood pH, bicarbonate, base deficit, and ten microbial acids implicated in acidosis in patients with severe malaria. (**c**) Boxplots of microbial acids implicated in acidosis. DAP is a component of Gram-negative cell walls; Hydroxyphenylacetic acid is a product of polyphenol metabolism by gut microbiota; Pipecolic acid is a product of lysine metabolism in intestinal bacteria.

Table 3.2 **Organic acids of a likely microbial origin identified by metabolomic profiling of plasma from acidotic patients with severe *P. falciparum* malaria.**

Name	HMDB ID	FC†	pKa‡	Metabolic pathway	Related bacteria	Reference
Dimethylglycine*	HMDB0000092	3.4	1.88	Gly	Bifidobacterium spp.	(Wang et al., 2011)
Diaminopimelic acid	HMDB0001370	3	1.7	Cell wall	Gram-negative bacteria	(Goodell and Schwarz, 1985)
Hydroxyphenylacetic acid*	HMDB00020	2.8	4	Phe	Clostridium spp., Bifidobacterium spp., Lactobacillus spp.	(Zheng et al., 2011)
Hydroxyphenyllactic acid*	HMDB0000755	2.7	3.58	Phe	Clostridium spp, Bifidobacterium spp., Lactobacillus spp.	(Beloborodova et al., 2012)
Phenyllactic acid*	HMDB0000779	2.5	4.02	Phe	Clostridium spp., Bifidobacterium spp., Lactobacillus spp.	(Svanstrom et al., 2013)
5-Aminopentanoic acid*	HMDB0003355	1.4	4.65	Lys	Firmicutes spp.	(Santoru et al., 2017)
Pipecolic acid*	HMDB0000070	3.6	2.06	Lys	E. coli	(Fujita et al., 1999)
Hippuric acid	HMDB0000714	4.1	3.6	Benzoate	Clostridium spp.	(Li et al., 2007)
L-Homoserine	HMDB0000719	1.9	2.22	Gly/Ser/Thr	Gram-negative bacteria	(Kumari et al., 2008)
Indoxyl-sulphate	HMDB0000682	3.7	-1.82	Trp	Clostridium sporogenes, E. coli	(Wikoff et al., 2009)

Gut microbial acids associated with acidosis among patients with severe malaria. Gly = glycine. Phe = phenylalanine. Lys = lysine. Ser = serine. Thr = threonine. Trp = tryptophan.

*Identity confirmed by chemical standards, otherwise annotated using HMDB, V4.0.

† Log2(Fold Change) compared to uncomplicated malaria controls as a clinically relevant reference group.

‡ As stated or predicted in HMDB, V4.0

3.6.4 Markers of intestinal barrier function

If intestinal leakage is driving the translocation of microbial acids into the circulation, we would expect markers of reduced gut barrier integrity in patients with malaria compared to healthy controls. To test this hypothesis, we quantified plasma concentrations of L-citrulline. We independently measured plasma levels of L-citrulline as a marker of enterocyte mass reflective of gut integrity in the study cohort, along with L-arginine as a correlate of endothelial dysfunction. L-citrulline is a non-proteogenic amino acid that is produced in intestinal cells (Van Wijck et al., 2014). A loss of enterocytes is reflected by reduced levels of plasma L-citrulline (Gutierrez et al., 2014; Piton et al., 2011), which has previously been associated with increased gut permeability and endotoxaemia (Grimaldi et al., 2013). We observed a significant loss of L-citrulline in patients with malaria compared to healthy controls ($p < 0.0001$), Figure 3.5. Furthermore, we observed a significant reduction in the concentration of plasma L-arginine ($p<0.001$), as described previously (Lopansri et al., 2003; Yeo et al., 2007).

3.6.5 Prognostic value of microbial acids

The temporal kinetics of newly identified organic acids were analysed using longitudinal data collected in patients with severe malaria over the first 48 hours of hospitalisation. Clearance curves are plotted for four compounds in patients who survived (n=40) and those who died (n=20) during in-hospital stay, Figure 3.6 (all ten newly identified microbial acids are described in the Supplementary Figure 9). All of these microbial acids were elevated in fatal cases and showed a delayed clearance over time in patients with a fatal course.

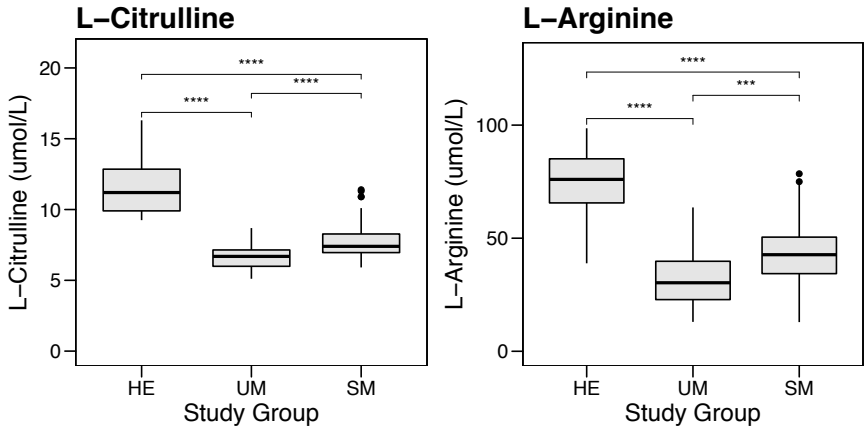


Fig. 3.5 **Plasma levels of L-Citrulline and L-Arginine in patients with malaria and healthy controls.** Plasma amino acids were quantified using targeted HPLC-MS/MS-MRM in plasma of healthy controls (HE), and patients with uncomplicated malaria (UM) and severe malaria (SM). (**a**) L-citrulline (μmol/L) is a marker of enterocyte mass reflective of gut wall integrity. (**b**) L-arginine (μmol/L) is a correlate of endothelial function.

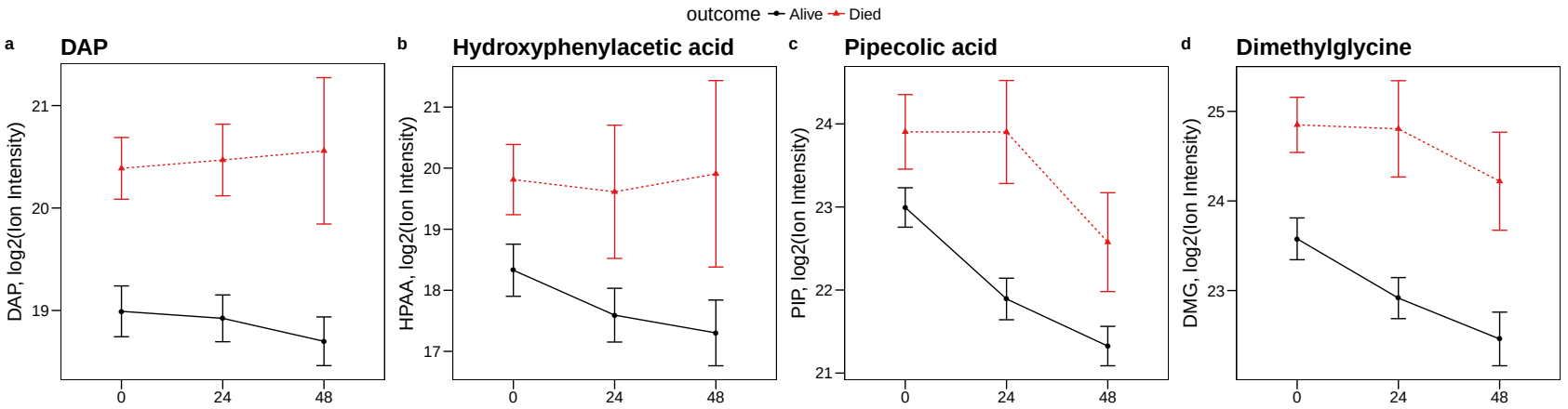


Fig. 3.6 **Delayed clearance of plasma markers of acidosis derived from a likely microbial origin.** The study hours are displayed on the x-axis. Means and standard errors of the mean are reported of the ion intensity of candidate acids. Serial sampling was done in patients with severe malaria (n=60) at 0, 24 and 48 hours and temporal dynamics of candidate compounds were compared between survivors (n=40, black lines) and patients who died (n=20, red lines). Shown here are four out of ten microbial acids identified in the plasma of acidotic patients with severe malaria. (**a**) DAP, Diaminopimelic acid. (**b**) p-Hydroxyphenylacetic acid. (**c**) Pipecolic acid. (**d**) Dimethylglycine.

3.7 Discussion

In this prospective observational study, we characterised circulating organic acids in patients with severe falciparum malaria from Bangladesh. Using high-resolution mass-spectrometry we detected increased levels of organic acids in patients with metabolic acidosis and found an over-representation of plasma free amino acids and organic acids from a microbial origin. Observed microbial compounds are likely related to the gut microbiota but it remains unclear if they might also be secreted into the circulation by *P. falciparum*. Longitudinal data show that the clearance of these acids was delayed in fatal cases of falciparum malaria highlighting their prognostic relevance. Moreover, patients with malaria had a reduction in enterocyte mass as reflected by a significant reduction in plasma L-citrulline levels. These findings suggest that a compromise in intestinal barrier function may contribute significantly to the pathogenesis of life threatening acidosis in severe falciparum malaria. In addition, we observe widespread amino acid dysregulation during acidosis. How malaria infection and acidosis modulate the plasma levels of free amino acids is not completely clear, and knowing this will be relevant for the interpretation of individual amino acid derangements.

Newly identified microbial acids might derive from the malaria parasite. It has been described before that parasitised red blood cells release L-lactate from anaerobic parasite glycolysis (Cranmer et al., 1995; Kanaani and Ginsburg, 1991; Pfaller et al., 1982; Senczuk et al., 2001). Similarly, *P. falciparum* parasites might release other low-molecular weight acids into the circulation, which is likely only in patients with a very high total body parasite biomass.

It is more likely that these microbial acids derive from intestinal bacteria. It has been established before that severe falciparum malaria predisposes for concomitant bacteremia (Bassat et al., 2009; Berkley et al., 1999; Bronzan et al., 2007; Enwere et al., 1998; Nyein et al., 2016; Pattanaik et al., 2012; Scott et al., 2011; Takem et al., 2014; Were et al., 2011). In patients with severe falciparum malaria, concomitant bacteremia is commonly due to cross-over of Gram-negative enteric bacteria into the blood circulation (Church and Maitland, 2014). Increased intestinal permeability in patients with severe malaria has been shown by sugar absorption tests (Molyneux et al., 1989b; Wilairatana et al., 1997). Endotoxemia, likely arising from intestinal bacteria, was found in children and adults with severe malaria (Olupot-Olupot et al., 2013; Usawattanakul et al., 1985).

The pathogenic mechanisms causing a compromise in intestinal barrier function in severe falciparum malaria are unclear. Intestinal injury may result from the sequestration of parasitised red blood cells in the mesenteric microcirculation (Pongponratn et al., 2003; Seydel et al., 2006). Microvascular perfusion abnormalities have been visualised *in vivo* (Dondorp et al., 2008b). Indeed, currently identified organic acids have been previously reported in the plasma of patients with mesenteric ischaemia (Khadaroo et al., 2017). Recent studies indicate a potential role for parasite induced histamine release related to increased intestinal permeability in patients with malaria (Chau et al., 2013; Potts et al., 2016).

If increased plasma concentrations of bacterial acids signify a loss of intestinal barrier function, these could be predictive for concomitant gut-derived bacteraemia. Unfortunately, we could not diagnose concomitant bacteraemia in our study population because quality-controlled blood culture facilities were unavailable at the study site. Cross-over of low-

molecular weight microbial acids into the blood circulation likely requires only subtle loss of gut barrier integrity. In addition, increased production of these acids by an altered gut microbiota could explain their increased plasma concentrations. However, it should be noted that although L-citrulline is considered a marker of enterocyte mass reflective of gut barrier integrity, the observed reduction in patients with malaria can be compounded by scavenging of L-citrulline by *P. falciparum* parasites, as described previously (Olszewski et al., 2009). Moreover, low L-citrulline levels have been described before in children with cerebral malaria from Malawi (Alkaitis et al., 2016). Additional measurements of markers reflecting intestinal damage (iFABP), intestinal translocation (16S rRNA in blood), and intestinal permeability (sugar probes) were unfortunately unsuccessful. Prospective follow-up studies may include these additional outcome measures to assess the role of gut integrity in the pathogenesis of acidosis in severe malaria.

Besides a contribution of newly identified acids to acidosis, individual metabolites may cause cellular toxicity. Previous studies identified increased CSF levels of quinolinic acid (QA), an excitotoxin, were associated with increased fatality in patients with cerebral malaria, although impaired renal clearance may have had a confounding effect in this relationship (Dobbie et al., 2000; Medana et al., 2003). Furthermore, indoxyl-sulphate is a uremic toxin (Motojima et al., 2002), and impaired renal clearance may worsen any existing kidney injury. Acute kidney injury could impair the clearance of currently detected organic acids as SBDc and creatinine correlated positively (Spearman's rho= 0.43, $p < 0.0001$). However, we observed that patients without renal failure also had marked acidosis and a similarly unexplained acid load, Supplementary Figure 7, and that many of the microbial acids already

showed a significant rise in patients without renal impairment (Figure 3.4c) suggesting that AKI was not the initial cause of their accumulation but rather a compounding factor.

In the presence of compromised intestinal barrier function, the composition of the gut microbiota may play a role in the pathogenesis of severe malaria acidosis. A previous mouse study showed increased intestinal permeability during malaria infection coinciding with changes in the composition of the gut microbiota (Taniguchi et al., 2015). Similar to our findings, a previous mouse study reported increased levels of pipecolic acid (in urine) which was linked to the gut microbiota (Li et al., 2008). Other recent studies implied a relationship between severity of disease and the composition of the gut microbiota (Villarino et al., 2016).

3.8 Conclusions

In conclusion, metabolomic profiling showed elevated plasma concentrations of potentially gut derived microbial acids in acidotic patients with *P. falciparum* malaria. These findings suggest a three-way interaction between the human host, the *P. falciparum* parasite, and the gut-microbiota. These data suggest a novel pathogenic pathway for acidosis in severe malaria, where loss of gut barrier function related to microcirculatory parasite sequestration may cause the translocation of microbial acids into the circulation. Further studies are required to determine if *P. falciparum* parasites release newly identified microbial acids, to further evaluate the relationship between acidosis and plasma free amino acid dysregulation, and to determine the extent of intestinal translocation and the role of the gut microbiota in the pathogenesis of acidosis.

Chapter 4

Metabolomic analysis of *Plasmodium falciparum in vitro*

4.1 Abstract

Background

Severe malaria is associated with a rapidly expanding *P. falciparum* parasite biomass inside the bloodstream. During the blood stage of infection *P. falciparum* parasites are metabolically active as they undergo growth and asexual replication. Parasite-derived metabolic acids may contribute to clinical acidosis. Metabolomic characterisation of acids released by *P. falciparum in vitro* could determine if there is an overlap between parasite metabolism and metabolic acids elevated in patients with acidosis.

Methods

An *in vitro* metabolomic analysis of the culture media of synchronised *P. falciparum* 3D7 strain parasites was conducted throughout the parasite intraerythrocytic life cycle. *P. falciparum* was maintained under standard culture conditions in RPMI 1640 medium, double synchronised using sorbitol, and split into subcultures at ascending levels of parasitaemia (1%, 2%, 3%) maintained at 5% haematocrit. Non-parasitised red blood cells from the same blood donor were maintained under the same culture conditions to function as negative controls. Temporal changes of metabolite levels in the culture media of *P. falciparum* were characterised using high-resolution mass-spectrometry.

Results

Using UHPLC-Orbitrap-MS we detected a total of 124 metabolites in the unmodified culture media of *Pf*3D7 parasitised cultures, of which 51 were chemically confirmed. Changes in the culture media reflected growth and development of the intracellular parasite with peak metabolic activity during the trophozoite stage. Throughout the life cycle there was an increase of glycolytic metabolites in the culture media (pyruvate, lactate, D-glyceraldehyde 3-phosphate) and a depletion of L-arginine. A total of 11 metabolic acids were detected in the culture medium of *Pf*3D7 that were previously identified in the plasma patients with metabolic acidosis, but only lactate significantly increased over time compared to non-parasitised red blood cell controls. None of the previously identified microbial acids were significantly elevated in the parasite culture medium over time.

Discussion

A metabolomics analysis of *P. falciparum* culture media shows changing levels of metabolites reflecting intracellular growth and replication. Previous observations of a release of lactate and a depletion of arginine were confirmed in the current study. Except for lactate, there was no evidence for an increased release of metabolic acids associated with acidosis in the media of parasitised cultures compared to non-parasitised red cell controls.

4.2 Background

Infection with the intracellular parasite *P. falciparum* is associated with severe metabolic acidosis. Metabolic perturbations in the human host primarily result from microvascular blood flow obstruction, intravascular haemolysis and tissue damage by parasitised red blood cells. However, bloodstream infection with *P. falciparum* might contribute to acidosis through parasite metabolism and release of parasite-derived organic acids in the blood.

During the blood stage of infection, parasites complete an intraerythrocytic developmental cycle (IDC) in approximately 48 hours. Under the microscope, a parasitised red blood cell resembles a multi-compartmental structure with on the outside the red blood cell membrane and on the inside the parasite with characteristic digestive vacuole and nucleus. *Plasmodia* are auxotrophes with a streamlined metabolism dependent on their host for the supply of nutrients required for growth. Unable to synthesize amino acids *de novo*, *P. falciparum* parasites acquire amino acids by the breakdown of haemoglobin in the digestive vacuole (Krugliak et al., 2002). Energy metabolism, largely constituted by glycolysis, is fuelled by

the uptake of glucose (Jensen et al., 1983). As parasites undergo maturation and replication, they rewire the intracellular metabolic networks of the red blood cell and introduce 'new permeability pathways' (Ginsburg et al., 1983). This allows the influx of nutrients (glucose) and the efflux of waste products (lactate) into the blood stream (Wu et al., 2015).

The potential contribution of *P. falciparum* metabolism to metabolic perturbations in the host was studied by measuring metabolites in *P. falciparum* culture media.

4.3 Hypothesis

This *in vitro* study examined the hypothesis that *P. falciparum* parasites release microbial acids identified previously in patients with severe malaria acidosis, measured by ultra-high-performance liquid-chromatography mass-spectrometry (UHPLC-Orbitrap-MS).

4.4 Objectives

The primary objective was to establish the release of previously identified microbial acids by *P. falciparum* 3D7 strain parasites *in vitro*, measured using UHPLC-Orbitrap-MS. Secondary objectives were:

- To characterise metabolite changes in the culture media of *P. falciparum* during the intraerythrocytic life cycle.
- To determine if *P. falciparum* parasites release metabolic intermediates from glycolysis, phenylalanine metabolism and L-arginine metabolism.

4.5 Methods summary

Detailed methods are described in Chapter 2.

Study Design

A metabolomic analysis of the spent culture media of *P. falciparum* throughout the intraerythrocytic life cycle and post-re-invasion.

P. falciparum cultures

Cultures of *P. falciparum* 3D7 strain (MR4, cat. no. MRA-102) were performed under standard culture conditions in a mycoplasma free environment (Trager and Jensen, 1976), as detailed in Section 2.2.1. In brief, an initial culture was expanded and double synchronised using 5% sorbitol (Sigma-Aldrich), which was then split into three subcultures diluted at 3%, 2%, and 1% parasitaemia and 2% haematocrit. Using blood from the same donor we maintained a non-parasitised red cell control culture at 2% haematocrit under the exact same culture conditions. Spent culture media were sampled at time points corresponding to the different intraerythrocytic stages of *P. falciparum*. Each time point a total of 100 µL of the spent media was collected and extracted in duplicate by immediately adding 900 µL of pre-chilled (4 °C) methanol spiked with an internal standard ($^{13}C_4$ - $^{15}N_1$ -aspartate). A metabolomic analysis was conducted of the extracted media samples by UHPLC-Orbitrap-MS, performed using the same analytical platform as a previous metabolomic analyses of patient plasma, as described in Chapter 3. Samples were normalized for haemocytometer and parasite cell counts and peak areas in blank control samples. Peak annotation was carried

out using the same method used for plasma samples (Chapter 3) using an in-house library of 292 chemically confirmed metabolic intermediates from major human and *P. falciparum* metabolic pathways. Unidentified peaks were putatively annotated using HMDB (V4.0) datasets (Wishart et al., 2018) with confidence at an *m/z* tolerance of 10 ppm.

Statistical analysis

Cumulative changes over time were determined for each metabolite by calculating the log2(fold change) of the parasitised cell line compared to the non-parasitised red blood cell control at the same time point. We visualised the change in concentrations in the culture media for the 3% parasitaemia cell line in a heat map with the sequential time points organised on the x-axis. The mean cumulative change and 95% confidence intervals were plotted for individual metabolites over time. We grouped the metabolites by their metabolic pathways, as described on the Malaria Parasite Metabolic Pathways database (MPMP) (compiled and maintained by Hagai Ginsburg, www.mpmp.huji.ac.il/).

4.6 Results

4.6.1 Temporal metabolite fluctuations in *P. falciparum* culture media

UHPLC-Orbitrap-MS detected an initial 644 molecular ion peaks in methanol extracted samples. Following removal of duplicates and quality control procedures, we identified 124 metabolites, of which 51 were chemically confirmed and the remainder were putatively annotated using HMDB data sets (V4.0).

Assays of the culture media during the trophozoite stage of highly synchronised *P. falciparum* showed a variety of metabolites accumulating in accordance with the parasite density in the culture. This was confirmed by principle component analysis which showed clear separation of the parasitised red blood cells from the red cell control, where 83.7% of the variation was explained by the incremental levels of parasitaemia, Figure 4.1a. We observed that a total of 22 metabolites had been released (Log2(fold change)>1) and 3 were depleted in the culture medium (Log2(FC)< -1) at the trophozoite stage in the 2% parasitaemia culture, Figure 4.1b. At this point in the parasite life cycle the vast majority of the metabolites detected (76%) were low-molecular weight anions between 100 and 300 Daltons, Figure 4.1c.

Repeated assays of the culture media of *P. falciparum* throughout the intraerythrocytic developmental cycle showed temporal fluctuations reflecting fundamental metabolic processes in the parasite, Supplementary Figure 10. The culture medium contained markers of intracellular parasite growth and replication. Growth was marked by increased levels of glycolytic intermediates in the culture medium. Replication was marked by elevated levels of orotate, an intermediate central to the biosynthesis of pyrimidines for DNA in *P. falciparum*.

Metabolic activity peaked during the trophozoite and schizont stage. We detected metabolites that have previously been described to be elevated in patients with severe metabolic acidosis, Supplementary Figure 10. In addition, an increase in the media concentration of glycolytic intermediates and phenylalanine/tyrosine metabolites was noted, and a depletion of arginine and glutamine from the culture medium.

Following completion of the IDC and re-invasion, we observed a sudden increase among a large number of metabolites presumably related to the release of contents from the red blood cell at schizont rupture, resembling the effects of intravascular haemolysis related to schizogony. Metabolomic changes observed during this phase were characterised by elevated levels of phenyl-compounds.

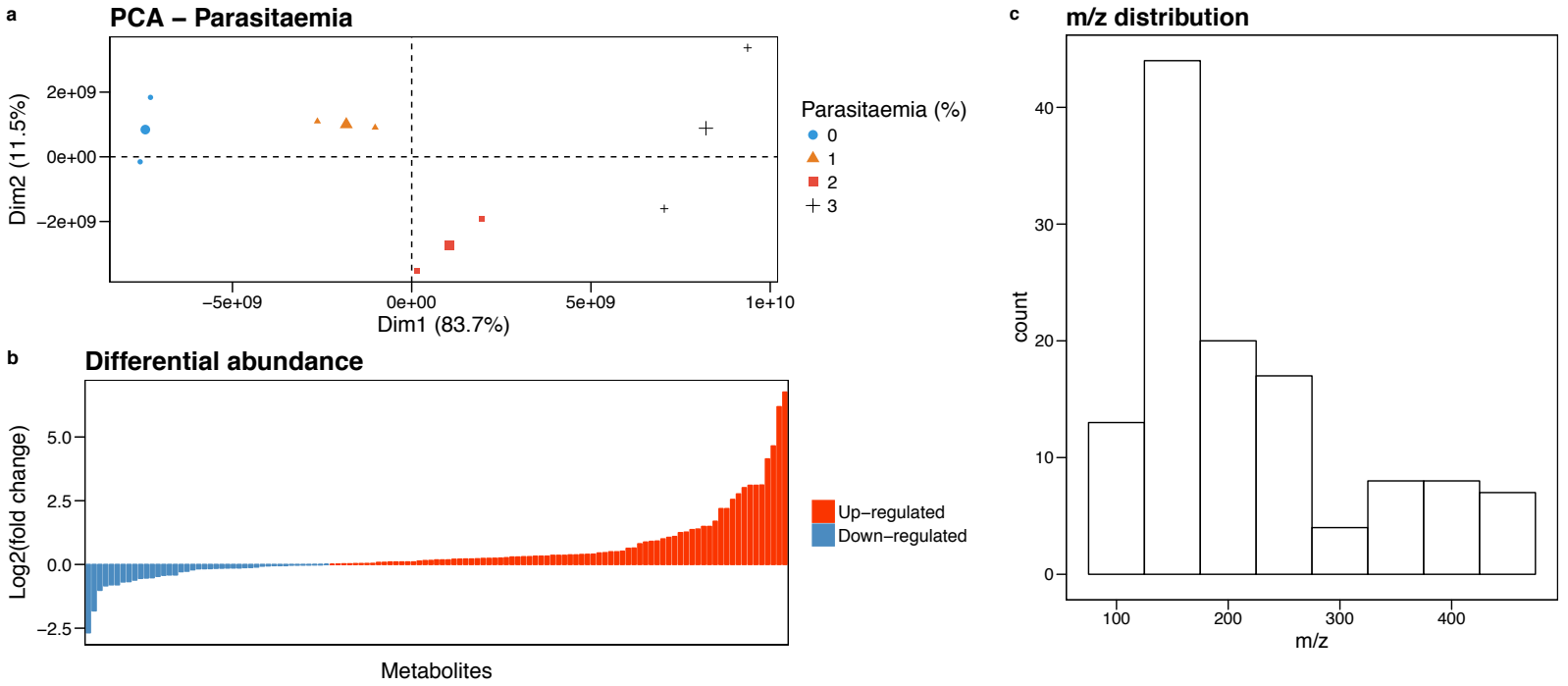


Fig. 4.1 **Metabolomic analysis of *Pf*3D7 culture media**. (**a**) Principle component analysis of different replicates at ascending levels of parasitaemia (0% (RBC control), 1%, 2%, and 3%). (**b**) Bar plot of differentially abundant metabolites during the trophozoite stage (3% parasitaemia replicate). (**c**) Distribution of the m/z of identified metabolites during the trophozoite stage (3% parasitaemia replicate).

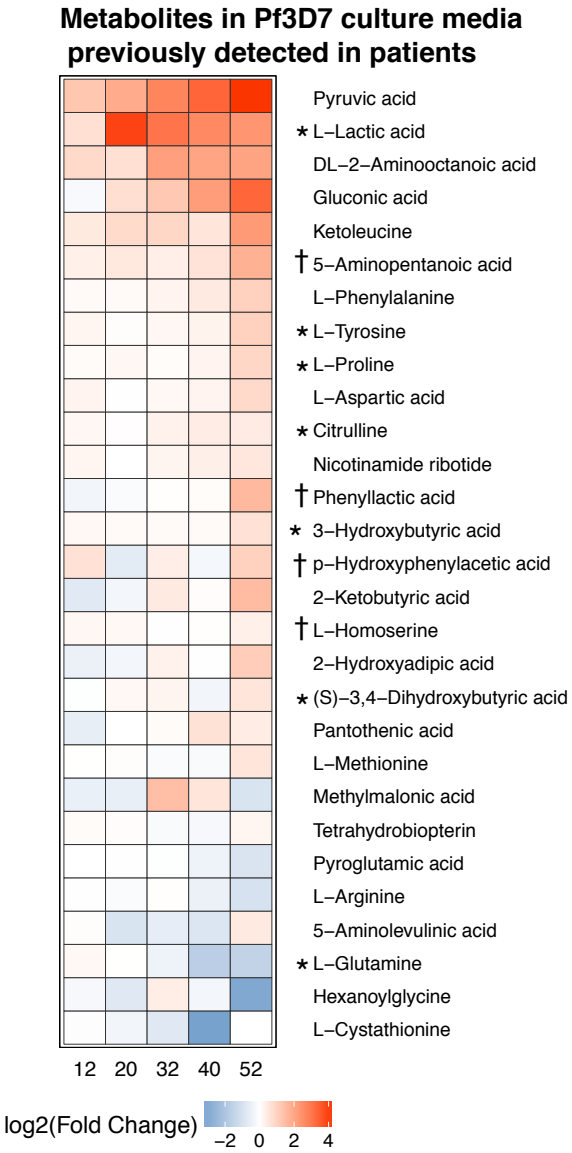


Fig. 4.2 **Metabolomic analysis of markers of acidosis detected in spent culture medium of synchronised *P. falciparum* 3D7 strain parasites sampled at different time points of the intraerythrocytic development.** Double synchronised cultures were adjusted to 5% haematocrit and 3% parasitaemia and kept in unchanged RPMI 1640 culture medium. The concentration of metabolites in spent media from parasitised red blood cells (pRBC) was compared to media from parallel cultured red blood cell controls (RBC) for each time point; relative changes were determined by calculating Log2(pRBC/RBC). *Metabolic acids that were also identified in the plasma of malaria patients with acidosis. †Metabolic acids previously identified in plasma of malaria patients with acidosis of a microbial origin.

4.6.2 Markers of acidosis detected in *Pf*3D7 culture media

A total of 29 metabolites that had previously been detected in the plasma of patients with severe malaria were also detected in the media of *Pf*3D7 cultures, Figure 4.2. Among these, we detected 11 markers of metabolic acidosis. During the intraerythrocytic stages of *P. falciparum*, we observed a moderate increase in two markers of acidosis over time (L-lactate, 5-aminopentanoic acid), while one marker was depleted (L-glutamine) from the culture media. Among the previously identified markers of acidosis from a likely microbial source, we only detected four in the culture media of *P. falciparum* (5-aminopentanoic acid, phenyllactic acid, p-hydroxyphenylacetic acid, L-homoserine). None of these microbial markers of acidosis significantly increased over time until red cell rupture, Figure 4.3.

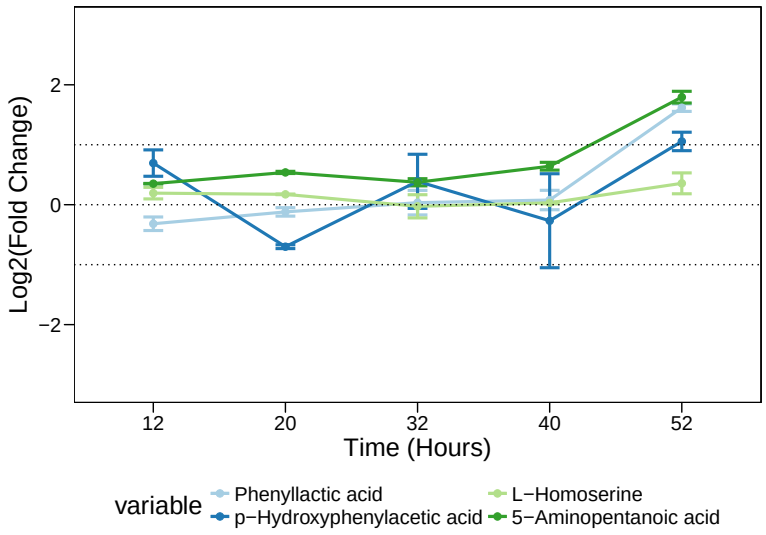


Fig. 4.3 **Temporal fluctuations of clinically prognostic markers of acidosis in spent culture medium of synchronised *P. falciparum* 3D7 strain parasites sampled at different time points of the intraerythrocytic development.** Metabolite concentrations measured in the media of highly synchronised parasitised red blood cells (pRBC) were compared to the media from non-parasitised red blood cells (uRBC) (same blood donor, 2% haematocrit) and normalised (log2(pRBC/uRBC)). Means and standard error are shown.

4.6.3 Release of intermediates from glycolysis

Metabolomic changes observed in the culture media were consistent with increasing rates of glycolysis with parasite maturation. Detectable intermediates of glycolysis included pyruvate, lactate, D-glyceraldehyde 3-phosphate (GADP), and glycerol-3-phosphate, Figure 4.4. GADP is an intermediate of glycolysis and links central carbon metabolism with parallel pathways in the parasite such as the pentose phosphate pathway and phosphatidylcholine metabolism. Metabolite levels increased during the late developmental erythrocytic stages, when the parasite has increased energy demands, Figure 4.4.

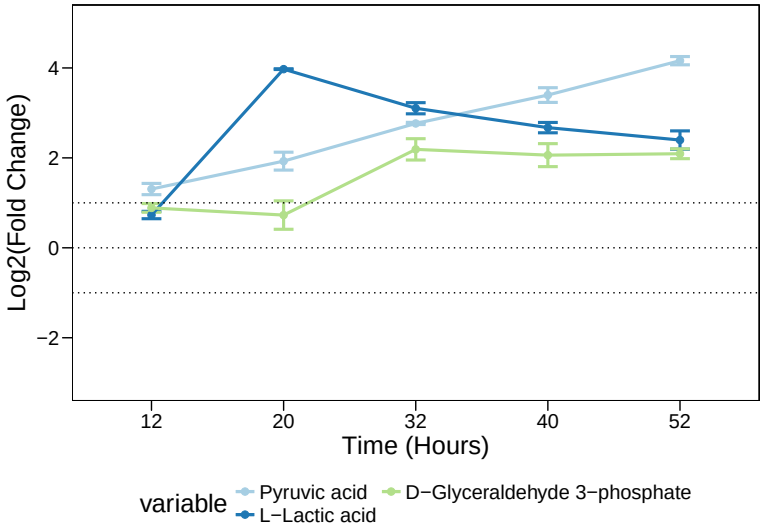


Fig. 4.4 **Temporal fluctuations of glycolysis metabolites in spent culture medium of synchronised *P. falciparum* 3D7 strain parasites sampled at different time points of the intraerythrocytic development.** Means and standard error of the log2 normalised fold changes (pRBC/uRBC) are shown.

4.6.4 Release of phenylalanine and tyrosine metabolites

Plasma levels of phenylalanine (Phe) and tyrosine (Tyr) amino acids and intermediates have previously been observed to increase during severe falciparum malaria infection, in particular hydroxyphenyllactate and hydroxyphenylacetic acid. We observed 6 metabolites from Phe/Tyr pathways in culture medium, Figure 4.5. Yet, no clear evidence was found of an increase of these amino acids during the IDC. We had specifically added two Phe and Tyr metabolites to the method (hydroxyphenyllactic acid, hydroxyphenylacetic acid) because they had previously been implicated in severe malaria acidosis, Chapter 3, however, both were undetectable. Notably, a marked but variable rise of Phe/Tyr metabolites was observed at 52 hours, potentially related to haemolysis of the sample, Figure 4.5.

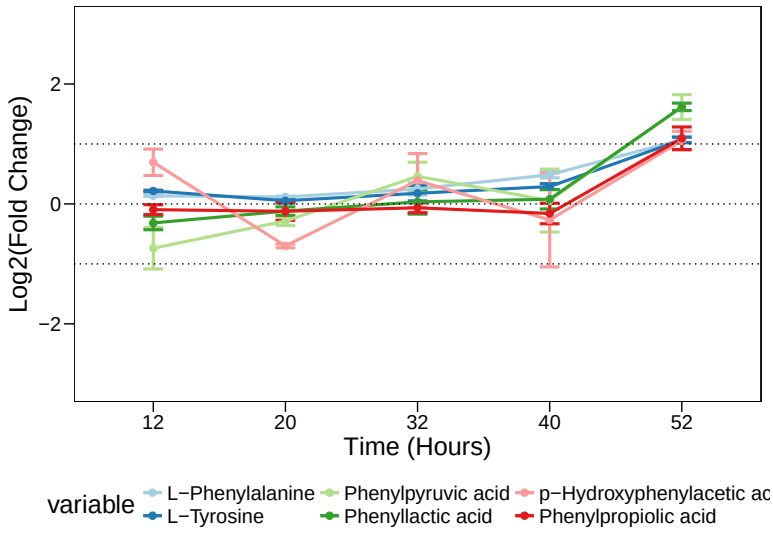


Fig. 4.5 **Temporal fluctuations of phenylalanine and tyrosine metabolites in spent culture medium of synchronised *P. falciparum* 3D7 strain parasites sampled at different time points of the intraerythrocytic development.** Means and standard error of the log2 normalised fold changes (pRBC/uRBC) are shown.

4.6.5 Depletion of arginine from the culture media

Arginine depletion was observed in the culture media of parasitised red blood cells with a concurrent rise of ornithine in the same media, Figure 4.5. These data are consistent with previous observations of arginine depletion from parasitised red blood cell culture media (Olszewski et al., 2009). Arginine is metabolised through several enzymes. The depletion of arginine with a simultaneous increase in ornithine suggests the activity of a parasite or erythrocyte-derived arginase. A reduction of arginine was related to a concomitant rise of citrulline, which may indicate breakdown of arginine through an erythrocyte nitric oxide synthase (NOS).

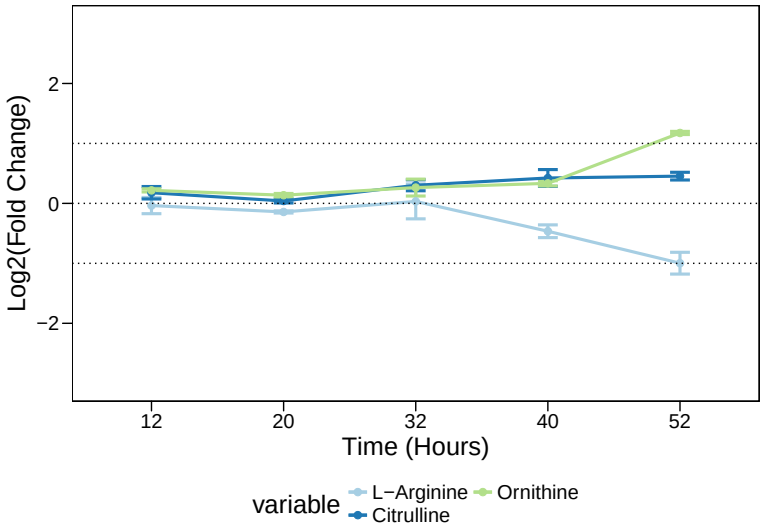


Fig. 4.6 **Temporal fluctuations of arginine metabolites in spent culture medium of synchronised *P. falciparum* 3D7 strain parasites sampled at different time points of the intraerythrocytic development.** Means and standard error of the log2 normalised fold changes (pRBC/uRBC) are shown.

4.7 Discussion

We analysed the spent media of highly synchronised *Pf* 3D7 cultures at different time points during the intraerythrocytic life cycle with the aim to determine if previously identified microbial markers of acidosis in patients with severe malaria are released by *P. falciparum in vitro*. Using UHPLC-Orbitrap-MS we detected four microbial derived components of acidosis, however, none of these metabolites increased significantly during the intraerythrocytic life cycle indicating that P. falciparum metabolism is unlikely to be the source of these organic acids. Other temporal fluctuations of the culture media were characterised by the complex metabolic interaction between the host cell and the parasite. We observed higher rates of glycolytic intermediates appearing in the culture media of parasitised red blood cells than in non-parasitised control red cells, which might reflect up-regulation of central carbon metabolism in the parasite. An increased extracellular concentration of orotate was observed, suggesting pyrimidine biosynthesis as part of parasite replication. Upon red cell lysis and re-invasion, we observed a release of phenylalanine and tyrosine molecules into the culture media. A previously reported depletion of arginine was confirmed in the current study (Olszewski et al., 2009). These findings suggest that although the metabolite composition of parasitised red cell culture media changes in relation to parasite metabolism, *P. falciparum* is unlikely to be the source of previously detected metabolic acids in patients with severe falciparum malaria.

Our findings are consistent with previous reports showing a high glycolytic turnover in parasitised red blood cells. Following infection with *P. falciparum*, red blood cell glucose uptake increases around 100-fold (Jensen et al., 1983). Between 62-89% of glucose is

converted to lactate depending on parasite stage and the production of lactate increases close to 18-fold in parasitised red blood cells compared to non-parasitised red blood cells (Jensen et al., 1983). Lactate production correlates with parasitaemia (Pfaller et al., 1982).

Phosphorylated species of glycolysis are usually contained within the healthy red blood cell. *P. falciparum* infection increases the permeability of the red cell membrane by the introduction of monocarboxylate transporters(Elliott et al., 2001; Marchetti et al., 2015; Wu et al., 2015), mediating the efflux of lactate (94%) and D-lactate (6%) into the extracellular space (Vander Jagt et al., 1990). Parasite derived 'new permeability pathways' allow small anions (but not cations) and possibly also phosphorylated glycolysis intermediates, such as the currently measured D-glyceraldehyde 3 Phosphate, to diffuse through parasite derived porins (Ginsburg et al., 1983).

We confirm the previously described depletion of arginine from the parasitised red cell medium which might be related to parasite arginases and could be a relevant contributor to low arginine in patients (Olszewski et al., 2009). First, it is unlikely that arginine is depleted from the medium because of parasite uptake as a nutritional requirement. During the intraerythrocytic cycle, *P. falciparum* digests up to 65% of the haemoglobin inside the red blood cell (Krugliak et al., 2002). Haemoglobin breakdown appears to provide sufficient amino acids for the parasite be able to grow in a nutrient depleted minimal media environment and except for glucose and isoleucine there are no requirements for the parasite to take up any other amino acids (Liu et al., 2006). The correlating increase of ornithine suggests metabolic turnover may be due to an arginase. Whether this is relevant *in vivo* is unclear as arginine catabolism by arginases was minimal in *P. berghei* ANKA parasitised mice, where instead

the appearance of citrulline and arginine was reduced suggesting impaired biosynthesis of these amino acids plays an important role in the development of hypoargininaemia *in vivo* (Alkaitis and Ackerman, 2016).

A possible limitation of the study is that the only culture strain used was *Pf*3D7, while repeating the experiment in additional laboratory or field parasite cell lines might have further supported the findings. The current study included different levels of parasitaemia of 1%, 2% and 3%, however, no higher levels of parasitaemia, which can be observed in patients. We did not study higher cultures at higher parasitaemia since we expected that hyperparasitaemia could lead to such high lactate production that it would create stress conditions which might inhibit growth or have other unexpected effects on metabolism.

Under the culture conditions used in this study we did not detect the release of metabolites that could explain the presence of newly identified organic acids in plasma of patients with severe malaria acidosis. Newly identified candidate microbial organic acids were not produced by the *P. falciparum in vitro* during the intraerythrocytic developmental cycle. Only phenyllactate became increased following completion of the IDC, but this was likely resulting from red blood cell haemolysis.

4.8 Conclusions

The source of potential microbial acids was investigated in an *in vitro P. falciparum* culture system. Metabolomic characterisation of temporal metabolite changes in the culture media of synchronised *P. falciparum* 3D7 strain parasites were analysed across the parasite intraery-

throcytic life cycle. We confirmed the release of lactate and the depletion of L-arginine from the culture media, as described previously. However, our data suggest that except for lactate, there is no evidence for an increased release of metabolic acids associated with acidosis in the media of parasitised cultures compared to non-parasitised red cell controls. *P. falciparum* is unlikely to be the source of previously identified microbial acids in acidotic patients with severe falciparum malaria.

Chapter 5

Amino acid derangements in severe falciparum malaria

5.1 Abstract

Background

Amino acid derangements are common in severe falciparum malaria and have been associated with endothelial dysfunction (L-arginine), metabolic acidosis (alanine and lactate), coma (phenylalanine and tryptophan metabolites), and other clinical presentations. Whether these amino acid perturbations reflect isolated pathogenic mechanisms or if they are part of overall changes in amino acid metabolism is unclear. To investigate this, we prospectively simultaneously quantified a broad range of plasma free amino acids (PFAA) using high-performance liquid chromatography tandem mass spectrometry with multiple reaction

monitoring (HPLC-MS/MS-MRM) in relation to presenting symptoms in patients with severe malaria, septicaemia, uncomplicated malaria, and healthy local controls.

Methods

A prospective observational study was conducted in a tertiary care hospital in Bangladesh. Clinical and parasitological data were collected on admission, and patients were followed until discharge or in-hospital death. Admission plasma free amino acids (PFAA) were simultaneously quantified from the same blood sample using HPLC-MS/MS-MRM. Amino acid concentration profiles were visualised and compared between study groups and clinical outcomes. Determinants of the total concentration of measured amino acids were assessed using multivariate regression analysis.

Results

A total of 295 participants were enrolled into the study, including patients with severe falciparum malaria (n=88), uncomplicated falciparum malaria (n=71), sepsis/SIRS (n=88), and local healthy controls (n=48). The total concentration of measured amino acids was significantly reduced in each of the patient groups when compared to normal levels observed in healthy local controls: uncomplicated malaria -54%, severe malaria -23%, and sepsis -32%, (p=<0.001). Inspection of amino acid profiles revealed that in each group the majority of amino acids was below normal levels, except phenylalanine. Within patients with severe malaria, overall amino acid levels were increased in patients with hyperlactataemia and those who died. Multivariate regression analysis showed that an increase in the total amino

acid concentration was independently associated with coma depth (p = 0.01) and L-lactate (p<0.001).

Discussion

We confirm previously described amino acid abnormalities in patients with severe falciparum malaria and show they are part of overall changes in the concentration of PFAA.

5.2 Background

Severe malaria, caused by infection with *Plasmodium falciparum*, remains an important contributor to premature death in endemic countries (Norheim et al., 2015). The World Health Organisation estimated 445,000 patients died from malaria infection in 2016, predominantly in sub-Saharan Africa (WHO, 2017). As parenteral artesunate is now established as the best drug to treat severe falciparum malaria (Dondorp, 2005; Dondorp et al., 2010), further gains in reducing case fatality may be achieved by the development of adjunctive therapies. To develop new adjunctive therapies for severe malaria increased understanding is required of the underlying pathophysiology of life-threatening complications.

The pathophysiology of severe falciparum malaria is linked to derangements in amino acid metabolism. Known biochemical abnormalities in falciparum malaria consist of L-arginine deficiency, associated with endothelial dysfunction (Lopansri et al., 2003; Yeo et al., 2007), elevated L-lactate and alanine, associated with metabolic acidosis (Day et al., 2000b; Pukrittayakamee et al., 2002), elevated phenylalanine (Enwonwu et al., 1999; Lopansri et al., 2006; Rubach et al., 2015; Yeo et al., 2015) and increased tryptophan metabolites (Dobbie

et al., 2000; Medana et al., 2003), both associated with coma, and low glutamine, a modulation of oxidative stress (Planche et al., 2002). Prior studies have described associations between specific amino acid derangements and clinical outcomes, and new adjunctive therapies are being developed to correct amino acid disorders. However, whether these amino acid derangements are isolated pathogenic mechanisms or if they are part of overall changes in amino acid metabolism is unclear.

The plasma concentration of free amino acids reflects a balance between amino acid uptake, *de novo* amino acid synthesis and protein turnover, which are under hormonal regulation, and modulated by the bioavailability of cofactors and disease (Nelson and Cox, 2008). During critical illness, the circulating concentration of PFAA may be affected by an energy deficit, hypoxia, and underlying malnutrition (Askanazi et al., 1980; Druml et al., 2001; Ghisolfi et al., 1978; Sprung et al., 1991). An acute energy deficit can develop because of reduced intake (coma) and elevated energy requirements (infection), associated with increased hepatic extraction of amino acids from the bloodstream (Druml et al., 2001; Fischer et al., 1995; Sprung et al., 1991). Tissue hypoxia increases cellular energy requirements because of incomplete anaerobic oxidation of glucose, and increases protein breakdown to allow amino acids to be converted to sugars or other energy substrates through oxidative deamination (Nelson and Cox, 2008). Underlying malnutrition may predispose and aggravate a negative energy balance (Ghisolfi et al., 1978).

During malaria infection, *Plasmodium falciparum* parasites adhere to the vascular endothelium of the microcirculation, where they sequester and compromise microvascular blood flow. Patterns of an obstructed microcirculation in patients with severe falciparum

malaria correlate strongly with hyperlactataemia (Dondorp et al., 2008a; Hanson et al., 2012). Abnormal L-lactate-to-pyruvate ratios in malaria suggest hyperlactataemia derives from anaerobic glycolysis (Day et al., 2000b), and that L-lactate is a marker of underlying tissue hypoxia. Thus in severe falciparum malaria, tissue hypoxia might increase the overall concentration of PFAA.

To determine the interrelationships between several individual amino acid disorders in patients with malaria we simultaneously quantified and profiled a broad range of amino acids in plasma by high-performance multiple-reaction-monitoring mass-spectrometry (HPLC-MS/MS-MRM). We hypothesised that amino acid derangements in patients with malaria are not isolated disorders but that they follow overall changes in amino acid metabolism, characterised by an overall reduction of the concentration of PFAA in the context of infection. While in severe falciparum malaria, we postulated, tissue hypoxia causes an increase in the total concentration of PFAA. These metabolomic studies might help improve the understanding of the pathogenesis of amino acid derangements in severe malaria, with potential implications for their adjunctive treatment.

5.3 Hypothesis

This study examined the hypothesis that tissue hypoxia increases the concentration of plasma free amino acids in patients with malaria.

5.4 Objectives

The primary objective of this study was to determine the clinical and laboratory factors associated with plasma free amino acid derangements in adult patients with severe falciparum malaria from Bangladesh. Secondary objectives include:

- To describe the amino acid profiles of patients with severe malaria, uncomplicated malaria, sepsis and healthy individuals.
- To describe the amino acid profiles associated with coma, acute kidney injury, hyperlactataemia and death among patients with severe malaria.
- To determine clinical and laboratory predictors of a change in the total concentration of plasma free amino acids among patients with severe malaria.

5.5 Methods summary

Detailed methods are described in chapter 2.

Ethics

This was a sub-study of an observational study protocol that was reviewed and approved by the local ethics review board, the Chittagong Medical College Ethics Committee, Bangladesh, and the Oxford Tropical Research Ethics Committee (OxTREC), in the United Kingdom. The trial was registered at *ClinicalTrials.gov* (Trial number: NCT02451904).

Study Design

A prospective observational case-control study in Chittagong Medical College Hospital, Bangladesh. The recruitment phase of the study spanned four wet-seasons and was from May-September 2014 and May-September 2017.

Study participants and procedures

We consecutively enrolled male and female adults (>12 years) with falciparum malaria and patients with sepsis. Patients with a blood slide positive for asexual blood stages of *P. falciparum* (including mixed infection with non-falciparum species) were eligible to participate in the malaria study group. Severe and uncomplicated falciparum malaria were strictly defined according the WHO criteria for severe malaria (World Health Organization, 2015), modified by Hien et al. (Hien et al., 1996), as detailed in Section 2.1.4. Septicaemia was defined using the SIRS criteria in combination with a suspected focus of bacterial infection (Dünser et al., 2012). Patients who had a confirmed negative blood slide for malaria were assessed for eligibility in the sepsis study group and consecutively enrolled. Healthy controls were required to have a blood slide negative for malaria, normal vital signs (no hypertension). Healthy controls were excluded if they were pregnant, had a chronic illness, took medication in the prior two months before enrolment.

Participants were informed about the study and asked to provide written informed consent. On enrolment, we took a medical history, a medication history, assessed vital signs, performed a physical examination and drew blood samples for venous blood gas analysis and amino acid analysis. Patients were followed daily until hospital discharge or in-hospital death.

Malaria cases were treated according to the antimalarial treatment regimens set out in Section 2.1. Laboratory assessments included haematology, which was done in a quality controlled external laboratory (Chevron Clinical Lab, Chittagong, Bangladesh). We used iStat analysers (Abbott, Chicago, Illinois) for biochemistry (Chem8+ cartridge) and blood gas and L-lactate analysis (CG4+ cartridge).

HPLC-MS/MS-MRM

A metabolomic analysis of amino acids was conducted on heparinised blood plasma. Amino acids were extracted using a solid-phase extraction technique (Phenomenex EZ:faast™), as described previously (Badawy et al., 2008), and outlined in more detail in section 2.2.2. Plasma free amino acids (PFAA) were separated on an AAA-MS™ column (250 x 3.0 mm, Phenomenex, Torrance, California) placed in a Thermo LC system. Mass spectrometry with multiple reaction monitoring was done in positive ion mode with electrospray ionization on an ABSciex API 5000 Triple Quadrupole LC-MS/MS Mass Spectrometer, detailed in Table 2.3.

Statistical analysis

Data analysis was carried out in R statistical software (V3.3.3). Baseline characteristics in the study groups and the plasma concentration of free amino acids were all presented as median with inter quartile ranges (IQR), unless stated otherwise. Categorical data were reported as number and proportions. Significance were estimated for multiple study groups (>2) using Kruskal-Wallis tests and corrected by a Benjamini-Hochberg procedure, where appropriate.

Associations were tested for using Spearman's rank correlation. The sum of all measured amino acids (per individual) was calculated to determine the total plasma concentration of free amino acids per individual. Amino acid profiles were visualised in radar plots using the 'fmsb' package in R, displaying the normalised amino acid concentrations (the log2 median fold change of patients compared to healthy controls). Significance tests between study groups and presence or absence of clinical complications was done using Mann-Whitney U tests corrected by a Benjamini-Hochberg procedure. We wanted to determine the potential relationship between plasma L-lactate on the total concentration of PFAA, and used multivariate regression analysis. We used a multivariate linear regression model to characterise the relationship between plasma L-lactate and the total measured PFAA. The standard linear regression was fitted using the function '*glm*' in R.

5.6 Results

5.6.1 Participants

A total of 295 adults were consecutively enrolled in a prospective observational study in Chittagong Bangladesh, between 2014-2017, including patients with severe malaria (n=88), septicaemia (n=88), uncomplicated malaria (n=71), and healthy controls (n=48). The overall case fatality rate of severe falciparum malaria was 30%, and that of sepsis was 23%, Table 1. Baseline characteristics of the study population are described in Table 5.1.

Table 5.1 **Baseline characteristics in patients and healthy controls (n=295) enrolled in a prospective observational study on amino acid derangements in severe falciparum malaria in Bangladesh**.

	Severe Malaria (n=88)		**Uncomplicated Malaria** (n=71)		**SIRS/Sepsis** (n=88)		**Healthy Controls** (n=48)		**p value**
Age (years)	28	(19 to 40)	29	(22 to 45)	35	(23 to 54)	29	(23.5 to 35)	0.016
Female sex	32	36	19	27	37	42	19	40	0.172
BMI	21	(19 to 24)	21	(19 to 24)	22	(20 to 25)	24	(21 to 26)	0.004
Duration of illness (days)	8	(7 to 10)	8	(6 to 11)	9	(5 to 14)	-	-	0.01
Anorexia before enrolment (days)	8	(6 to 10)	7	(5 to 11)	7	(4 to 14)	-	-	0.025
Mortality	9	-	0	-	0	-	0	-	<0.001
Clinical examination									
Temperature (°C)	38.3	(37.3 to 39.1)	37.4	(37 to 38.4)	38.4	(38 to 38.9)	36.6	(36.3 to 36.8)	<0.001
Coma depth*	9	(7 to 14)	15	(15 to 15)	15	(11 to 15)	15	(15 to 15)	<0.001
Pulse rate (per minute)	110	(96 to 130)	98	(89 to 109)	107	(92 to 120)	82	(73 to 88)	<0.001
Respiratory rate (per minute)	34	(28 to 42)	24	(24 to 28)	32	(26 to 39)	18	(16 to 20)	<0.001
Oxygen saturation (%)	96	(95 to 97)	97	(96 to 98)	96	(94 to 98)	98	(97 to 98)	<0.001
Mean arterial blood pressure (mm Hg)	81	(72 to 88)	78	(71 to 84)	85	(77 to 98)	93	(89 to 97)	<0.001
Laboratory assessments									
Parasitaemia (parasites per µL)†	46705	(25541 to 85406)	4321	(1901 to 9819)	0	-	0	-	<0.001
*Pf*HPR2 (ng/dL)†	4165	(3101 to 5594)	640	(426 to 962)	0	-	0	-	<0.001
Glucose (mmol/L)	6.6	(5.2 to 8.3)	6.6	(5.6 to 7.9)	7	(6.1 to 9.4)	5.8	(5.2 to 6.3)	<0.001
Sodium (mmol/L	135	(131 to 139)	135	(132 to 138)	134	(130 to 138)	140	(139 to 141)	<0.001
Potassium (mmol/L)	3.9	(3.5 to 4.3)	3.4	(3.3 to 3.6)	3.5	(3.2 to 3.9)	3.7	(3.5 to 3.9)	<0.001
Chloride (mmol/L)	105	(100 to 110)	103	(98.5 to 106)	98.5	(94.8 to 103)	103	(102 to 105)	<0.001
Creatinine (µmol/L)	122	(82 to 317)	82	(65 to 103)	75.5	(57.5 to 113.3)	72.5	(57.8 to 81.3)	<0.001
Blood urea nitrogen (mmol/L)	40	(25 to 78)	16	(11 to 25)	11.5	(7 to 21.3)	8	(5.8 to 10)	<0.001
Haemoglobin (g/L)	83	(64 to 108)	97	(78 to 115.8)	118	(104 to 133)	134	(120 to 148)	<0.001
White blood cells ($x10^3$/µL)	9.1	(6.6 to 12.8)	6	(4.6 to 7.9)	11.1	(7 to 15)	7.7	(6.3 to 8.8)	<0.001
Platelets ($x10^3$/µL)	32	(19 to 47)	47	(35 to 96)	200	(121 to 277)	228	(191 to 289)	<0.001
pH	7.39	(7.35 to 7.44)	7.43	(7.39 to 7.46)	7.42	(7.38 to 7.45)	7.37	(7.35 to 7.39)	<0.001
pCO_2 (mm Hg)	31	(27 to 34)	35	(31 to 36)	34	(31 to 39)	47	(42 to 52)	<0.001
HCO_3^- (mmol/L)	18.8	(16.3 to 21.1)	22.5	(20.7 to 25)	22.9	(20.1 to 25.1)	26.7	(24.9 to 28.6)	<0.001
Plasma SBD (mmol/L)	6	(3 to 10)	1.5	(-1 to 4)	1	(-0.3 to 5)	-1	(-3 to 0)	<0.001
Anion gap (mmol/L)	16	(14 to 18.8)	15	(13 to 16)	17	(15 to 19)	16	(14 to 17)	<0.001
L-lactate (mmol/L)	3.1	(2 to 4.8)	1.5	(1.1 to 1.8)	1.5	(1.1 to 2)	1.3	(1.1 to 1.6)	<0.001

Data are number (%) or median (IQR) unless otherwise indicated. BMI = body mass index. *Pf*HRP2 = Plasma *P. falciparum* Histidine Rich Protein 2. pCO_2 = partial pressure of carbon dioxide. HCO_3^- = bicarbonate. SBD = standard base deficit.

*Depth of coma was assessed by Glasgow coma scale (3-15)

† geometric mean, 95% geometric confidence interval.

5.6.2 Plasma free amino acids

Using HPLC-MS/MS-MRM a total of 30 amino acids were measured in the plasma of study participants. Amino acids were excluded from the final analysis if they were detected in only a small number of cases (n<50). We excluded 1-methylhistidine, alpha-aminoadipic acid, threonine, homocysteine, sarcosine, thiaproline, alpha-aminopimelic acid, and hydroxylysine, as these were generally below limit of detection.

In addition, γ-aminobutyric acid (GABA) was excluded, which could be quantified in only ten cases. However, among these patients we observed high rates of neurological impairment (8/10), renal impairment (9/10), and case fatality (6/10); the individual patient data are tabulated in Supplementary Table 3.

The final data set included 21 plasma free amino acids (PFAA) that could be accurately quantified in the plasma of study participants, Table 5.2 and Supplementary Figure 11. The total PFAA concentration was calculated for each participant and compared between patient groups. We observed a significant reduction of the median concentration of PFAA in all patient groups when compared to healthy individuals, particularly in patients with uncomplicated malaria, Figure 5.1. The total concentration of PFAA in healthy controls was 6.4 mmol/L (IQR 5.4 to 7.4), uncomplicated malaria 2.9 mmol/L (IQR 1.9 to 3.7; -54%), severe malaria 4.9 mmol/L (IQR 3.9 to 6.2; -23%), and sepsis 4.4 mmol/L (IQR 3.3 to 5.6; -32%).

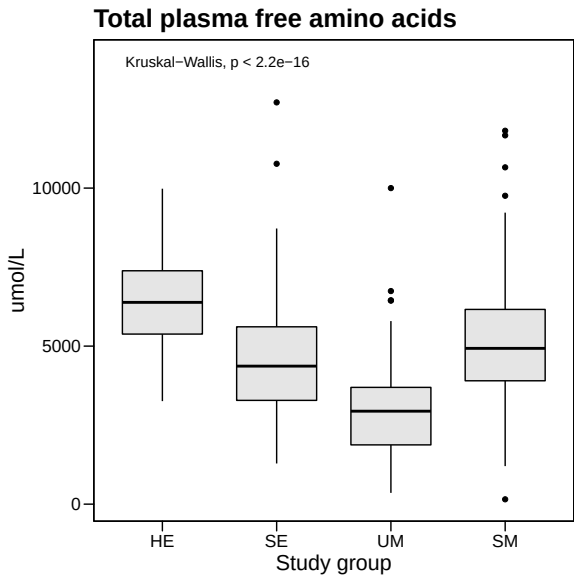


Fig. 5.1 **Total concentration of admission plasma free amino acids in the study groups.** Median and interquartile ranges are shown for local healthy controls (HE) (n=48), patients with SIRS/sepsis (SE) (n=88), uncomplicated falciparum malaria (UM) (n=71), and severe falciparum malaria (SM) (n=88) from Bangladesh.

Table 5.2 **Plasma concentrations of 21 free amino acids in patients and healthy controls (n=295) from Bangladesh.**

Name	**Abbrev**	**Severe Malaria (n=88)** (µmol/L)		**Uncomplicated Malaria (n=71)** (µmol/L)		**SIRS/sepsis (n=88)** (µmol/L)		**Healthy controls (n=48)** (µmol/L)	
Alanine	Ala	549	(350 to 858)	178	(10 to 407)	445	(343 to 622)	625	(413 to 784)
Arginine	Arg	43	(33 to 51)	32	(24 to 44)	36	(28 to 45)	75	(59 to 85)
Asparagine	Asn	19.7	(16.4 to 25.2)	14.7	(11.7 to 18.5)	13.9	(11.9 to 19)	19.7	(17 to 21.9)
Citrulline	Cit	7.4	(6.9 to 8.2)	6.7	(6.4 to 7.4)	8	(6.9 to 9.1)	11.4	(10.1 to 13.3)
Cysteine	Cys	44	(19 to 59)	28	(5 to 41)	42	(33 to 56)	56	(53 to 61)
Glutamate	Gln	200	(175 to 252)	190	(148 to 228)	208	(175 to 255)	259	(239 to 284)
Glutamine	Glu	190	(116 to 305)	130	(61 to 294)	302	(147 to 434)	718	(469 to 954)
Glycine	Gly	55	(37 to 72)	26	(8 to 47)	47	(36 to 68)	82	(64 to 100)
Glycine-Proline	Gly-Pro	343	(93 to 789)	184	(38 to 361)	493	(304 to 935)	536	(425 to 700)
Histidine	His	262	(200 to 327)	144	(101 to 202)	155	(105 to 210)	290	(266 to 337)
Hydroxyproline	Hyp	8.3	(6.5 to 10.8)	5.9	(5 to 6.9)	5.5	(5 to 6.4)	7.7	(6.8 to 8.9)
Leucine	Leu	57	(49 to 72)	38	(24 to 53)	48	(37 to 63)	51	(45 to 64)
Lysine	Lys	1150	(897 to 1410)	814	(580 to 1130)	802	(516 to 1148)	1460	(1265 to 1660)
Methionine	Met	20.2	(18.1 to 22.5)	19.4	(18.1 to 21.8)	18.5	(17.3 to 19.7)	19.7	(19 to 20.7)
Ornithine	Orn	196	(106 to 377)	129	(22 to 239)	219	(94 to 333)	519	(437 to 663)
Phenylalanine	Phe	85	(65 to 109)	57	(49 to 70)	52	(40 to 68)	37	(33 to 40)
Proline	Pro	551	(417 to 696)	277	(128 to 443)	376	(274 to 477)	540	(455 to 594)
Serine	Ser	27.5	(20.8 to 34.4)	24.1	(17.9 to 33.1)	26.6	(19 to 33.3)	42	(35.5 to 48)
Tryptophan	Trp	64	(27 to 136)	36	(0 to 107)	26	(0 to 82)	266	(235 to 300)
Tyrosine	Tyr	67	(43 to 98)	34	(19 to 63)	57	(41 to 76)	91	(83 to 107)
Valine	Val	790	(603 to 968)	387	(128 to 611)	579	(446 to 799)	704	(524 to 936)

Data are median (IQR).

5.6.3 Amino acid derangements in malaria and sepsis

To determine the patterns of change of individual amino acids, PFAA were profiled by calculating the fold change of each amino acid compared to healthy controls and plotting the log2 normalised changes in a radar plot, Figure 5.2. With the exception of phenylalanine, we observed that in each of the study groups the majority of amino acids were below normal levels.

Patients with severe malaria showed an average -23% reduction of the total concentration of PFAA. This reduction was largely due to lower concentrations of amino acids in the arginine-NO pathway, aromatic amino acids (with the exception of phenylalanine), and glycogenic amino acids.

Uncomplicated malaria was associated with a -54% reduction of the total concentration of PFAA. All amino acids, except phenylalanine, were below normal levels. Particularly metabolites from the arginine-NO pathway were low, indicating a deficiency of L-arginine and L-arginine precursors in uncomplicated malaria.

Sepsis was associated with a -32% reduction in the total PFAA concentration. A widespread reduction in amino acids was observed, particularly characterised by a reduction in tryptophan, while phenylalanine was increased.

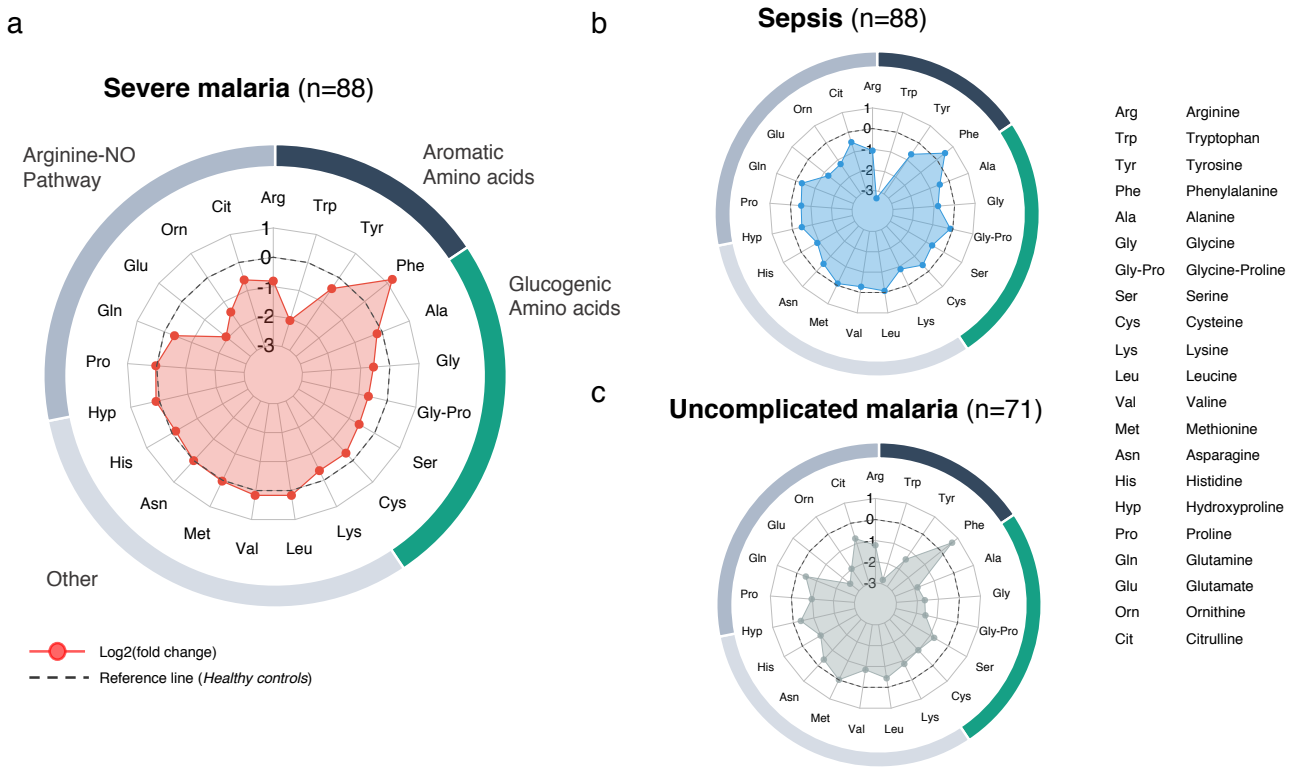


Fig. 5.2 **Amino acid profiles of admission plasma free amino acids in the study groups.** (**a**) severe falciparum malaria (n=88). (**b**) SIRS/Sepsis (n=71). (**c**) Uncomplicated Malaria (n=88). The log2 normalised medium fold change of each amino acid was calculated compared to the normal levels measured in healthy controls and visualised in a radar plot.

5.6.4 Association of amino acid profiles with coma, AKI, and hyperlactataemia

The amino acid profiles of 88 patients with severe malaria were analysed in relation to major clinical manifestations: coma, acute kidney injury, and hyperlactataemia, Figure 5.3.

Coma, as defined by a Glasgow Coma Scale <11 (World Health Organization, 2015), was observed in a total of 30 (34%) patients with severe falciparum malaria. Having coma, using a binary cut-off, did not significantly alter the plasma free amino acid profile, except for a decrease in valine (Benjamini Hochberg (BH) adjusted p-value = 0.037). There was no significant correlation between GCS and phenylalanine (Spearman's rho = -0.17, p = 0.13).

Acute kidney injury, defined by a serum creatinine >265 μmol/L (3mg/dL) (World Health Organization, 2015), was found in 29 (33%) patients. Patients with acute kidney injury had significantly higher levels of hydroxyproline (BH adj. p = 0.0037) and cysteine (BH adj. p = 6.4 x 10^{-4}).

Hyperlactataemia, as defined by a venous L-lactate >5 mmol/L (World Health Organization, 2015), was diagnosed in a total of 21 (23%) of patients with severe malaria. Incremental changes in venous L-lactate were associated with an increasing total concentration of plasma free amino acids, Figure 5.3. L-lactate correlated with plasma alanine (Spearman's rho = 0.57, p = 3.5 x 10^{-8}), and phenylalanine (Spearman's rho = 0.37, p = 4.5 x 10^{-4}), and proline (Spearman's rho = 0.39, p = 2.4 x 10^{-4}).

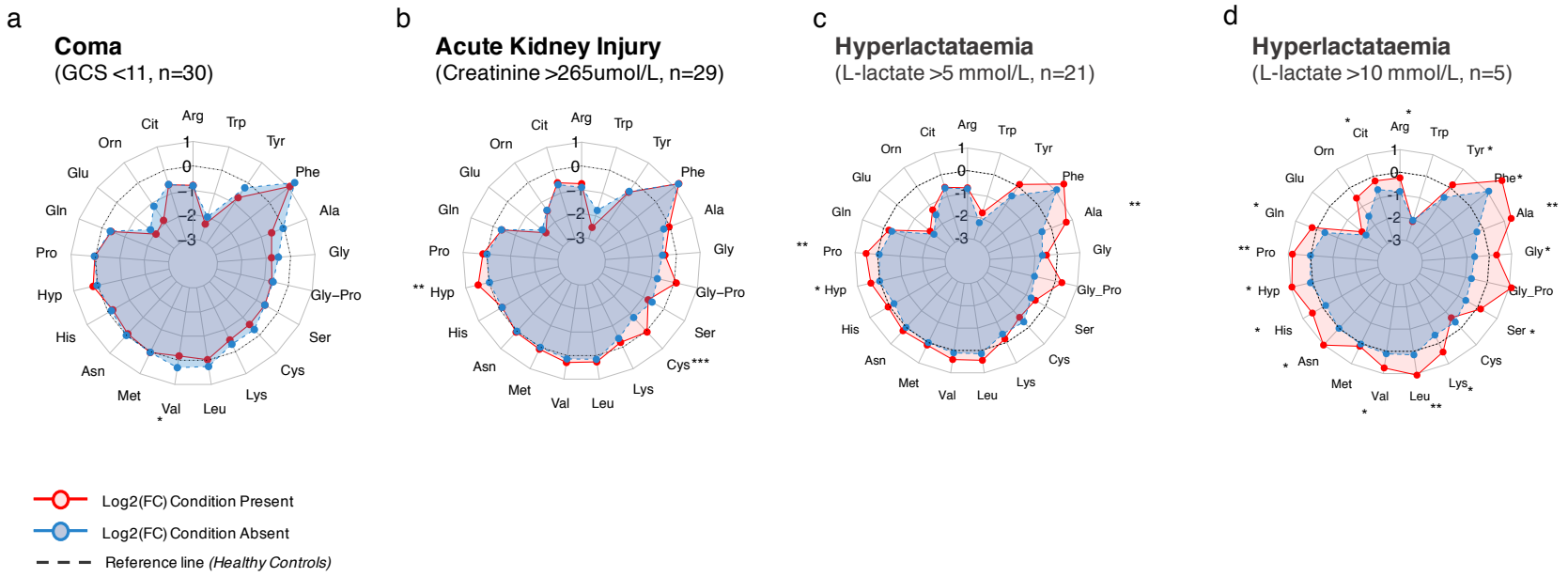


Fig. 5.3 **Admission amino acid profiles associated with major clinical syndromes in patients with severe falciparum malaria (n=88) from Bangladesh.** (**a**) Coma, as defined by a GCS <11. (**b**) Acute kidney injury, serum creatinine >265 μmol/L. (**c**) Hyperlactataemia, defined lactate >5mmol/L. (**d**) Hyperlactataemia, defined lactate >10mmol/L. The log2 normalised medium fold change of each amino acid was calculated compared to the normal levels measured in healthy controls and visualised in a radar plot.

5.6.5 Amino acid profiles associated with death

In patients with severe malaria, the amino acid profiles at study enrolment in patients who died compared to survivors generally showed an overall increase in the wide range of amino acids, Figure 5.4. The altered amino acid profiles observed in patients who died resembled the patterns observed in patients with hyperlactataemia. A total of 10 out of 21 measured amino acids were significantly elevated in patients who died from severe malaria compared to those who survived their infection. These changes included a significant increase in plasma alanine (BH adj. p = 5 x 10^{-4}), phenylalanine (BH adj. p = 5 x 10^{-4}), and proline (BH adj. P= 0.011). In contrast, among patients with sepsis there were no significant differences between the levels of plasma amino acids between patients who survived and those who died.

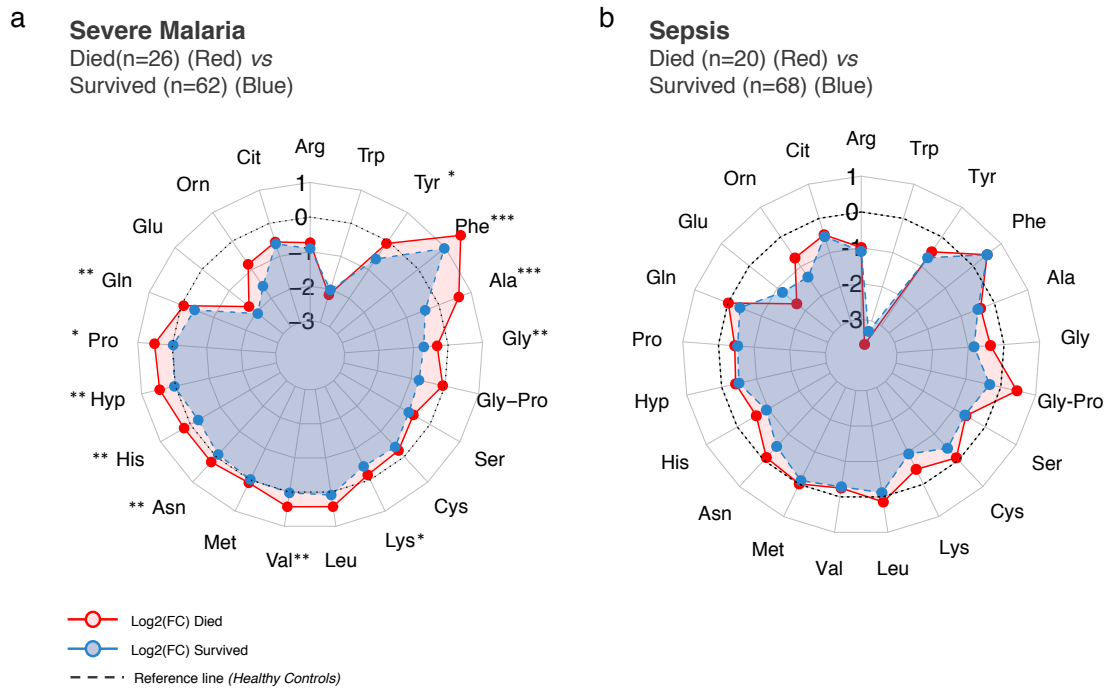


Fig. 5.4 **Admission amino acid profiles associated with mortality in patients with severe falciparum malaria and septicaemia.** (**a**) Amino acids were compared between patients with severe falciparum malaria who survived their infection and patients who died. (**b**) Patients with septicaemia. The log2 normalised medium fold change of each amino acid was calculated compared to the normal levels measured in healthy controls and visualised in a radar plot.

5.6.6 Determinants of the total PFAA concentration in severe falciparum malaria

We modelled the effects of plasma L-lactate on the total concentration of PFAA using multivariate regression analysis, adjusting for factors that may be related to an energy deficit or (acute) malnutrition, including duration of illness, coma depth (GCS), blood glucose levels (hypoglycaemia), and body mass index (BMI). We adjusted for the total parasite biomass by including log10 normalised plasma *Pf*HRP2. Renal elimination of amino acids was adjusted for by including serum creatinine.

Based on our multivariate linear regression model, and if our assumptions are correct, coma depth (GCS) and L-lactate are independent predictors of total PFAA, Table 5.3. We observed incremental levels of L-lactate were significantly associated with increased total concentrations of PFAA. Patients who were in a deeper coma were also more likely to have higher total PFAA levels.

5.7 Discussion

In this prospective observational cohort of patients with malaria and sepsis we investigated the patterns and determinants of plasma free amino acid derangements during severe infection. Using HPLC-MS/MS-MRM we quantified 21 free amino acids in the plasma of 295 study participants. All patient groups showed a general reduction in the total concentration of amino acids on enrolment compared to normal levels observed in local healthy controls. Patients with uncomplicated malaria showed the largest decrease of PFAA (-54%). We analysed the

Table 5.3 **Multivariate linear regression model to predict changes in the total concentration of plasma free amino acids (µmol/L) in patients with severe malaria (n=88) in Bangladesh.**

Variable	Estimate	SE	p-value	
BMI	68.8	62.3	0.273	
Duration of illness*	2.8	48.2	0.953	
Coma depth†	−125.3	55.9	0.028	*
*Pf*HRP2‡ (ng/dL)	−598.7	505.1	0.240	
Creatinine (µmol/L)	1.6	1.1	0.148	
Glucose (mmol/L)	61.8	58.3	0.293	
L-lactate (mmol/L)	342.3	72.6	1.3^{-5}	***

Regression coefficients (estimates) and standard errors (SE) are reported.
BMI = body mass index. PfHRP2 = *Plasmodium falciparum* Histidine Rich Protein 2.
*Duration of illness before enrolment in days.
†Depth of coma was assessed by the Glasgow Coma Scale (3-15).
‡ Log10 Normalized.

amino acid profiles in relation to clinical manifestations and outcome in patients with severe malaria. Although the total concentration of PFAAs was on average decreased compared to normal levels (on average -23%), hyperlactataemia was associated with widespread increase, likely because this reflects tissue hypoxia. We used a multivariate regression model to determine the relation between L-lactate and the plasma concentration of free amino acids in patients with severe falciparum malaria and observed that after adjusting for potential confounders, L-lactate remained a significant cause of an increased total concentration of PFAA.

The total plasma free amino acid concentration reflects a balance between uptake of exogenous amino acids, *de novo* amino acid production, protein catabolism, and extraction of amino acids by vital organs and peripheral tissues. Previous studies show increased hepatic extraction of a majority of plasma free amino acids during severe illness, such as during

sepsis, which manifests in a decreased total plasma amino acid concentration (Druml et al., 2001; Forli et al., 2000; Freund et al., 1979). It has been proposed that increased hepatic extraction contributes to the synthesis of acute-phase proteins (Druml et al., 2001).

Concentrations of PFAA were generally decreased in patients with severe malaria, but also in sepsis and uncomplicated malaria. Previously described individual amino acid derangements are in part explained by this, and are therefore less likely to be specific for certain presenting syndromes. In cases with malaria and sepsis, we observe amino acids are depleted from plasma, which is likely due to increased hepatic amino acid extraction from blood, as has been shown before in patients with inflammation and sepsis (Druml et al., 2001; Fischer et al., 1995; Sprung et al., 1991). The relationship between increased plasma L-lactate and raised PFAA may be due to increased protein catabolism during hypoxia, possibly aggravated by impaired liver blood flow with hepatic tissue dysoxia (Day et al., 2000a; Pukrittayakamee et al., 1994), leading to a reduction of hepatic PFAA extracted from plasma.

Our data are consistent with previous reports of L-arginine deficiency in patients with malaria, and we show this is already present in uncomplicated malaria. Previously, this deficiency was attributed to increased metabolism of L-arginine following intravascular nitric oxide depletion (Weinberg et al., 2008) and by arginases from host (Yeo et al., 2010) or parasite (Cobbold et al., 2016; Olszewski et al., 2009). It has recently been proposed that L-arginine deficiency might arise from low bioavailability of its precursors (Alkaitis et al., 2016). We measured two L-arginine precursors, L-ornithine and L-citrulline, and observed both were significantly depleted in uncomplicated malaria. Our findings support

the hypothesis that low-bioavailability of precursors contributes to L-arginine deficiency in malaria, early during the disease process, and probably because of an overall negative nitrogen balance.

We confirm previous findings of a correlation between L-lactate and plasma alanine in severe falciparum malaria (Pukrittayakamee et al., 2002). As tissue hypoxia develops, and anaerobic glycolysis causes L-lactate levels to rise, concomitant muscle protein catabolism increases nitrogen levels. Nitrogen is shuttled to the liver through the Cahill cycle in the form of alanine, where it is utilized in gluconeogenesis. However, high levels of L-lactate may reduce the hepatic disposal alanine and of other glucogenic amino acids (Marliss et al., 1972; Pukrittayakamee et al., 2002), which likely explains their strong correlation. In addition, impaired hepatic perfusion in patients with severe falciparum malaria may reduce hepatic disposal of plasma alanine (Day et al., 2000b).

We observe hyperphenylalaninaemia in adults with severe malaria and confirm previous work showing that patients with sepsis and uncomplicated malaria also have higher levels of phenylalanine (Enwonwu et al., 1999; Lopansri et al., 2006; Rubach et al., 2015). Previous studies have shown elevated hyperphenylalaninaemia in patients with uncomplicated malaria, and a further increase in patients with severe malaria with a potential relation to the development of coma (Enwonwu et al., 1999, 2000; Lopansri et al., 2006; Rubach et al., 2015). Hyperphenylalaninaemia has been attributed to reduced activity from phenylalanine-hydroxylase due to a deficiency of its co-factor tetrahydrobiopterin BH_4 (Enwonwu et al., 1999; Rubach et al., 2015; Yeo et al., 2015) which affects the metabolism of aromatic amino acids (Arg, Trp, Phe, Tyr) and the biosynthesis of neurotransmitters from the phenylala-

nine/tyrosine pathways. A limitation of the current study is that we did not measure BH_4 levels and therefore cannot assess whether changes in aromatic amino acids might have been related to a cofactor deficiency. Within patients with severe malaria, we did not observe a relationship between phenylalanine and coma depth (Spearman's rho = -0.17, p = 0.13), as shown previously in both adult and pediatric severe malaria (Lopansri et al., 2006). Although it is not completely clear what causes hyperphenylalaninaemia in severe falciparum malaria, it is not specific to malaria itself.

5.8 Conclusions

In conclusion, the concentrations of PFAA are decreased in patients with severe malaria, but also in sepsis and uncomplicated malaria. Previously described individual amino acid derangements are in part explained by this, and less likely specific for certain presenting syndromes. Hyperlactataemia increased the total amino acid concentration in patients with severe malaria, likely because this reflects tissue hypoxia.

Chapter 6

The role of the 16S rRNA gut microbiota composition in the pathogenesis of acidosis in severe falciparum malaria

6.1 Abstract

Background

In severe *Plasmodium falciparum* malaria, mature stage infected red blood cells sequester in the intestinal microvasculature and reduce gut barrier integrity, with subsequent cross-over of Gram-negative bacteria into the circulation. We hypothesised that low-molecular weight bacterial acids translocate into the bloodstream and are associated with changes in the gut microbiota composition.

Methods

A prospective observational study was conducted in a tertiary care hospital in Bangladesh, to determine markers of gut permeability and to profile the 16S rRNA composition of the gut microbiota in patients with falciparum malaria. Clinical and parasitological data were collected on admission, and patients were followed until discharge or in-hospital death. Plasma samples were collected for the measurement of soluble markers reflecting gut barrier integrity, including L-citrulline as a marker of enterocyte mass and D-lactate as a bacterial-derived organic acid. In addition, we collected faeces samples for the profiling of the gut microbiota composition by Illumina sequencing of the V4 region of the 16S rRNA gene in faecal DNA. Gut microbiota profiles were compare between study groups and the relationship with plasma D-lactate was assessed for genus levels changes of the gut microbiota.

Results

A total of 89 individuals was enrolled, including patients with severe falciparum malaria (n=29), uncomplicated falciparum malaria (n=23), and local healthy controls (n=34). Patients with malaria had significantly lower levels of plasma L-citrulline and significantly elevated plasma D-lactate, suggesting impaired intestinal barrier function. We observed changes in the gut microbiota on the phylum level consistent with severity of disease and in relation to parasite biomass. On the genus level the composition of the gut microbiota markedly changed in patients with severe malaria compared to healthy controls, characterised by a significant enrichment with potentially pathogenic species. The levels of D-lactate in plasma

correlated with coincidental changes in the abundance of (DL)-lactate producing species in the gut microbiota.

Discussion

Patients with severe falciparum malaria have signs of intestinal injury and increased gut permeability. For the first time the gut microbiota has been profiled in patients with severe malaria. We observed a pathogenic host-parasite-microbe interaction, highlighting the gut microbiota as a potential target for adjunctive therapies.

6.2 Background

Severe malaria remains a major cause of mortality in the tropical world. The World Health Organization estimated a total of 216 million cases of malaria worldwide in 2016, leading to approximately 445,000 deaths (WHO, 2017). The majority of malaria attributable deaths (99%) are caused by *P. falciparum*. Despite optimal antimalarial treatment, case fatality of severe malaria remains high, mainly depending on the number of organs affected by the disease (Dondorp et al., 2008b; von Seidlein et al., 2012). New adjunctive therapies are urgently needed to improve outcomes.

Malaria, caused by infection with *P. falciparum*, is characterised by a progressively expanding parasite biomass inside the bloodstream. The majority of the circulating parasitised red blood cells will sequester in the microcirculation of vital tissues and organs through cytoadhesion to the endothelial wall, precipitating diffuse microvascular obstruction and organ dysfunction (Leech et al., 1984; Luse and Miller, 1971). Autopsy reports show

preferential binding by parasitised red blood cells to the vascular endothelium in the brain, spleen, kidneys and intestines (Pongponratn et al., 1991, 2003; Seydel et al., 2006). Video microscopy has allowed direct visualisation of sequestration in the rectal microcirculation in patients with severe malaria (Dondorp et al., 2008b).

Mesenteric sequestration may contribute to the observed loss of gut barrier integrity in severe falciparum malaria (Church et al., 2016). This includes translocation of bacterial organisms, as shown by an increased risk of concomitant Gram-negative bacteraemia, mainly reported in children (Bassat et al., 2009; Berkley et al., 1999; Bronzan et al., 2007; Enwere et al., 1998; Scott et al., 2011; Takem et al., 2014; Were et al., 2011), but also in adults (Nyein et al., 2016). In addition, increased intestinal permeability for smaller compounds has been documented, including sugars (Molyneux et al., 1989a; Wilairatana et al., 1997). Increased plasma concentrations of endotoxins in severe malaria could have an intestinal origin (Olupot-Olupot and Maitland, 2013; Usawattanakul et al., 1985).

The pathogenic consequences of a compromised gut barrier might depend on the composition of the gut microbiota and their metabolic products. In Chapter 3 we observed raised levels of bacterial organic acids in the plasma of acidotic patients with severe falciparum malaria suggesting a role for the gut microbiota in the pathogenesis of severe disease. Previous studies have shown that changes in gut microbiota were associated with an increased risk of clinical falciparum malaria in African children and with disease severity in experimental mouse models (Taniguchi et al., 2015; Villarino et al., 2016; Yilmaz et al., 2014; Yooseph et al., 2015). The composition of gut microbiota has not been characterized in patients with severe falciparum malaria.

We postulated that patients with falciparum malaria have increased gut permeability and changes in the gut microbiota changes in relation to severity of disease and parasite biomass. We hypothesised that patients with malaria have decreased plasma concentrations of L-citrulline, a marker of enterocyte mass reflecting gut integrity, and raised plasma levels of D-lactate, a bacterial organic acid. In the presence of a leaky gut, the plasma D-lactate concentration is associated with the relative abundance of (DL)-lactate producing bacteria in the gut as measured based on the 16S rRNA gut microbiota composition. To investigate the role of this three-way interaction between host, parasite, and gut microbes, we conducted a prospective observational study in Bangladesh.

6.3 Hypothesis

This study tested the hypothesis that patients with falciparum malaria have lower L-citrulline and higher D-lactate than healthy controls and patients with uncomplicated malaria. We also hypothesised that plasma D-lactate is related to the abundance of (DL)-lactate producing species in the gut microbiota.

6.4 Objectives

The primary objective of this study was to describe the role of the 16S rRNA gut microbiota composition in the pathogenesis of metabolic acidosis in adult patients with severe falciparum malaria from Bangladesh. Secondary objectives were:

- To compare the concentration of plasma L-citrulline and D-lactate between patients with falciparum malaria and healthy controls.

- To determine the gut microbiota diversity and composition in patients with malaria compared to local healthy controls, by sequencing the V4 region of the 16S rRNA gene in DNA extracted from faecal samples.

- To determine the relationship between parasite biomass, quantified by *Pf*HRP2, and changes in the 16S rRNA gut microbiota composition.

- To describe changes in the 16S rRNA gut microbiota composition on the phylum and genus level.

- To determine the relationship between plasma D-lactate, measured enzymatically, and changes in the 16S rRNA gut microbiota composition.

6.5 Methods summary

Detailed methods are described in Chapter 2.

Ethics

This was a sub-study of an observational study protocol that was reviewed and approved by the local ethics review board, the Chittagong Medical College Ethics Committee, Bangladesh, and the Oxford Tropical Research Ethics Committee (OxTREC), in the United Kingdom. The trial was registered at *ClinicalTrials.gov* (Trial number: NCT02451904).

Study Design

A prospective observational case-control study in Chittagong Medical College Hospital, Bangladesh. The recruitment phase of the study spanned two wet-seasons and was from May-September 2015 and May-September 2016.

Study participants and procedures

Consecutive male or female adults (>12 years) with falciparum malaria were enrolled as well as a group of healthy controls. Patients were screened for malaria and were considered eligible if they had a blood slide positive for asexual blood stages of *P. falciparum* (including mixed infection with non-falciparum species). Severe and uncomplicated falciparum malaria were strictly defined according the WHO criteria for severe malaria (World Health Organization, 2015), modified by Hien et al. (Hien et al., 1996), as detailed in Section 2.1.4. Alongside patients with malaria we recruited local healthy controls. Participants enrolled in the healthy control group were required to have a blood slide negative for malaria, normal vital signs (no hypertension) and were excluded if they were pregnant, had a chronic illness, or took medication in the prior two months before enrolment.

All study participants gave informed consent before study enrolment. On enrolment, we took a medical history, a medication history, assessed vital signs, performed a physical examination and drew blood samples for venous blood gas analysis. All malaria cases were treated according to the World Health Organisation Guidelines for the Treatment of Malaria (World Health Organization, 2015) and antimalarial treatment regimens set out in Section 2.1, and followed them until hospital discharge or in-hospital death. Laboratory assessments

included haematology, which was done in a quality controlled external laboratory (Chevron Clinical Lab, Chittagong, Bangladesh). We used the portable iStat system (Abbott, Chicago, Illinois) for biochemistry (Chem8+ cartridge), venous blood gas, and L-lactate analysis (CG4+ cartridge).

Plasma markers of parasite biomass and intestinal integrity

Blood for the analysis of plasma markers of parasite biomass and gut integrity was drawn from a new catheter in the forearm, and was collected in lithium-heparin tubes. Samples were immediately centrifuged, and plasma was separated and flash-frozen in liquid-nitrogen. Samples were shipped on dry ice to the Mahidol-Oxford Tropical Medicine Research Unit. Plasma *Pf*HRP2 was quantified using a commercially available sandwich ELISA assay (Cellabs, Sydney, Australia), as described previously (Dondorp et al., 2005; Hendriksen et al., 2012) and in Section 2.2.3. Plasma D-lactate concentrations were determined using commercially available colorimetric ELISA assays (Sigma-Aldrich (MAK058)), Section 2.2.3. Plasma L-citrulline was quantified using HPLC-MS/MS-MRM as described in more detail in Section 2.2.2.

16S rRNA gut microbiota analysis

Stool samples for 16S sequencing were collected within the first 3 hours after study enrolment and immediately flash-frozen in liquid-nitrogen. During the first year of this sub-study we collected faeces samples but following a protocol amendment we switched to using rectal swabs (FLOQ swabs; COPAN, CA, USA) in the subsequent year for practical purposes.

Samples were shipped on dry ice to the Department of Microbiology at the University of Wageningen, the Netherlands.

We profiled the human gut microbiota composition of patients and healthy individuals by sequencing the V4 region of the 16S rRNA gene in DNA extracted from stool samples. In summary, I extracted DNA using a bead-beating method (Yu and Morrison, 2004) and a Maxwell 16 Tissue LEV Total DNA purification kit (Promega, Leiden, The Netherlands). A 2-step PCR strategy was used to generate barcoded amplicons from the V4 region of 16S rRNA genes (Caporaso et al., 2011). Purified amplicon pools were 250 bp paired-end sequenced using Illumina Miseq (sequenced commercially at GATC Biotech, Konstanz, Germany). Sequence processing was done by our collaborators at the University of Amsterdam, details can be found in Section2.2.4. Samples were de-multiplexed and trimmed (removing barcodes, primers and linker sequences. Amplicon sequence variants were inferred using Dada2 (v1.5.2)(Callahan et al., 2017) and aligned against the Silva database (v128) using Mothur (Schloss et al., 2009). Population diversity indices included Shannon, Chao1 and observed species, for which the Alpha diversity was calculated using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline (Caporaso et al., 2010b).

Statistical analysis

The baseline characteristics in the study groups are presented as median with inter quartile ranges (IQR), unless stated otherwise. Categorical data were reported as number and proportions. Statistical analysis was carried out using R software (v3.3.3) and Canoco software (v5.0). Multivariate statistics and redundancy analyses (RDA) were performed in Canoco

software (v5.0) on the relative abundances of the genus level. We assessed significant differences of the phylum and genus level between multiple study groups using Kruskal-Wallis tests. The Benjamini-Hochberg procedure was used to correct for multiple comparisons where appropriate. Correlations were done by calculating Spearman's correlation coefficient.

6.6 Results

6.6.1 Participants

A total of 86 participants were consecutively enrolled in this prospective sub-study in Chittagong Bangladesh, between 2015-2016. Among them were 29 patients with severe malaria, 23 patients with uncomplicated malaria, and 34 were healthy local controls. The overall case fatality rate of severe falciparum malaria was 17%. Baseline characteristics of the study population are described in Table 6.1. The majority of patients with malaria were young males, with a median duration of illness of one week, Table 6.1. Gastrointestinal symptoms before enrolment were frequently reported both by patients with severe malaria and uncomplicated malaria.

6.6.2 Plasma markers of parasite biomass and intestinal integrity.

Parasite load was quantified through the measurement of the plasma concentration of *P. falciparum* Histidine Rich Protein 2 (*Pf*HRP2) which reflects the total parasite biomass in the body (Dondorp et al., 2005). *Pf*HRP2 was significantly higher in patients with severe malaria compared to patients with uncomplicated malaria ($p<0.0001$), Figure **??**.

L-citrulline is a non-proteogenic amino acid that is produced in enterocytes (Chau et al., 2013; Piton and Capellier, 2016). Plasma L-citrulline levels reflect enterocyte mass, and can be used as a correlate of gut barrier integrity. A reduction of plasma L-citrulline has been previously described to correlate with intestinal injury (Gutierrez et al., 2014; Piton et al., 2011), increased intestinal permeability and endotoxaemia (Grimaldi et al., 2013). Here, plasma L-citrulline levels were significantly lower in patients with malaria ($p<0.0001$), indicating a reduction in enterocyte mass compared to healthy controls, Figure 6.1.

The cross-over of bacterial organic acids from the intestines into the blood circulation was measured by the quantification of plasma D-lactate in plasma. Plasma levels D-lactate were significantly higher in patients with severe malaria ($p<0.0001$), Figure **??**, indicating a transfer of bacterial products into the circulation of patients with severe malaria. We furthermore observed a positive correlation between plasma D-lactate and standard base deficit (Spearman's rho 0.42, $p = 0.038$) among patients with severe malaria.

Table 6.1 **Baseline characteristics in patients and healthy controls (n=86) enrolled in a prospective observational study on the 16S rRNA gut microbiota composition in Bangladesh.**

	Severe Malaria (n=29)		Uncomplicated Malaria (n=23)		Healthy Controls (n=34)		p value
Age (years)	26	(19 to 35)	27	(22 to 42)	29	(24 to 36)	0.741
Female sex	7	(24%)	6	(26%)	8	(24%)	0.759
BMI	21	(18 to 24)	23	(19 to 25)	23	(21 to 25)	0.166
Duration of illness (days)	7	(6 to 10)	7	(6.3 to 12)	0	-	0.643
Antibiotics before enrolment	13	(45%)	5	(23%)	1	(4%)	0.003
Mortality	5	(17%)	0	(0%)	0	(0%)	0.015
Abdominal symptoms before enrolment							
Nausea	19	(66%)	19	(86%)	0	-	0.094
Anorexia	20	(69%)	11	(50%)	0	-	0.174
Vomiting	17	(59%)	15	(68%)	0	-	0.489
Diarrhoea	10	(34%)	4	(18%)	0	-	0.201
Abdominal pain	14	(48%)	13	(59%)	0	-	0.448
Clinical examination							
Temperature (°C)	37.8	(36.9 to 38.8)	37.3	(37 to 38.4)	36.6	(36.2 to 36.8)	<0.001
Coma depth*	9	(8 to 15)	15	(15 to 15)	15	(15 to 15)	<0.001
Pulse rate (per minute)	108	(95 to 119)	98	(84 to 112)	81	(73 to 85)	<0.001
Respiratory rate (per minute)	34	(28 to 40)	27	(24 to 32)	18	(17 to 20)	<0.001
Mean arterial blood pressure (mm Hg)	97	(95 to 98)	97	(96 to 98)	98	(98 to 98)	0.004
Laboratory assessments							
Parasitaemia (parasites per µL)†	19972	(6535 to 61034)	3097	(836 to 11475)	0	-	0.007
*Pf*HPR2 (ng/dL)†	4723	(2933 to 7606)	733	(484 to 1109)	0	-	<0.001
Glucose (mmol/L)	6.7	(5.7 to 9.5)	6.1	(5.2 to 6.5)	5.8	(5.5 to 6.2)	0.044
Sodium (mmol/L	134	(132 to 139)	136	(133 to 141)	140	(139 to 141)	0.003
Potassium (mmol/L)	3.9	(3.5 to 4.2)	3.4	(3.3 to 3.6)	3.6	(3.4 to 3.8)	0.004
Chloride (mmol/L)	105	(100 to 109)	104	(100 to 106)	104	(102 to 106)	0.793
Creatinine (µmol/L)	115	(90 to 446)	77	(63 to 99)	73	(62 to 77)	<0.001
Blood urea nitrogen (mmol/L)	35	(25 to 72)	16	(11 to 23)	8	(6 to 10)	<0.001
Haemoglobin (g/L)	83	(63 to 103)	93	(78 to 136)	132	(116 to 148)	<0.001
White blood cells ($x10^3$/µL)	8.8	(6.2 to 11.5)	6.8	(5.7 to 9.8)	7.3	(6.3 to 8.6)	0.373
Platelets ($x10^3$/µL)	32	(17 to 58)	36	(34 to 111)	228	(191 to 301)	<0.001
pH	7.38	(7.36 to 7.45)	7.43	(7.41 to 7.46)	7.37	(7.35 to 7.39)	<0.001
pCO2 (mm Hg)	31	(28 to 35)	35	(32 to 37)	45	(41 to 52)	<0.001
HCO_3^- (mmol/L)	20	(16.3 to 21.6)	22.6	(21.7 to 24.7)	25.8	(24.7 to 28.3)	<0.001
Plasma SBE (mmol/L)	5	(2 to 9)	2	(-1 to 3)	-0.5	(-3 to 0)	<0.001
Anion gap (mmol/L)	17	(15 to 19)	16	(15 to 17)	16	(13.8 to 17)	0.117
L-lactate (mmol/L)	2.6	(1.8 to 3.6)	1.3	(1.1 to 1.8)	1.3	(1.1 to 1.6)	<0.001
Intestinal markers							
D-Lactate (mmol/L)	0.7	(0.5 to 0.9)	0.2	(0.1 to 0.3)	0.2	(0.1 to 0.3)	<0.001
L-Citrulline (µmol/L)	7.7	(7 to 8.3)	6.7	(6.3 to 7.2)	11.5	(10.3 to 13.6)	<0.001

Data are number (%) or median (IQR) unless otherwise indicated. BMI = body mass index. *Pf*HRP2 = Plasma *P. falciparum* Histidine Rich Protein 2. pCO2 = partial pressure of carbon dioxide. HCO_3^- = bicarbonate. SBE = standard base excess.

*Depth of coma was assessed by the Glasgow Coma Scale (3-15)

† geometric mean, 95% geometric confidence interval.

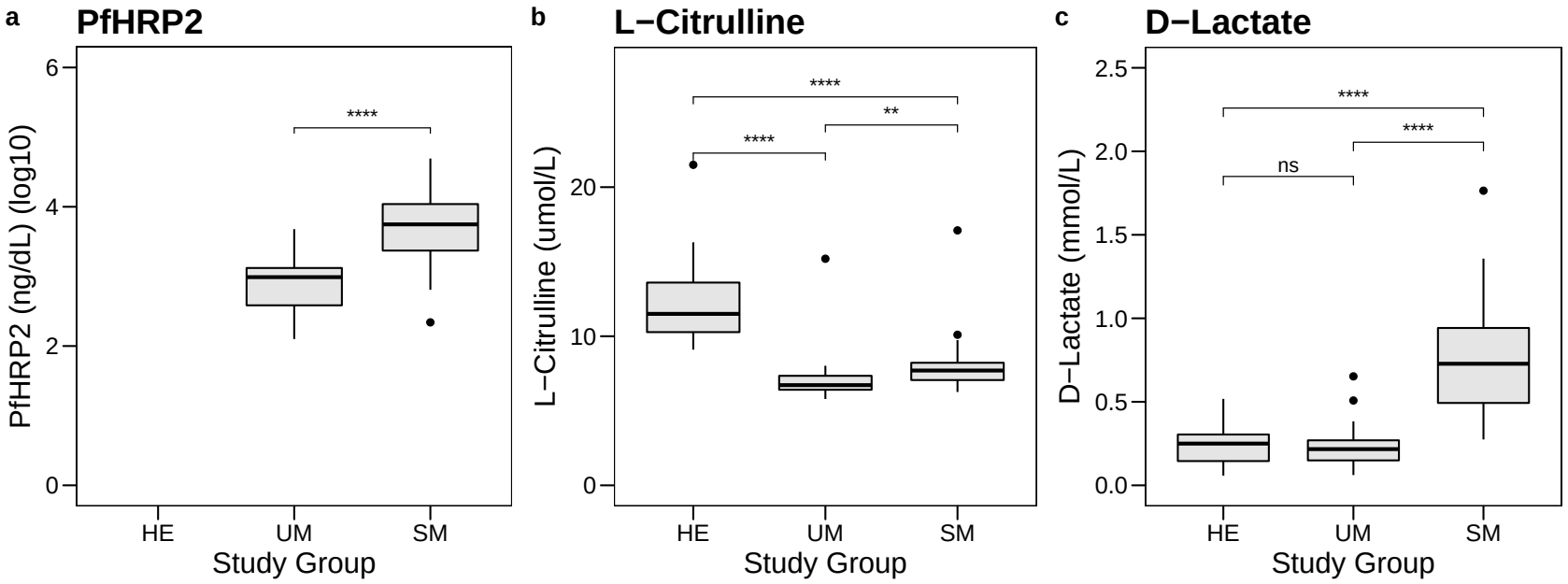


Fig. 6.1 **Plasma markers of parasite biomass and gut barrier function in patients in whom the 16S rRNA gut microbiota composition was profiled.** Study groups include healthy local controls (HE) (n=34), uncomplicated Malaria (UM) (n=23), and severe malaria (SM) (n=29). (**a**) *P. falciparum* Histidine Rich Protein 2 (PfHRP2) (ng/dL). (**b**) L-citrulline (µmol/L). (**c**) D-lactate (mmol/L). Significance tested for using Kruskal-Wallis tests (**: p<0.01, , ****: p 0.0001).

6.6.3 16S rRNA profiling of gut microbiota

Gut microbiota diversity

Population diversity of the 16S rRNA composition of gut microbiota was significantly reduced in patients with malaria compared to healthy controls ($p<0.001$) based on Shannon and Observed diversity indices, Figure 2. A multivariate redundancy analysis (RDA) was performed to explore predictors of change of the composition of the gut microbiota in all participants. In a visual depiction of the RDA we observed that the strongest predictor of a difference between the gut microbiota of individual participants was whether they were healthy or had malaria or, Figure 6.3 and Supplementary Figure 13. We did note an overlap between patients with severe and uncomplicated malaria.

We performed a partial RDA, where the type of sample, year of sampling and antibiotic exposure were taken along as co-variates, to see the effect of the other variables on the microbiota composition. Antibiotic exposure, type of sample and year of sampling together explained 10.4% of the variation (where type of sample explained most, 7.6%), and antibiotic exposure 1.8%, Supplementary Figure 13.

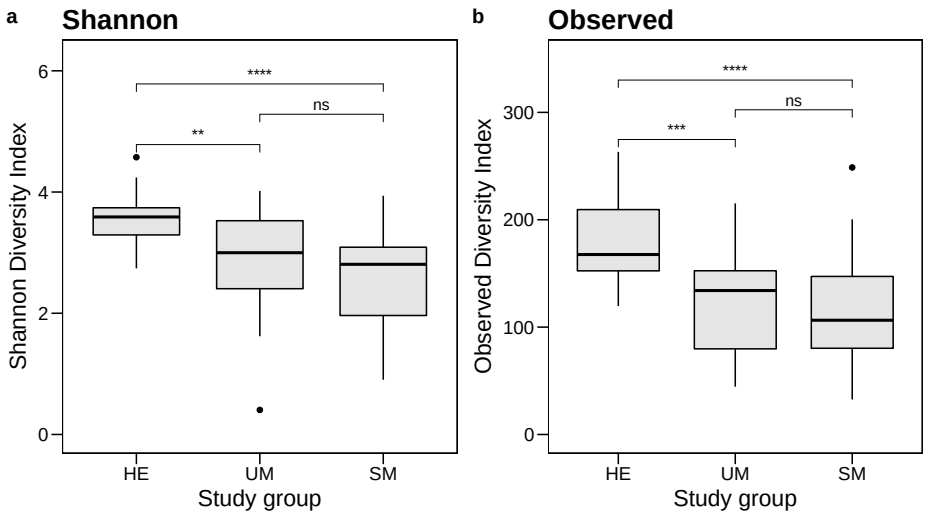


Fig. 6.2 **Population diversity of the 16S rRNA gut microbiota composition in the study groups.** (**a**) Shannon diversity index. (**b**) Observed species diversity index. Significance was tested for using Kruskal-Wallis tests (**: p<0.01, , ****: p 0.0001).

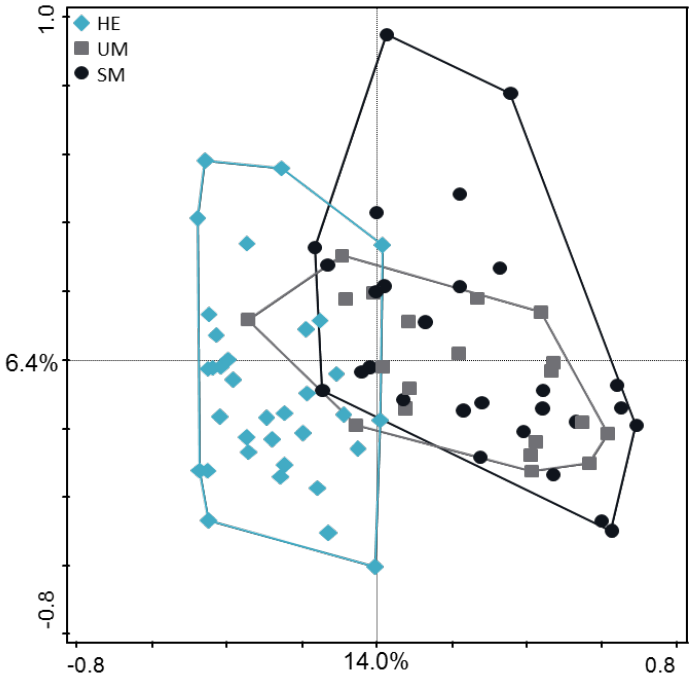


Fig. 6.3 **Partial redundancy analysis (RDA) of the 16S rRNA gut microbiota composition on the genus level.** This RDA included both clinical and laboratory variables as well as gut microbiota signatures. We explored predictors of microbiota composition based on variables included in Table1. The total variation explained by the partial redundancy model was 42%; patient groups separated from healthy individuals based on the composition of their gut microbiota, responsible for 10% variation, Supplementary Table 13.

Compositional changes on the phylum level are associated with disease severity

At the microbiota phylum level, the 16S rRNA gut microbiota composition changed according to severity of disease, Figure 6.4a. We observed that patients with malaria showed a significant reduction in *Firmicutes* compared to healthy controls (p=0.018), 6.4b. In contrast, in patients with malaria, the gut microbiota were enriched in *Proteobacteria* (p = 0.012). *Proteobacteria* correlated significantly with *Pf*HRP2 based estimates of parasite biomass (Spearman's rho 0.3, p=0.036), See Figure 6.4c. In addition, we observed non-significant increases of *Bacteroidetes* and *Fusobacteria* in patients with malaria.

Compositional changes on the genus level show enrichment for pathogenic species

At the microbiota genus level, major differences were observed between patients with malaria and healthy controls, See Figure 6.5. A gradual shift in composition according to disease severity was characterised by an inverse relationship between the most abundant genera composing the gut microbiota, with a large increase of *Bacteroides* species at the expense of *Prevotella* species. Importantly, potentially invasive bacterial species were more abundant in patients with severe malaria, including *Enterococcus* ($p < 0.0001$) and *Escherichia/Shigella* ($p < 0.0001$), See Figure 6.5. In patients with malaria, *Pf*HRP2 correlated significantly with microbiota enrichment of *Enterococcus spp.* (Spearman's rho 0.32, p = 0.022) and *Escherichia/Shigella spp.* (Spearman's rho 0.31, p = 0.029). Among patients with severe malaria, neither depth of coma nor survival were associated with changes in the composition of the gut microbiota.

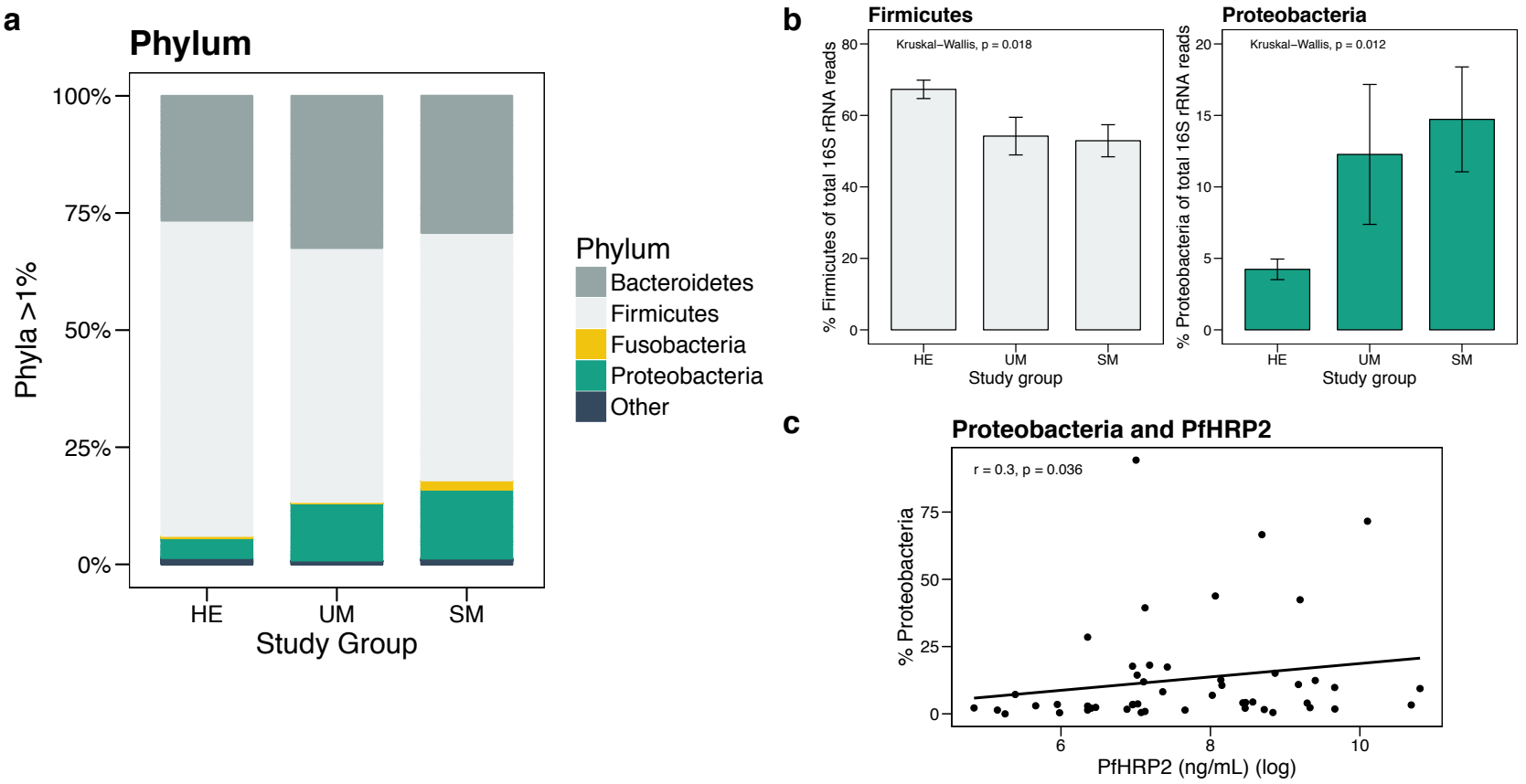


Fig. 6.4 **Relative abundance analysis of the 16S rRNA gut microbiota composition on the phylum level.** (**a**) Relative abundance of phyla representing >1% of the total gut microbiota composition (below 1% were combined as 'Other'). (**b**) Significant up and down regulated phyla in the study groups - significance testing was done by Kruskal-Wallis. (**c**) Relationship between *Pf*HRP2 parasite biomass and *Proteobacteria* using Spearman's rank correlation.

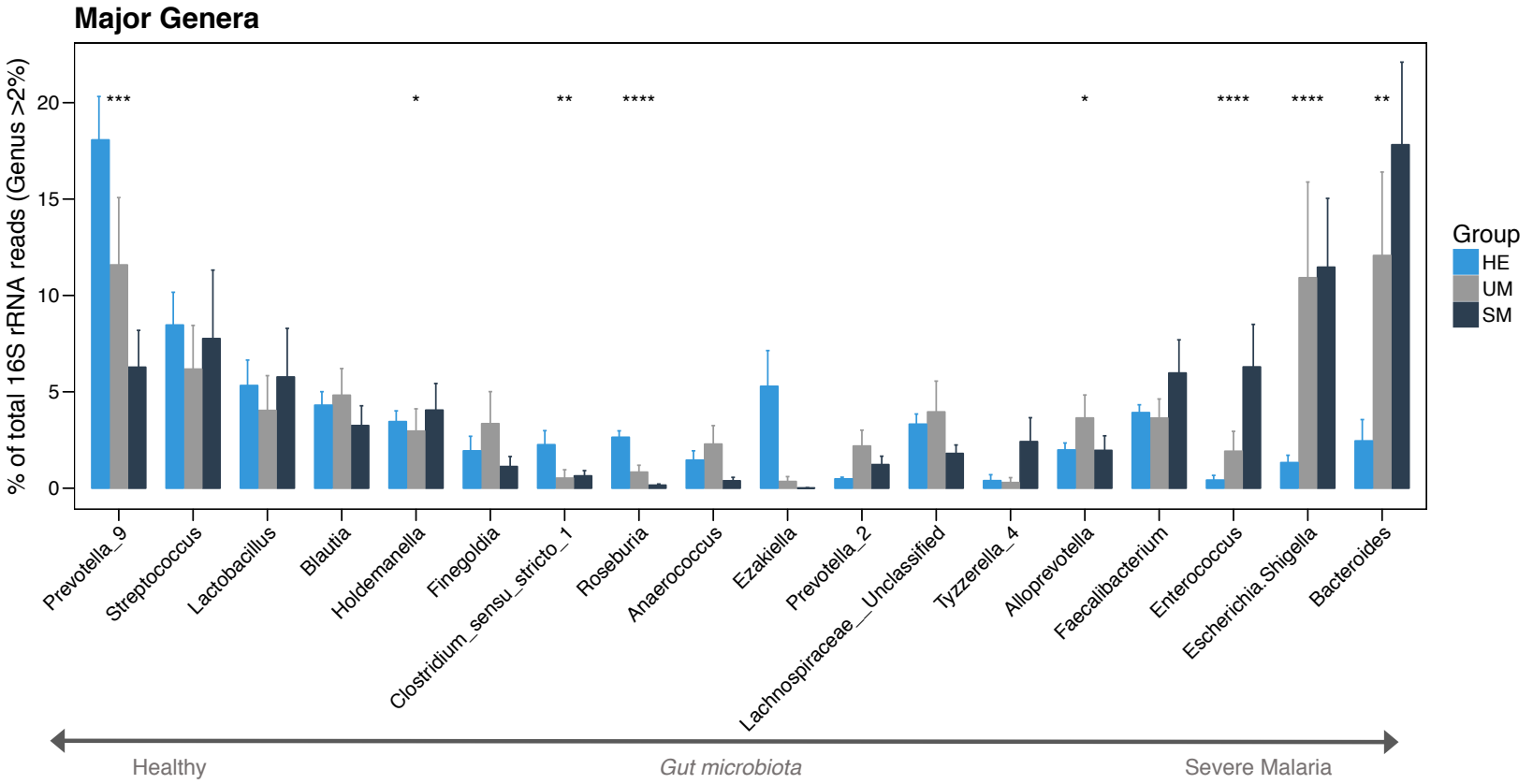


Fig. 6.5 **Major changes in the 16S rRNA gut microbiota composition on the genus level comparing healthy individuals and patients with malaria.** Clustered bar plots show the mean and standard error of the percentage of total 16S rRNA reads of the most abundant genera (contributing >2% to the gut microbiota). Significance tests using Kruskal-Wallis tests were adjusted by a Benjamini-Hochberg procedure: (*:p<0.05, **: p<0.01, ***: p<0.001, ****: p<0.0001).

Relation between plasma D-lactate and 16S rRNA gut microbiota composition

We investigated the relationship between the bacterial product D-lactate in the circulation and the constituent populations of gut microbiota in patients with severe malaria, Figure 6.6. Individuals with a high plasma D-lactate had a gut microbiota depleted for *Bacteroides* and enriched with (DL)-lactate producing species. Plasma D-lactate concentrations correlated positively with the relative abundance of *Prevotella* (Spearman's rho 0.47, p = 0.021), a negative significant association was found with the relative abundance of *Bacteroides* (Spearman's rho -0.41, p = 0.047).

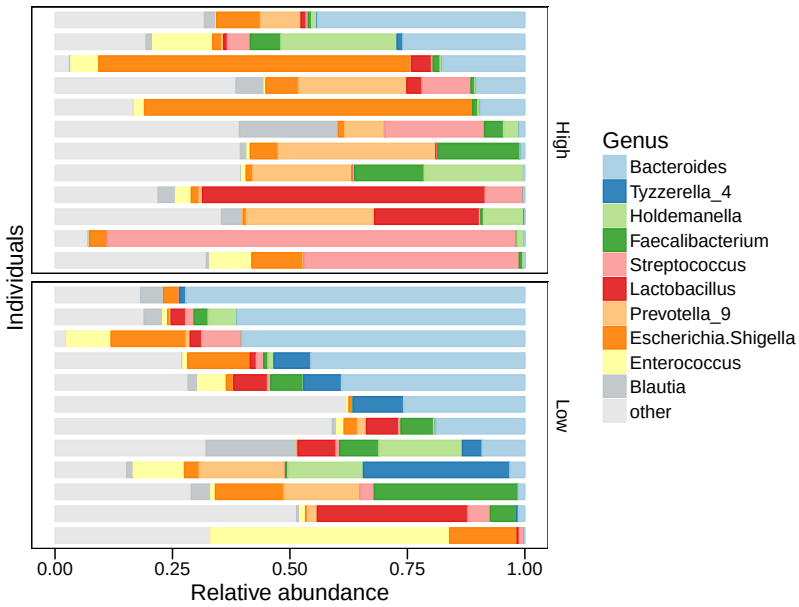


Fig. 6.6 **Changes in the gut microbiota composition visualised per individual patient with severe falciparum malaria.** Bar plots of the 16S rRNA gut microbiota composition on the genus level (>1% of the total abundance) by individuals with a high plasma D-lactate (>0.7 mmol/L) and a low plasma D-lactate ($\leq$0.7 mmol/L).

6.7 Discussion

We report for the first time the human gut microbiota profiles of patients with severe falciparum malaria by sequencing the V4 region of the 16S rRNA gene from faecal DNA. We found gut microbiota population diversity was reduced in patients with malaria compared to healthy local controls. We identified major shifts in the microbiota composition at both the phylum and genus level and identified that the gut microbiota of patients with severe malaria was enriched with potentially invasive bacterial species, including *Escherichia/Shigella spp.* and *Enterococcus spp*. We observed that patients with severe malaria had signs of a compromised intestinal barrier function and higher blood levels of D-lactate, indicating translocation of bacterial products into the blood circulation. Moreover, plasma levels of bacterial D-lactate correlated with (DL)-lactate producing species in the gut. These data show a pathogenic host-parasite-gut microbe interaction, highlighting the gut microbiota as a potential target for adjunctive therapies.

Malaria infection is known to predispose for life-threatening bacteraemia. Evidence for an increased incidence of bacteraemia in patients with malaria comes from Mendelian randomisation studies in Kenyan children. Scott et al. found a significant protective association between having a sickle haemoglobin phenotype (HbAS) and bacteraemia (Scott et al., 2011), indicating sickle cell (HbAS) protects against bacteraemia because it protects against severe malaria. Furthermore, these studies showed the protective effect of HbAS for (Gram-negative) sepsis disappears as malaria incidence goes down.

By which mechanisms does malaria infection predispose individuals to bacteraemia? The majority of pathogens that cause bacterial co-infection in patients with malaria are Gram-

negative enteric organisms, including non-typhoidal salmonella and *Escherichia coli* species (Chau et al., 2013; Nyein et al., 2016; Scott et al., 2011), which are probably translocated from the gut. The translocation of Gram-negative enteric micro-organisms likely relates to increased intestinal permeability caused by the malaria infection itself. Parasitised red blood cells sequester in the mesenteric microcirculation (Pongponratn et al., 1991, 2003; Seydel et al., 2006; Taniguchi et al., 2015) and lead to microcirculatory blood flow abnormalities, which have been directly visualized *in vivo* in the rectal microcirculation of patients with severe malaria (Dondorp et al., 2004a).

Mesenteric sequestration by parasitised red blood cells may cause intestinal injury in patients with severe falciparum malaria (Church et al., 2016). Diffuse gut ischaemia may cause desintegration of enterocyte tight junctions, thereby making the gut more permeable for bacterial products and whole bacteria. Recent studies in mice showed that intestinal permeability in malaria may be related to parasite induced mastocytosis with local release of histamines, which damages enterocyte tight junctions (Chau et al., 2013; Potts et al., 2016). It has been suggested that L-arginine deficiency predisposes for this mast-cell associated damage (Chau et al., 2013). Oral supplementation of L-arginine or L-citrulline may improved gut barrier function in mice (Chau et al., 2013), whether this is the case in humans remains to be studied (Alkaitis et al., 2016).

The concentration of L-citrulline was already significantly reduced in patients with uncomplicated and severe malaria, however, only in patients with severe malaria a significant increase was observed in the levels of D-lactate increase. Possibly, dense parasite seques-

tration associated with severe malaria was required for translocation of D-lactate, or other currently unmeasured factors.

In this study, we were unable to assess the incidence of concomitant bacteraemia and/or sepsis because of an absence of quality controlled microbiology laboratories at the study site. We studied translocation by measuring gut derived D-lactate, and found increased levels in severe malaria. In the patients with high D-lactate, acidosis may have been compounded even further by the simultaneous translocation of other low-molecular weight acids such as short-chain fatty acids and phenyllactates not assessed in this study. The levo-rotatory isomer of lactate (L-lactate) is produced by human cells and is commonly elevated in patients with severe malaria due to anaerobic glycolysis, and is the main acid contributing to acidosis. While the dextro-rotatory isomer of lactate (D-lactate) is a glycolytic end-product produced by bacterial populations existing in the colon and its contribution to acidosis was previously unknown. Potentially, D-lactate might have been overestimated due to background concentrations of LDH (Martí et al., 1997). Possibly, a part of the measured D-lactate could also have been produced by *P. falciparum* parasites, as approximately 6-7% of all (DL)-lactate produced by the parasite is dextro-rotatory(Vander Jagt et al., 1990).

6.8 Conclusions

In this cohort of patients with severe falciparum malaria, uncomplicated malaria and healthy controls we characterised soluble markers of gut permeability and profile the composition of the gut microbiota by sequencing the 16S rRNA gene in faecal DNA. Patients with

severe falciparum malaria show signs of reduced enterocyte mass, based on lowered plasma L-citrulline, and increased gut permeability, based on increased plasma D-lactate. The composition of the gut microbiota of patients with severe malaria is enriched with potentially pathogenic species. Our data suggest there might be a relation between plasma D-lactate and enrichment of (DL)-lactate producing species in the intestines of patients with severe malaria.

Chapter 7

Summary

7.1 Discussion

Below follows a discussion of the main study findings, limitations, and potential implications of the current work.

Summary of findings

The current work characterises the components of metabolic acidosis in severe falciparum malaria. A prospective observational study was conducted to characterise the non-lactic acid load in patients with severe malaria from Bangladesh. Metabolomic characterisation using high-resolution mass spectrometry indicated that patients with acidosis have elevated plasma levels of acids from a potential microbial origin, and a dysregulated amino acid metabolism. Clearance of newly identified microbial acids was delayed in fatal cases. Patients with severe

malaria had signs of reduced gut barrier integrity, suggesting the source of newly identified microbial candidate acids could be bacterial.

The source of these microbial acids was further investigated *in vitro*. Temporal metabolite changes in the culture media of synchronised *P. falciparum* 3D7 strain parasites were characterised using the same analytical mass spectrometry platform. We confirmed the release of lactate and the depletion of L-arginine from the culture media, as described previously. We show that, except for lactate, there is no evidence for an increased release of metabolic acids associated with acidosis in the media of parasitised cultures compared to non-parasitised red cell controls.

The relationship between acidosis and amino acid dysregulation was further studied through simultaneous quantification of plasma free amino acids in plasma in a cohort of patients with severe malaria, uncomplicated malaria, sepsis, and healthy controls. We confirmed previously described individual amino acid derangements and showed that they are part of widespread amino acid deficiencies in uncomplicated and severe falciparum malaria, and thus not specific for the previously described amino acid depletions. We note that hyperlactataemia increases the total amino acid concentration in plasma in patients with severe malaria, which likely reflects the effects of tissue hypoxia on amino acid metabolism, and may be aggravated by impaired hepatic blood flow with reduced hepatic extraction of L-lactate and PFAA from plasma.

Finally, in an independent cohort of patients we characterised soluble markers of intestinal permeability and profiled the composition of the gut microbiota by sequencing the 16S rRNA gene in faecal DNA. Patients with severe falciparum malaria showed markers of reduced

intestinal integrity, based on reduced plasma L-citrulline, and intestinal gut permeability, based on increased plasma bacterial D-lactate. The composition of the gut microbiota of patients with severe malaria was enriched with potentially pathogenic species. We concluded there may be a relation between plasma bacterial D-lactate and enrichment of (DL)-lactate producing bacterial species in the intestines of patients with severe malaria.

These findings suggest that acidosis in severe falciparum malaria may be related to a compromise in intestinal barrier function and transfer of intestinal products into the blood circulation. Further studies are now warranted to determine if increased gut permeability also plays a role in the development of acidosis in children, which molecular mechanisms contribute to increased gut permeability, and if outcomes can be improved by adjunctive therapies aiming to improve gut barrier function of change the composition of the gut microbiota.

Limitations

The current work has several limitations. It was not possible to assess the incidence of concomitant bacteraemia because there were no quality controlled microbiology facilities at the study site. Although previous studies have been able to set-up dedicated microbiology labs at the study site for the duration of clinical studies (Maude et al., 2016), this was not within the scope or capacity of the current studies. More efforts are under way to improve the blood culture facilities in Chittagong in a collaboration with the Bangladesh Institute for Tropical Infectious Diseases and to build local capacity over time. Therefore, it was difficult to distinguish whether microbial acids transfer from the intestines into blood, or

if they might have been associated with bacteria circulating in the bloodstream. In either case, the presence of these bacterial acids indicated increased intestinal permeability with translocation of pathogenic products from the intestinal tract.

It might be argued that the candidate acids described in Chapter 3 are largely related to acute kidney injury. Some of these metabolites are uremic toxins, such as indoxyl-sulfate, or related to renal failure, such as quinolinic acid and hippuric acid. The candidate microbial acids might also be normally present in blood at undetectable levels and accumulate during renal failure, thus appearing as a uremic toxin. However, we observed that these microbial metabolites are also elevated in patients with a normal renal function, for example the levels of DAP were already increased in patients with uncomplicated malaria. Metabolic acidosis and acute kidney injury are intertwined conditions. In severe malaria, accumulation of organic acids is likely aggravated by impaired clearance due to acute kidney injury.

UHPLC-Orbitrap-MS is semi-quantitative and has limited capability to distinguish between molecular isomers. Although these methods are useful for detection of small molecules, there is only a partial role in their quantification. We did assess the relation between UHPLC-Orbitrap-MS methods compared to bedside L-lactate measured using the iStat system and found a strong correlation, Supplementary Figure 5. We considered the possibility to design new LCMS methods to quantify each of the suspected microbial acids individually and to separate the isomers using chiral LC columns, however, these steps are time consuming and expensive.

As a practical solution, I directly measured the concentration of D-lactate in plasma using commercially available enzymatic assays. These methods allow better reproducibility of our

results as they does not depend on bespoke LCMS. D-lactate is a low-molecular weight acid that is representative for other bacterial metabolic acids that fall within the same molecular weight category. We found that D-lactate levels in patients with severe malaria ranged between 0.5 and 1.5 mmol/L. Therefore, microbial acids are likely to make an important quantitative contribution to acidosis in severe malaria. However, these findings may require confirmation in a larger population of patients with severe falciparum malaria, preferably in other ages and at different study sites.

The use of L-citrulline as a marker of functional enterocyte mass might be limited in the presence of other amino acid deficiencies. Although plasma L-citrulline is expected to represent functional enterocyte mass, we also note in the amino acid analysis in Chapter 5 that L-citrulline deficiency occurs in the context of an overall negative nitrogen balance. Low plasma L-citrulline should be interpreted with caution as there may be other causes of this deficiency, such as low precursor bioavailability (proline and glutamine).

I have attempted to determine additional markers of gut permeability, however, some of these efforts were unsuccessful. A commercially available I-FABP assay was done but the results were considered unreliable because many cases were below the limit of detection and the results had high coefficients of variation, I therefore decided not to include these results in this thesis. Furthermore, 16S rRNA was quantified in plasma as a marker of intestinal translocation using qPCR in a total of 80 EDTA plasma samples, as described previously (Kramski et al., 2011). Although some of the cases with sepsis had higher levels of 16S rRNA in plasma, we could not reach an adequately low limit of detection to infer conclusions on the quantity of 16S rRNA in plasma. Although this method has important potential to

quantify intestinal translocation, I decided not to include these preliminary results in this thesis.

The metabolomic signatures described here are based on measurements from peripheral blood samples and from parasite clones of *Pf*3D7, studied under standard culture conditions. Although these methods are relevant for investigating the role of unmeasured metabolites in the systemic circulation, the findings do not necessarily reflect what happens in the micro-environment of sequestered tissue. Understanding the localised metabolic changes induced by parasite sequestration on the tissue-level is challenging but could be highly informative. Re-creating the micro-environment of a sequestered blood vessel in an experimental model has not been convincingly done before. Animal models do not always reflect the pathophysiology as shown in humans, as complications do not occur similarly (White et al., 2010) .

The current methods could have been expanded by including additional parasite lines other that *Pf*3D7, to provide an opportunity to interrogate intra-strain variability in metabolism in relation to cellular adhesion. For example, *P. falciparum* phenotypes associated with endothelial adhesion, such as clones ITO4 or A4, may have allowed an experimental design to investigate potential changes in the local production of organic acids by parasites that have undergone cytoadhesion. In addition, culture conditions could have been changed to mimic the potential micro-environment of a sequestered parasite, for example by inducing relative dysoxia, increasing pCO_2, and depleting glucose, in a slightly acidic culture medium. Although the exact microenvironmental changes at the tissue-level related to sequestration *in vivo* are in fact not completely clear, modelling the expected conditions *in vitro* may have

revealed a metabolic signature of *P. falciparum* reflective of its adaptation to cytoadhesion and sequestration.

Alternatively, localised effects of tissue sequestration might be studied *in vivo* through continuous micro-dialysis of skeletal muscle tissue with high-density sampling of unbound analytes in the extracellular fluid, as has been done before in sepsis (Levy et al., 2005). Although insertion of micro-dialysis catheters may provide logistical challenges this could reveal a tissue-gradient of lactate and pyruvate compared to peripheral blood samples, and could provide an indication of other locally accumulating, and potentially toxic, metabolites.

Other alternatives to study the localised effects of tissue sequestration might include imaging modalities such as magnetic resonance spectroscopy (MR-S), functional magnetic resonance imaging (fMRI), or positron-emision tomography (PET). However, these imaging modalities also pose major logistical challenges as they require high-resource facilities and equipment, which are currently and to my knowledge not available at any of the field sites around the world where there is regular access to patients with severe malaria (Although there might be access to MRI facilities in Rourkela India, Chittagong, Bangladesh and Blantyre, Malawi, these specific imaging protocols might not be available at these sites). In addition, these scans require patients to be immobilised for an extended period of time , necessitating on-site access to oxygen and intensive care support for cases with severe malaria. Furthermore, pilot studies would need to be carried out first to determine the optimal scanning sequences, to select which metabolites to measure (in case of MR-S), and to determine optimal scanning zones (if single voxel proton spectroscopy is applied). A solution

may be to apply these imaging modalities using animal models (Kawai and Sugiyama, 2010); only if these infection models are also characterised by tissue sequestration.

Potential implications

These studies identify the gut as a potential source of non-lactate acidosis in severe falciparum malaria, and implicate a role for the composition of the gut microbiota in the development of severe malaria. Further studies are now warranted to confirm these findings in larger patient cohorts and in children with severe malaria. If indeed it is consistently found that bacterial acids contribute to acidosis, then this could have potential clinical implications. Plasma bacterial acids in patients with severe falciparum may be predictive of concomitant bacterial sepsis, which associated with high case fatality in severe malaria. Novel adjunctive therapies might be considered with the aim to improve intestinal barrier function or to change the composition of the gut microbiota in order to reduce the impact of acidosis and concomitant enteric bacterial sepsis. These strategies may potentially benefit patients by improving the treatment of acidosis and concomitant sepsis, which could ultimately contribute to a reduction in mortality from severe falciparum malaria.

7.2 Future work

Future studies are now warranted and should aim to answer the following questions:

1. Do children children with severe falciparum malaria have increased plasma levels of bacteria acids, and do these markers predict concomitant bacteraemia?

2. What are the underlying molecular mechanisms responsible for increased intestinal permeability in severe falciparum malaria?

3. Is there a role for increased intestinal permeability in the pathogenesis of acidosis in non-malarial critically ill patients admitted to general intensive care units?

4. Can outcomes in patients with severe falciparum malaria be improved by adjunctive treatments aimed to increase gut barrier function or reduce intestinal bacterial load?

Laboratory models of infection or post-mortem patient studies can be helpful to further study the structural changes in the intestinal wall during infection with *Plasmodium falciparum*.

Clinical observational studies could determine if children with severe falciparum malaria and non-malarial critically ill patients are also affected by translocation of bacterial content in the presence of increased intestinal permeability.

Randomized placebo controlled trials could provide evidence for the efficacy and safety of selective intestinal decontamination (SID) in severe malaria. Such an RCT could include multiple treatment arms: 1) SID; 2) SID + pro-biotic; 3) L-citrulline; and 4) a control arm. Outcome measures in such an RCT should include acidosis clearance rates, incidence of bacteraemia, and mortality. Findings could be supported by the measurement of blood markers of intestinal translocation, such as L-citrulline, sugar probes, plasma 16S rRNA, gut bacterial acids, IFABP, and endotoxins; with longitudinal sampling of the gut microbiota.

7.3 Publications and presentations

Publications

Stije J Leopold, Aniruddha Ghose, Erik L Allman, Hugh W F Kingston, Amir Hossain, Asok Kumar Dutta, Katherine Plewes, Kesinee Chotivanich, Nicholas P J Day, Joel Tarning, Markus Winterberg, Nicholas J White, Manuel Llinás, Arjen M Dondorp, Identifying the Components of Acidosis in Patients With Severe Plasmodium falciparum Malaria Using Metabolomics, The Journal of Infectious Diseases, 19 december 2018, jiy727.

Stije J. Leopold, Siribha Apinan, Aniruddha Ghose, Hugh W. Kingston, Katherine Plewes, Amir Hossain, Asok Kumar Dutta, Anupam Barua, Sujat Paul, Joel Tarning, Nicholas P.J. Day, Markus Winterberg, Nicholas J. White, and Arjen M. Dondorp. Amino acid derangements in severe falciparum malaria. [*Submitted*].

Stije J. Leopold, Aniruddha Ghose, Floor Hugenholtz, Hugh W.F. Kingston, Katherine Plewes, Amir Hossain, Ashok Kumar Dutta, Joel Tarning, Markus Winterberg, Nicholas P.J. Day, Nicholas J. White, Willem M. de Vos, Arjen M. Dondorp, W. Joost Wiersinga. Gut microbiota composition is associated with severity of disease in patients with *P. falciparum* malaria. [*Submitted*].

Presentations and posters

Oral

Stije J. Leopold. The role of the human gut microbiome in severe malaria acidosis, Symposium on the pathophysiology and treatment of severe malaria, 67th Annual Meeting of the American Society of Tropical Medicine and Hygiene, USA, 2018.

Stije J. Leopold. Contributors to metabolic acidosis in falciparum malaria, First Malaria World Congress, Melbourne, Australia, 2018.

Stije J. Leopold. Acidosis in severe malaria, Joint International Tropical Medicine Meeting, Bangkok, Thailand, 2016.

Posters

Leopold et al. Identifying the Components of Severe Malaria Acidosis by Metabolomics, 66th Annual Meeting of the American Society of Tropical Medicine and Hygiene, USA, 2017.

Leopold et al.Pulmonary manifestations of malaria and sepsis detected by point-of-care lung ultrasound, 66th Annual Meeting of the American Society of Tropical Medicine and Hygiene, USA 2017.

Leopold et al. Gut microbiome and disease severity in malaria, Radboud symposium on Microbiome, the Netherlands, 2017.

Leopold et al. Lactic acidosis in severe malaria may be aggravated by *P. falciparum*-derived lactate, 64th Annual Meeting of the American Society of Tropical Medicine and Hygiene, USA 2015.

Leopold et al. The role of previously unmeasured organic acids in the pathogenesis of severe malaria, 63rd Annual Meeting of the American Society of Tropical Medicine and Hygiene, USA, 2014.

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Lewis Thomas, on interstellar communication

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Appendix A: Supplementary data

Supplementary data

Fig. S1. **Metabolomics data analysis and variable selection pipeline.**

Fig. S2. **Base peak chromatograms of a metabolomic analysis (UHPLC-Orbitrap-MS) of plasma collected from participants (n=152) from Bangladesh**. (**a**) Solid phase extraction (SPE). (**b**) Methanol (MeOH) extraction.

Fig. S3. **Base peak chromatograms of a metabolomic analysis of *Plasmodium falciparum* 3D7 strain culture media** . Samples were extracted using a methanol (MeOH) extraction technique and analysed by UHPLC-Orbitrap-MS.

Fig. S4. **Example of QC robust LOESS signal correction (QC-RLSC) of a plasma metabolite measured through UHPLC-Orbitrap-MS**. (**a**) Before QC-RLSC correction. (**b**) After QC-RLSC correction.

Fig. S5. **Correlation between L-lactate measured using the iStat system and by UHPLC-Orbitrap-MS.** L-lactate measurements at the study site were performed <15minutes after blood

collection using a $CG4^{+}$ cartridge on an iStat blood gas analyser (Abbott, Chicago, Illinois).

Fig. S6. **Acid-base measures in patients with uncomplicated malaria, severe malaria, and healthy controls from Bangladesh.** (**a**) standard base deficit corrected for albumin and phosphate. (**b**) pH (**c**) Strong ion gap (SIG). (**d**) L-lactate.

Fig. S7. **Correlation of acid-base measures in patients with severe falciparum malaria (n=60) from Bangladesh.** The upper panel shows correlation with standard base deficit corrected for albumin and phosphate (SBDc): (**a**) Anion gap. (**b**) Strong ion gap (SIG). (**c**) Creatinine. The lower panel shows the correlations with creatinine: (**d**) Anion gap. (**e**) Strong ion gap. (**f**) L-lactate. Spearman's correlation coefficients are shown.

Fig. S8. **Partitioning of standard base deficit in patients with severe falciparum malaria (n=60) from Bangladesh.** The attributable fractions of L-lactate are shown. (**a**) Patients with acute kidney injury (n=2, n=12, n=15). (**b**) Patients without acute kidney injury (n=7, n=16, n =8).

Fig. S9. **Structure, chemical formula, and average molecular weight (MW) of suspected microbial acids detected in plasma of acidotic patients with severe falciparum malaria from Bangladesh.** DAP = diaminopimelic acid. (Chemical data and structures © 2018 HMDB, V4.0).

Fig. S10. **Clearance kinetics of suspected microbial markers associated with acidosis.** Study hours are displayed on the x-axis. Means and standard errors of the mean are reported of the ion intensity of candidate acids. Serial sampling was done in patients with severe malaria (n=60) at 0,

24 and 48 hours and temporal dynamics of candidate compounds were compared between survivors (n=40, black lines) and patients who died (n=20, red lines).

Fig. S11. **Temporal fluctuations of metabolites measured in the culture media of highly synchronised *Plasmodium falciparum* 3D7 strain parasites**. The x-axis states the hours post infection at which sampling was done. Metabolite concentrations measured in the media of highly synchronised parasitised red blood cells (pRBC) (*Plasmodium falciparum* 3D7 strain, 3% parasitaemia, 2% haematocrit) were compared to the media from non-parasitised red blood cells (uRBC) (same blood donor, 2% haematocrit) and normalised (log2(pRBC/uRBC)); means and standard deviation are shown.

Fig. S12. **Free amino acid concentrations measured by UHPLC-MS/MS-MRM in plasma of study participants (n=295) from Bangladesh**. Boxplots with medians and inter quartile ranges are shown.

Fig. S12 (Continued). **Free amino acid concentrations measured by UHPLC-MS/MS-MRM in plasma of study participants (n=295) from Bangladesh (Continued).** Boxplots with medians and inter quartile ranges are shown.

Fig. S13. **Redundancy analysis of the 16S rRNA composition of gut microbiota in the study groups.** Patients with severe malaria (n=29) (SM), uncomplicated malaria (n=23), and healthy local volunteers (n=34). (**a**) Clinical and laboratory variables included in multivariate RDA analysis. (**b**) Major components predicting a change in the composition of the 16S rRNA gut microbiota in patients; patients separate by study group. (**c**) Analysis of the potential effects of antibiotic exposure and year

of sampling on the microbiota composition. (**d**) Analysis of the potential effects of sampling method.

Table S1. **Metabolites detected in a metabolomic analysis (UHPLC-Orbitrap-MS) of plasma collected at study enrolment in study participants (n=152) from Bangladesh.**

Table S1 (Continued). **Metabolites detected in a metabolomic analysis (UHPLC-Orbitrap-MS) of plasma collected at study enrolment in study participants (n=152) from Bangladesh (Continued).**

Table S2. **Differential abundance analysis of metabolites detected in plasma from patients with uncomplicated malaria (n=47), severe malaria (n=60), and healthy controls (n=45) in a metabolomic analysis (UHPLC-Orbitrap-MS) of plasma.**

Table S2 (Continued). **Differential abundance analysis of metabolites detected in plasma from patients with uncomplicated malaria (n=47), severe malaria (n=60), and healthy controls (n=45) in a metabolomic analysis (UHPLC-Orbitrap-MS) of plasma (Continued).**

Table S3. **Plasma γ-aminobutyric acid (GABA) measured in patients (n=10) from Bangladesh.**

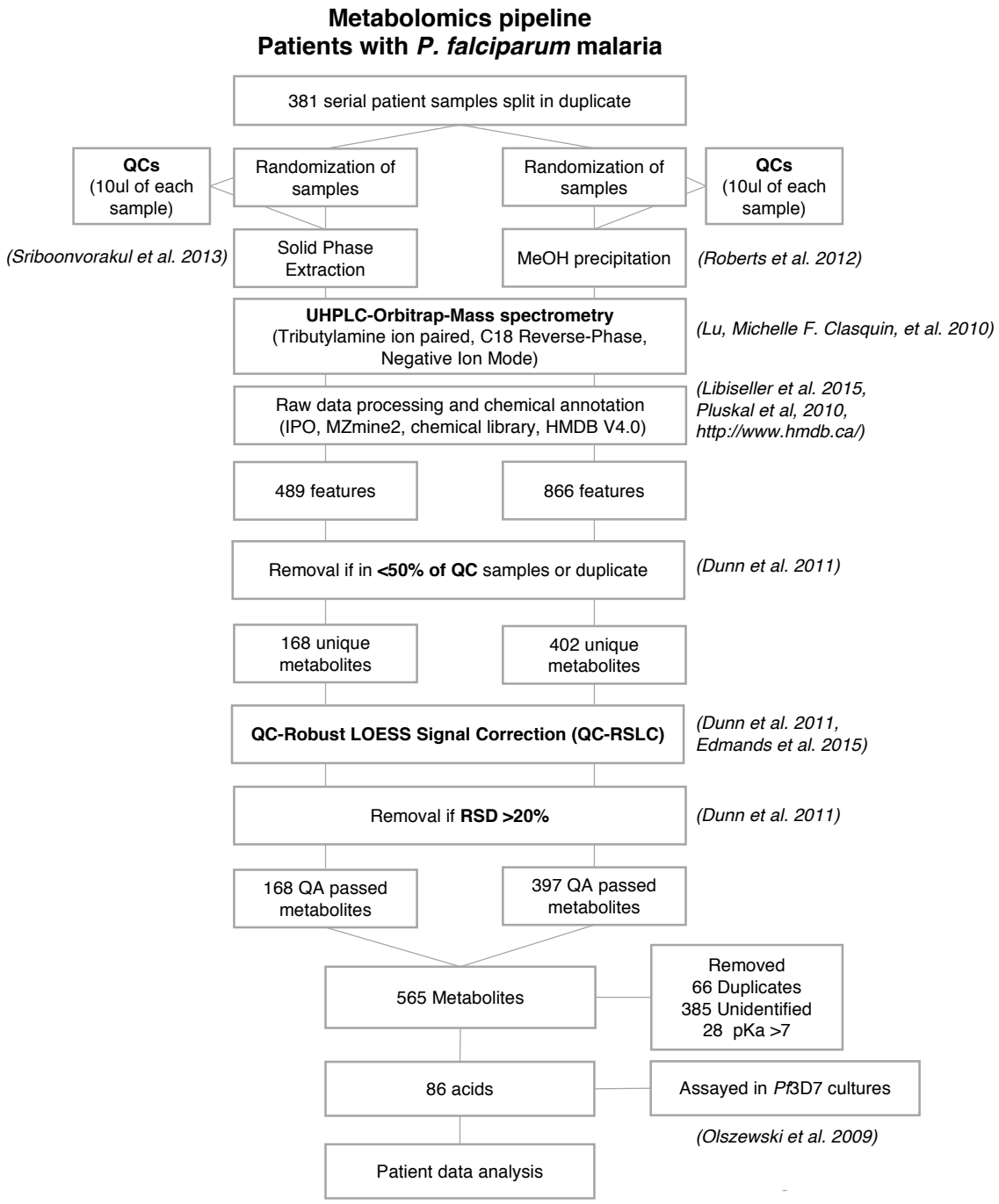


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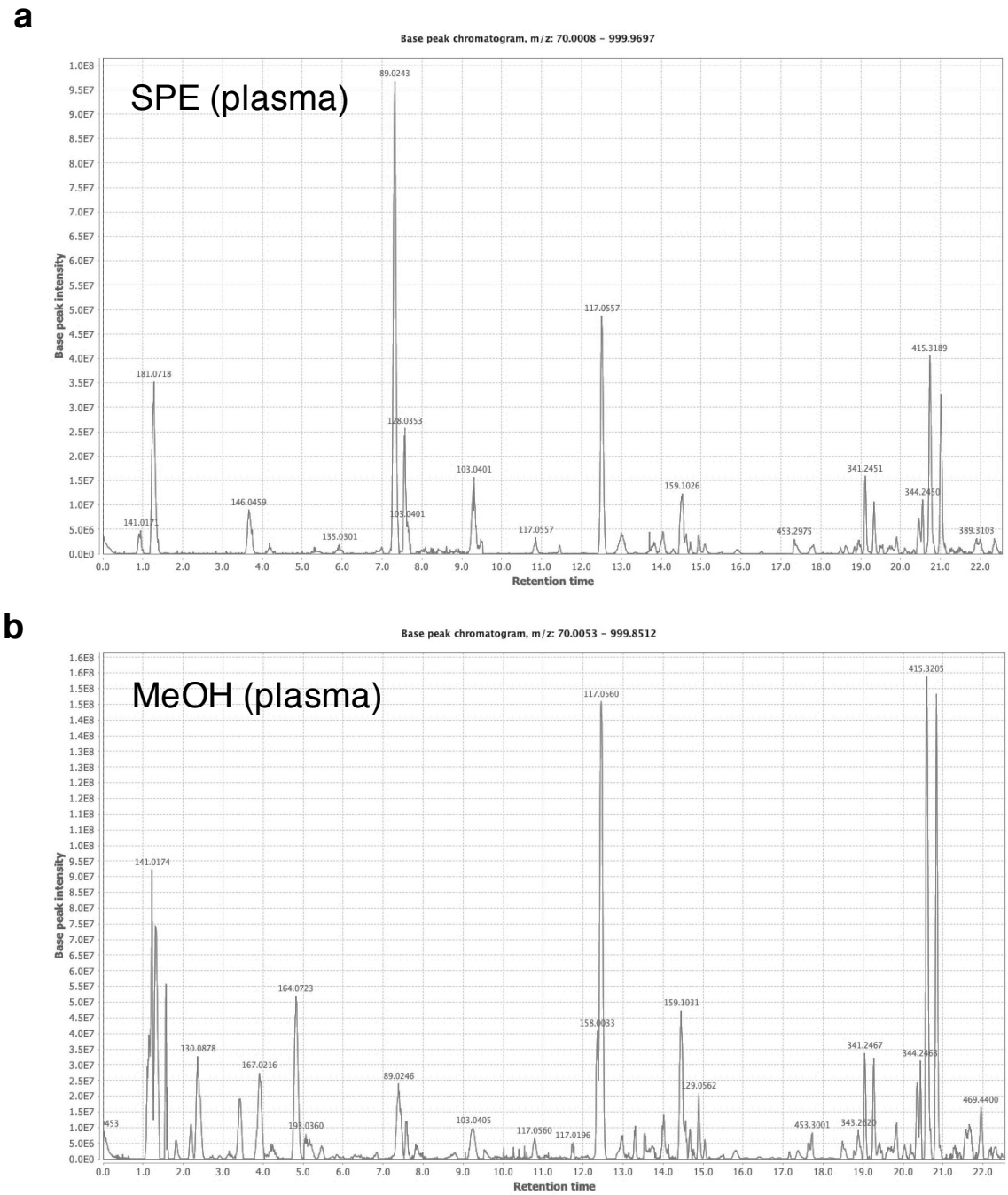


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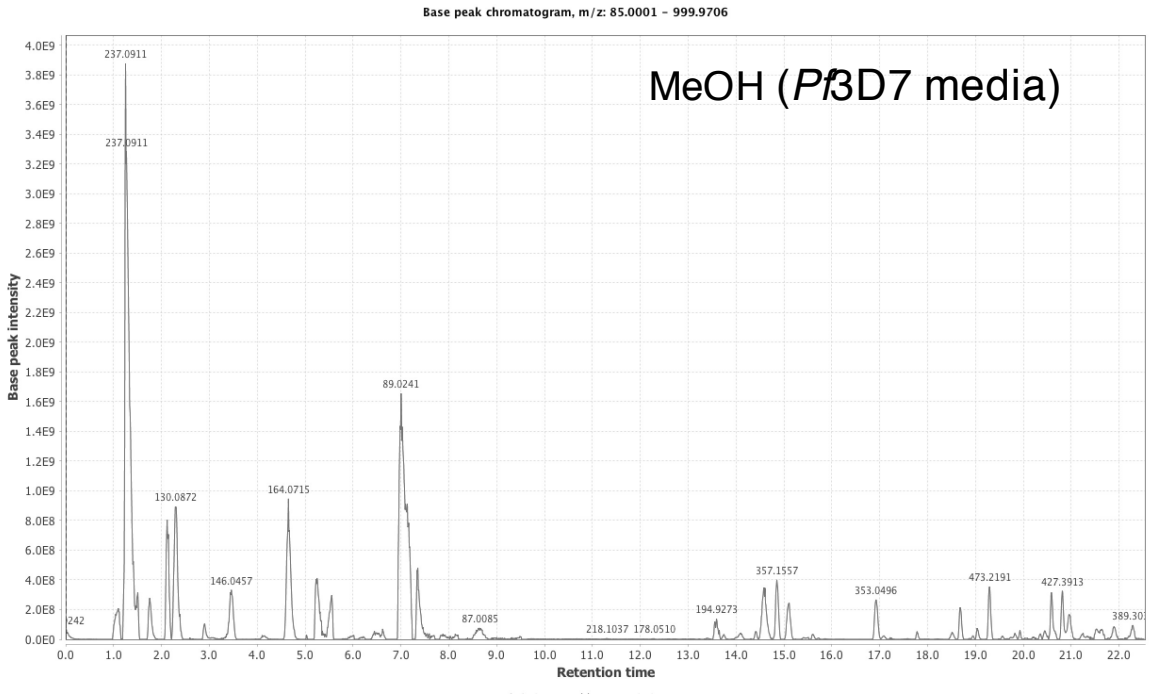


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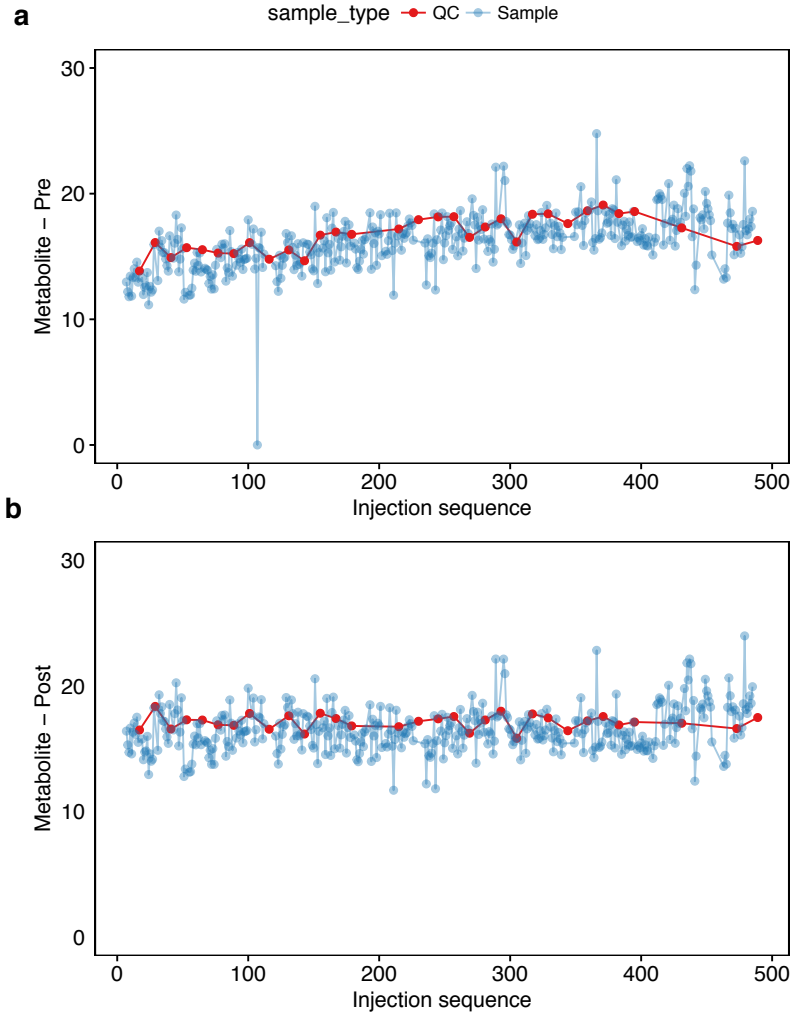


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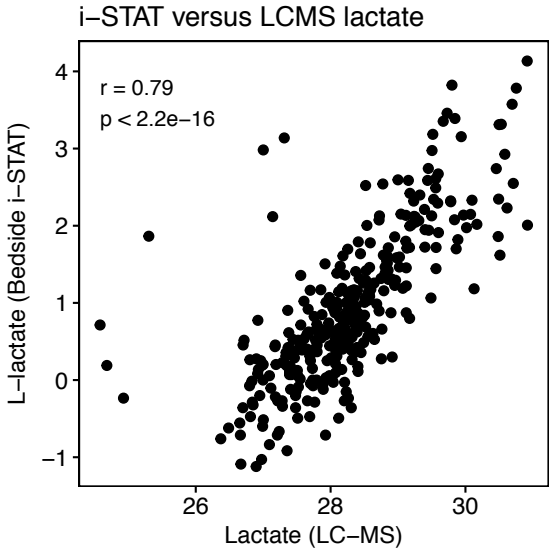


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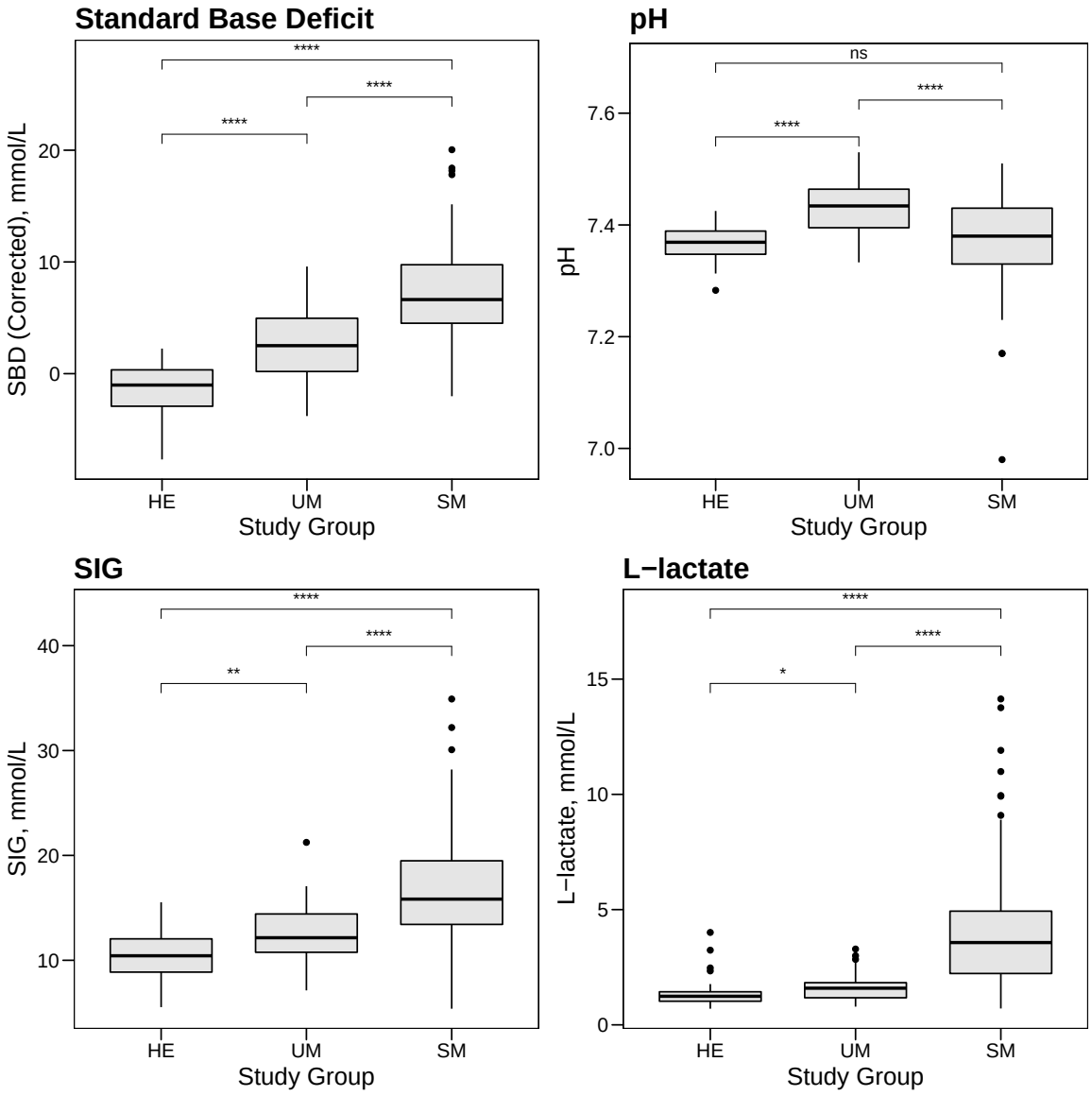


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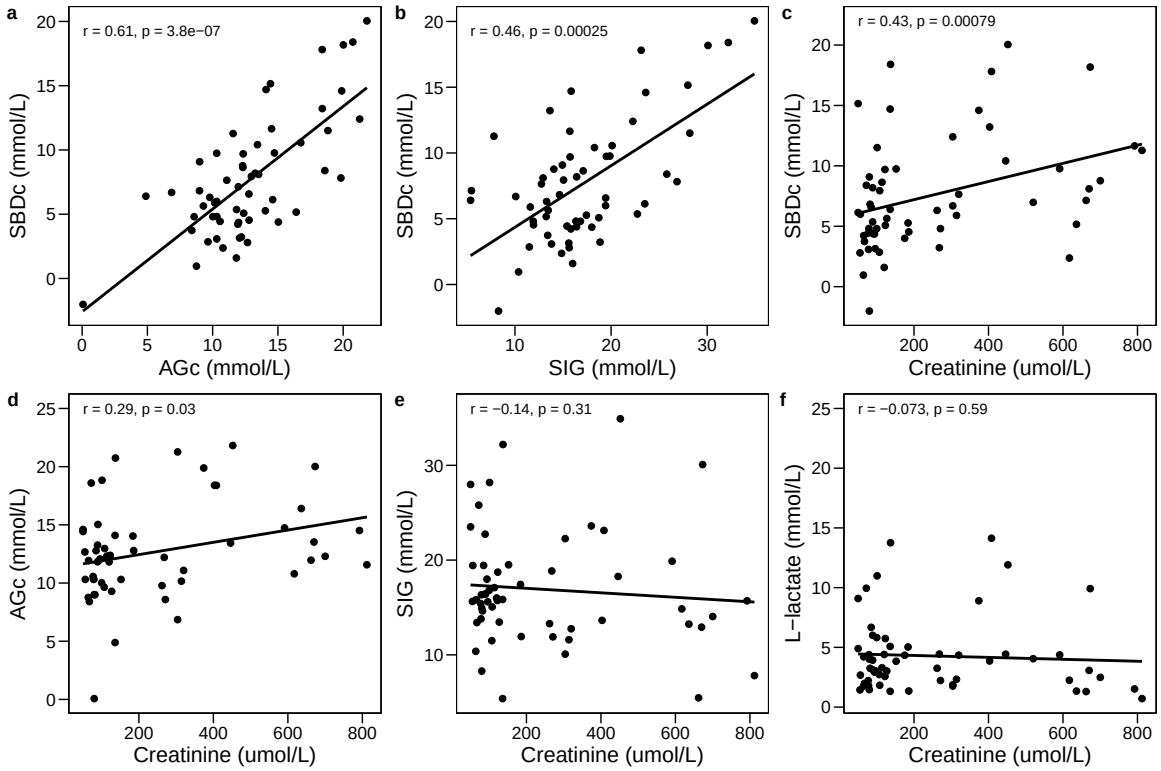


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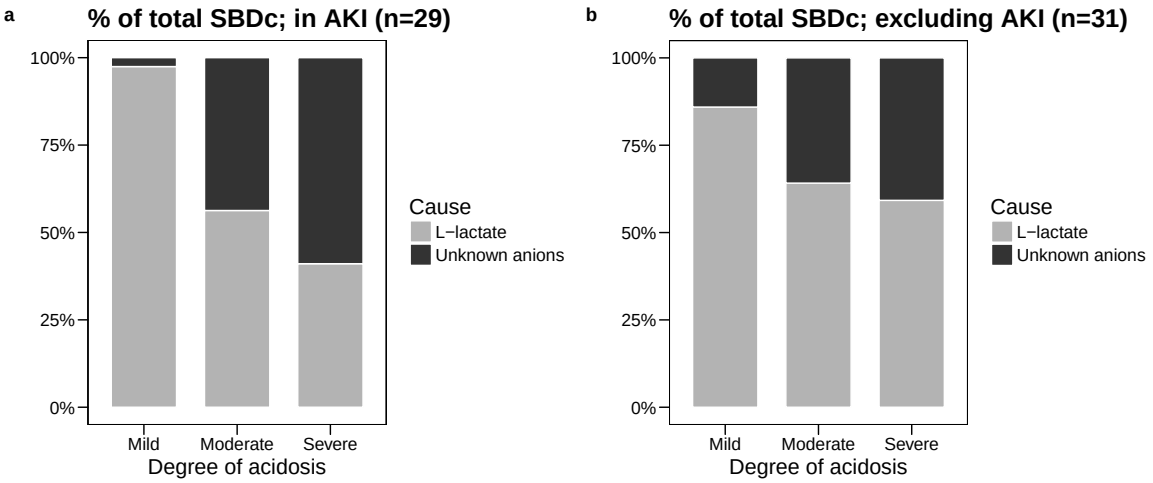


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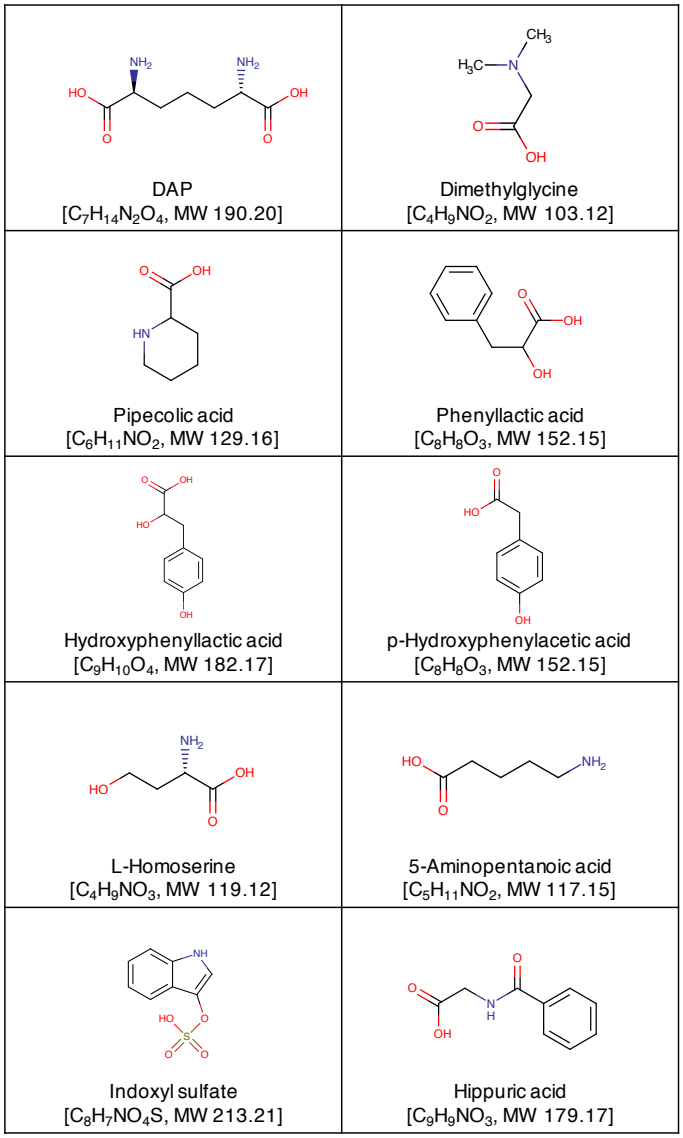


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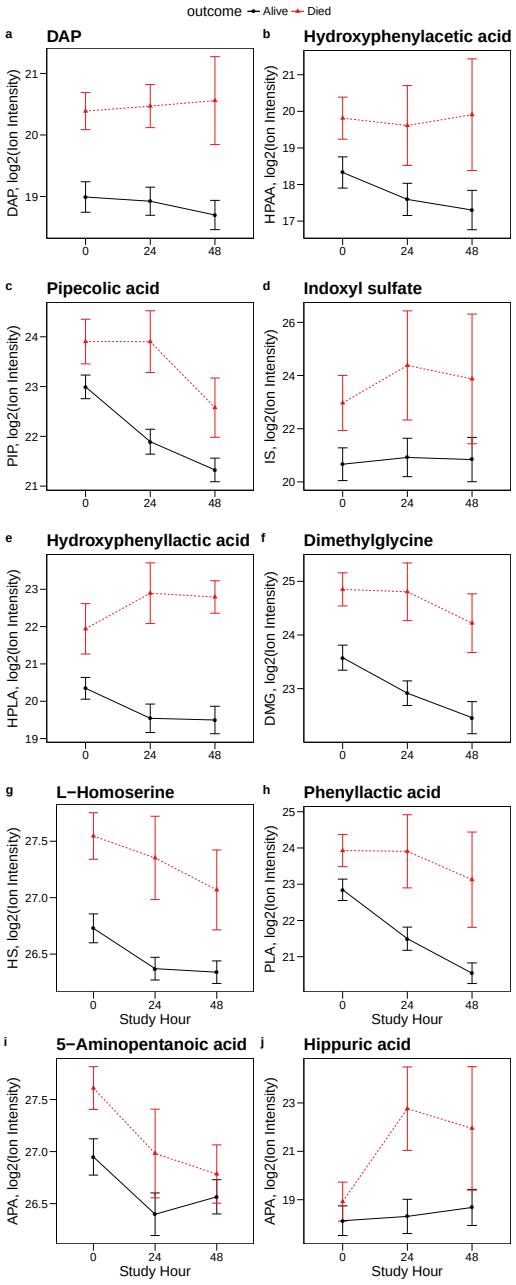


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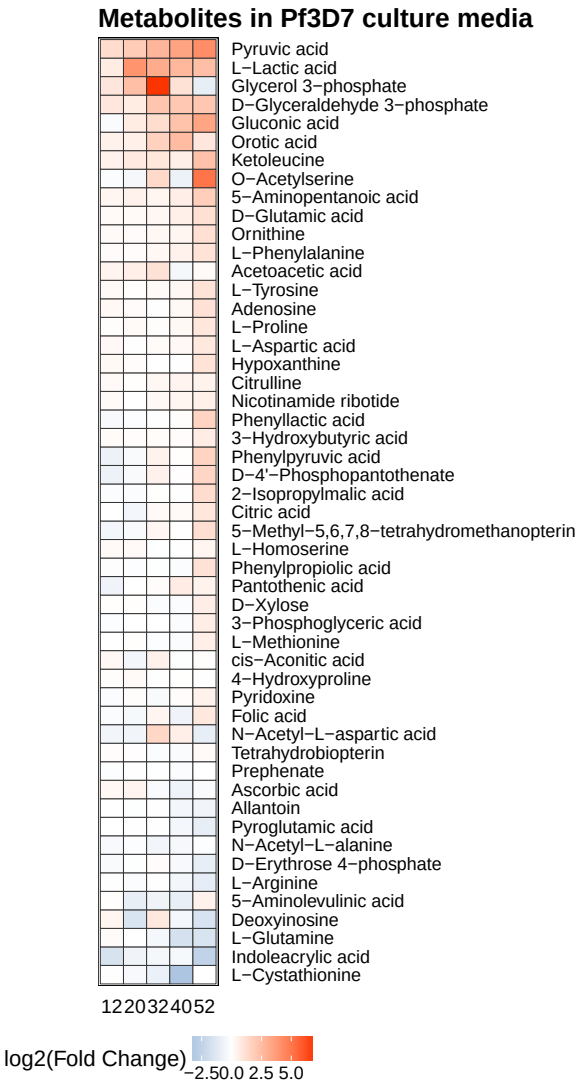


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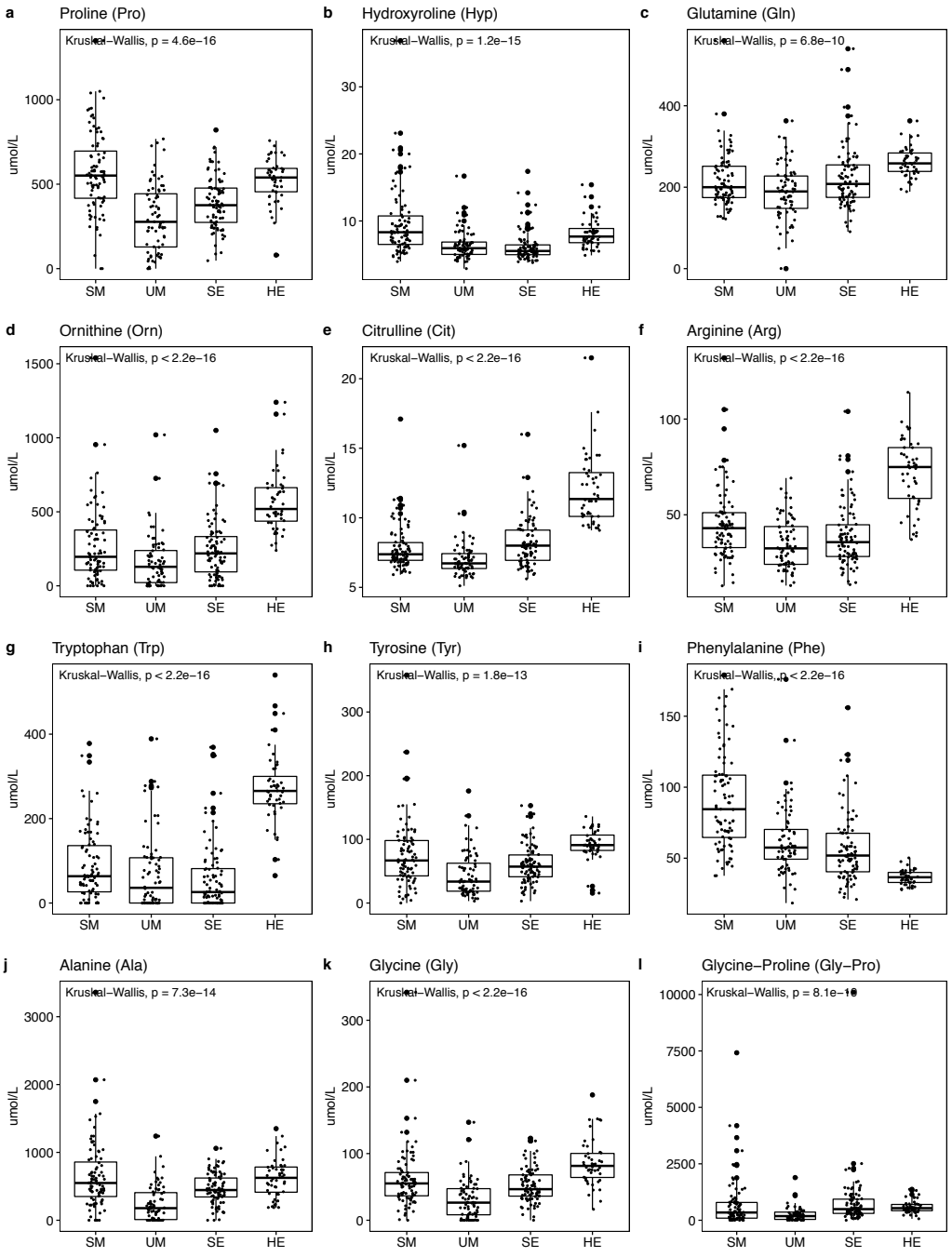


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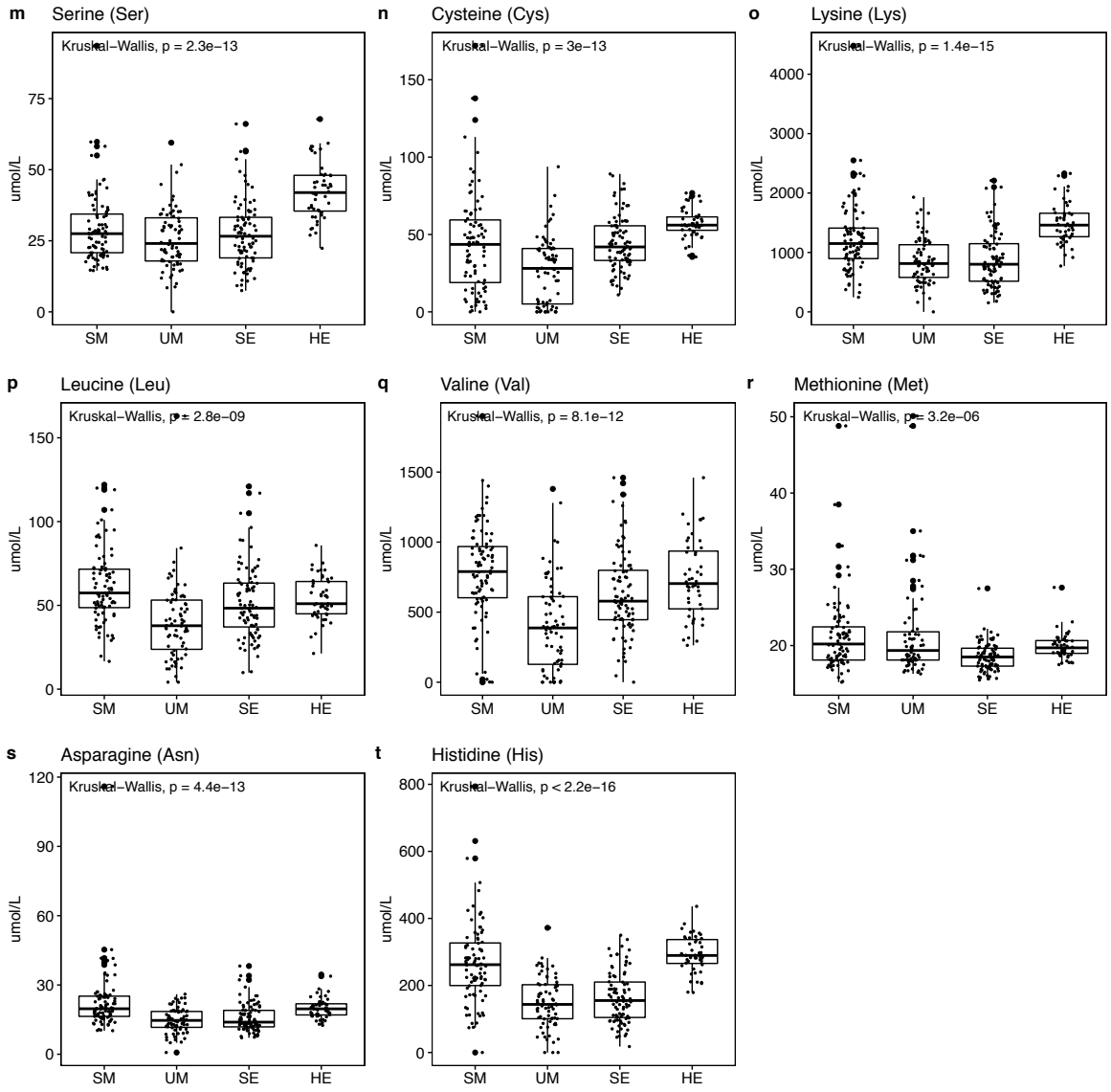


Fig. S12 (Continued). **Free amino acid concentrations measured by UHPLC-MS/MS-MRM in plasma of study participants (n=295) from Bangladesh (Continued).** Boxplots with medians and interquartile ranges are shown.

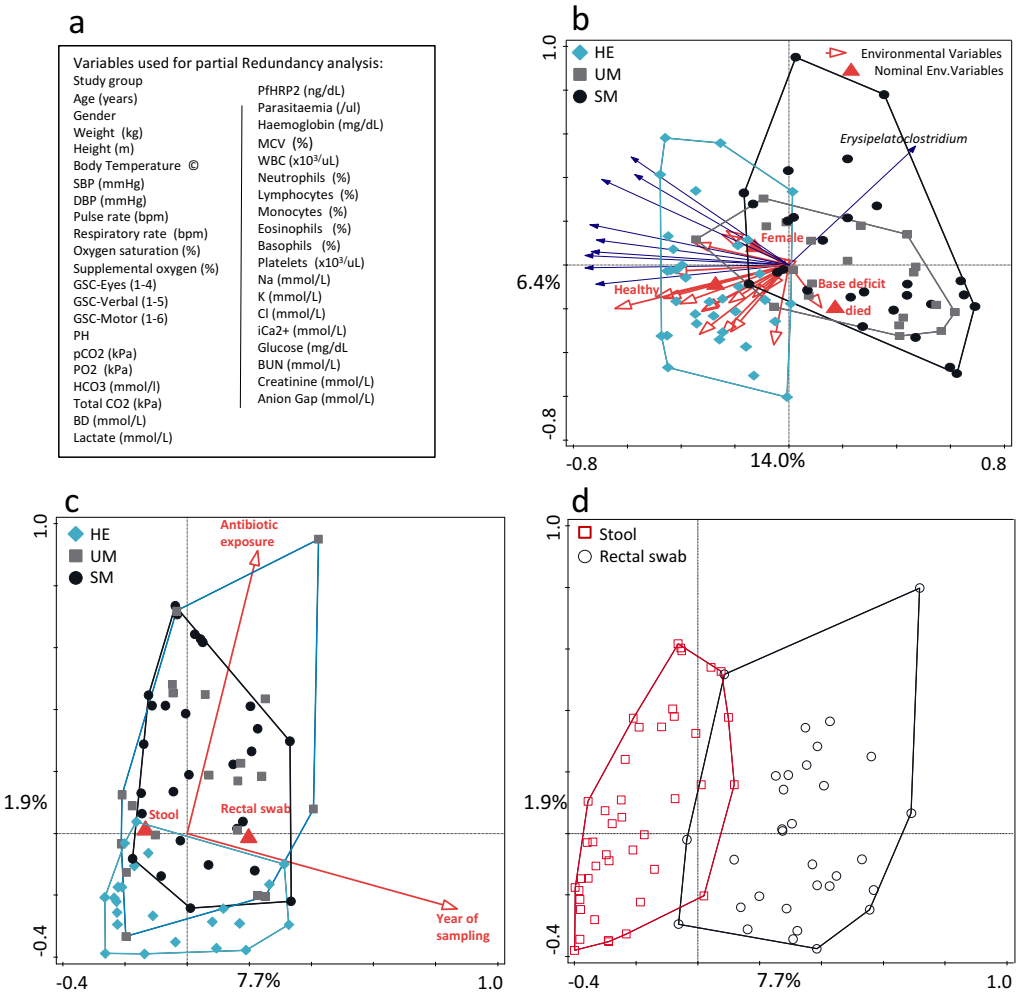


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Identifiers						Chemistry		HMDB V4.0 Biological origin			
Match	HMDB	PubChem	ChEBI	KEGG	METLIN	pKa	method	Human	Microbial	Food	Drug
(S)-3,4-Dihydroxybutyric acid	HMDB0000337	150929	86371			4.1	S	Yes			
2-Hydroxyadipic acid	HMDB0000321	193530	17023	C02360	5310	3.4	M	Yes			
2-Hydroxybutyric acid	HMDB0000008	11266	1148	C05984	3783	4.0	S	Yes			
2-Ketobutyric acid	HMDB0000005	58	30831	C00109		3.2	M	Yes			
2-trans,4-cis-Decadienoylcarnitine	HMDB0013325	53481669				4.1	M	Yes		Yes	
2,5-Furandicarboxylic acid	HMDB0004812	76720	84212		7076	2.8	S	Yes	Yes		
3-Hydroxybutyric acid	HMDB0000357	441	20067	C01089	125	4.4	S	Yes			
4-Pyridoxic acid	HMDB0000017	6723	17405	C00847	239	2.5	M	Yes			
5-Aminolevulinic acid	HMDB0001149	137	17549	C00430	6037	4.1	M	Yes			
5-Aminopentanoic acid	HMDB0003355	138	15887	C00431	6902	4.7	M	Yes	Yes		
5-Hydroxyindoleacetic acid	HMDB0000763	1826	27823	C05635	2975	4.2	M	Yes			
Acetaminophen glucuronide	HMDB0010316	83944	32636			3.2	M	Yes			Yes
Alanyltryptophan	HMDB0013209	53481655		C00806		3.9	S	Yes			
Alpha-ketoisovaleric acid	HMDB0000019	49	16530	C00141	5091	3.4	M	Yes		Yes	
Aminoadipic acid	HMDB0000510	469	37024	C00956	5496	2.0	S	Yes			
Azelaic acid	HMDB0000784	2266	48131	C08261	5750	4.1	S	Yes			
Beta-Alanine	HMDB0000056	239	16958	C00099	5119	4.1	M	Yes			
Bilirubin	HMDB0000054	5280352	16990	C00486	81	4.0	M	Yes			
Biotin	HMDB0000030	171548	15956	C00120	243	4.4	M			Yes	
Cinnamic acid	HMDB0000567	5372954	35699	C10438	310	4.0	M	Yes			
Citramalic acid	HMDB0000426	1081	15584	C00815	5415	3.3	S		Yes		
Citrulline	HMDB0000904	9750	16349	C00327	16	2.3	M	Yes			
D-Urobilinogen	HMDB0004158	440784		C05791	7021	4.1	M	Yes	Yes		
Dehydroepiandrosterone sulfate	HMDB0001032	12594	16814	C04555	4095	-1.4	M	Yes		Yes	Yes
Diaminopimelic acid	HMDB0001370	439283	16026	C00666	352	1.8	M		Yes		
Dimethylglycine	HMDB0000092	673	17724	C01026	277	1.9	M	Yes	Yes		
DL-2-Aminooctanoic acid	HMDB0000991	69522	75145		5923	2.9	M	Yes			
Gluconic acid	HMDB0000625	10690	33198	C00257	345	3.4	S	Yes			
Glyceric acid	HMDB0000139	439194	32398	C00258	280	3.4	M	Yes			
Glycerophosphocholine	HMDB0000086	71920	16870	C00670	370	1.9	M	Yes		Yes	
Glycocholic acid	HMDB0000138	23617285	29746	C01921	5169	3.8	M	Yes	Yes	Yes	
Glycolic acid	HMDB0000115	757	17497	C00160	3219	3.5	M	Yes			
Glyoxylic acid	HMDB0000119	760	16891	C00048	3213	2.6	M	Yes			
Guanidoacetic acid	HMDB0000128	763	16344	C00581	5163	3.4	M	Yes			
Hexanoylglycine	HMDB0000701	99463	64390		5669	4.3	M	Yes			
Hippuric acid	HMDB0000714	464	18089	C01586	1301	3.6	M	Yes	Yes		Yes
Hydroxyphenyllactic acid	HMDB0000755	9378	17385	C03672	5723	3.6	M	Yes	Yes		
Indoxyl sulfate	HMDB0000682	10258	43355		524	-1.8	M	Yes	Yes		
Inodxyl glucuronide	HMDB0010319	2733785		C03033		3.5	M	Yes			
Inosine	HMDB0000195	6021	17596	C00294	84	6.9	M	Yes			Yes
Ketoleucine	HMDB0000695	70	48430	C00233	5663	3.5	M	Yes		Yes	

Table S1. **Metabolites detected in a metabolomic analysis (UHPLC-Orbitrap-MS) of plasma collected at study enrolment in study participants (n=152) from Bangladesh.**

Match	HMDB	PubChem	ChEBI	KEGG	METLIN	pKa	method	Human	Microbial	Food	Drug
L-Aspartic acid	HMDB0000191	5960	17053	C00049	5206	1.7	S	Yes			
L-Cystathionine	HMDB0000099	439258	17482	C02291	39	1.8	M	Yes			Yes
L-Dopa	HMDB0000181	6047	15765	C00355	42	1.7	M	Yes			
L-Glutamic acid	HMDB0000148	33032	16015	C00025	5174	1.9	M	Yes			
L-Glutamine	HMDB0000641	5961	18050	C00064	5614	2.1	M	Yes			
L-Histidine	HMDB0000177	6274	15971	C00135	21	1.8	M			Yes	Yes
L-Homoserine	HMDB0000719	12647	15699	C00263	5687	2.2	M	Yes	Yes		
L-Kynurenine	HMDB0000684	161166	16946	C00328	72	1.2	M	Yes			
L-Lactic acid	HMDB0000190	107689	422	C00186	5205	3.8	S	Yes			
L-Lysine	HMDB0000182	5962	18019	C00047	5200	2.7	M			Yes	Yes
L-Malic acid	HMDB0000156	222656	30797	C00149		3.2	M	Yes			
L-Methionine	HMDB0000696	6137	16643	C00073	5664	2.5	M	Yes		Yes	Yes
L-Phenylalanine	HMDB0000159	6140	17295	C00079	28	2.5	M			Yes	Yes
L-Proline	HMDB0000162	145742	17203	C00148	29	1.9	M	Yes			Yes
L-Serine	HMDB0000187	5951	17115	C00065	5203	2.0	M	Yes			
L-Tyrosine	HMDB0000158	6057	17895	C00082	34	2.0	M	Yes			
LysoPC(16:1(9Z))	HMDB0010383	24779461		C04230		1.9	S	Yes		Yes	
Methionine sulfoxide	HMDB0002005	847	49033		6428	1.7	M	Yes			
Methylmalonic acid	HMDB0000202	487	30860	C02170	3712	2.5	M	Yes			
N-Acetylneuraminic acid	HMDB0000230	445063	45744	C19910	3321	3.0	M	Yes			
Nicotinamide ribotide	HMDB0000229	14180	16171	C00455	5238	1.2	M	Yes			
Nicotinic acid	HMDB0001488	938	15940	C00253	6272	2.8	M	Yes		Yes	Yes
p-Hydroxyphenylacetic acid	HMDB0000020	127	18101		130	4.0	M		Yes	Yes	
Pantothenic acid	HMDB0000210	988	7916	C00864	241	4.4	M			Yes	Yes
Pelargonidin	HMDB0003263	67249	28510	C05904	3399	6.0	M	Yes			
Phenyllactic acid	HMDB0000779	3848	25998	C01479	5745	4.0	M	Yes	Yes		
Phosphoric acid	HMDB0002142	1004	26078	C00009		1.8	S	Yes			
Pipecolic acid	HMDB0000070	849	17964	C00408	50	2.1	M	Yes	Yes		
Propionic acid	HMDB0000237	1032	30768	C00163	3215	4.8	M		Yes	Yes	
Pyroglutamic acid	HMDB0000267	7405	18183	C01879	3251	3.6	S	Yes			
Pyrophosphate	HMDB0000250	644102	18361	C00013	3306	1.7	M	Yes			
Pyruvic acid	HMDB0000243	1060	32816	C00022	117	2.9	M	Yes	Yes		
Quinolinic acid	HMDB0000232	1066	16675	C03722	330	0.3	M	Yes			
Salicyluric acid	HMDB0000840	10253	9008	C07588	617	3.3	M	Yes	Yes		
Sarcosine	HMDB0000271	1088	15611	C00213	51	2.1	M	Yes			
Shikimic acid	HMDB0003070	8742	16119	C00493	338	4.1	S	Yes	Yes		
Succinylacetone	HMDB0000635	5312	87897		5608	4.2	S	Yes			
Sulfolithocholylglycine	HMDB0002639	53477756		C11301	6725	-1.4	M	Yes	Yes	Yes	
Taurine	HMDB0000251	1123	15891	C00245	31	-1.5	M	Yes			
trans-trans-Muconic acid	HMDB0002349	5356793	27036		6639	3.9	M	Yes		Yes	
Tuftsin	HMDB0005770	53477795	88947			3.4	M	Yes			
Uridine 5'-diphosphate	HMDB0000295	6031	17659	C00015		1.8	M	Yes			
Urocanic acid	HMDB0000301	736715	30817	C00785	298	3.8	M	Yes			
Vanillylmandelic acid	HMDB0000291	736172	1127735	C05584	697	3.1	M	Yes			

Table S1 (Continued). **Metabolites detected in a metabolomic analysis (UHPLC-Orbitrap-MS) of plasma collected at study enrolment in study participants (n=152) from Bangladesh (Continued).**

	Uncomplicated Malaria (n=47) *vs* Healthy Controls (n=45)		Severe Malaria (n=60) *vs* Uncomplicated Malaria (n=47)		Severe Malaria (n=60) *vs* Healthy Controls (n=45)	
Name	**Log2(FC)**	**Benjamini-Hochberg Adjusted P value**	**Log2(FC)**	**Benjamini-Hochberg Adjusted P value**	**Log2(FC)**	**Benjamini-Hochberg Adjusted P value**
Acetaminophen glucuronide	7.43	8.27E-17	0.37	6.53E-01	7.87	2.22E-21
Quinolinic acid	2.97	1.79E-13	2.92	2.40E-09	5.95	3.66E-25
Phenyllactic acid	2.07	7.06E-09	2.09	2.46E-07	4.19	1.77E-23
Biotin	2.08	1.72E-07	1.92	7.33E-08	3.87	2.51E-24
Nicotinic acid	1.13	1.76E-04	2.74	4.04E-08	3.82	1.50E-13
Dimethylglycine	1.25	7.21E-06	2.15	5.70E-10	3.52	3.66E-25
L-Kynurenine	1.71	1.52E-07	1.62	2.25E-05	3.45	6.33E-15
Hydroxyphenyllactic acid	1.23	3.27E-02	1.88	8.70E-04	3.21	1.49E-11
Diaminopimelic acid	1.28	1.11E-04	1.83	7.65E-08	3.10	2.71E-20
Methylmalonic acid	1.97	1.15E-07	0.98	5.82E-03	2.94	4.28E-21
5-Hydroxyindoleacetic acid	1.28	4.18E-05	1.65	2.05E-05	2.93	8.16E-13
LysoPC(16:1(9Z))	2.32	1.52E-07	0.62	1.96E-01	2.92	3.04E-12
Pyrophosphate	0.72	1.76E-03	2.08	8.16E-09	2.89	2.46E-14
Pipecolic acid	0.37	3.44E-01	2.44	5.25E-10	2.89	1.69E-15
N-Acetylneuraminic acid	0.89	8.07E-03	1.85	5.76E-06	2.81	1.04E-10
p-Hydroxyphenylacetic acid	0.46	2.95E-01	2.23	1.87E-06	2.78	7.77E-08
Glycocholic acid	2.28	3.53E-08	0.27	5.62E-01	2.60	8.61E-11
2-Hydroxybutyric acid	1.85	1.34E-15	0.69	3.01E-06	2.54	1.43E-23
D-Urobilinogen	1.79	7.88E-06	0.61	1.82E-01	2.43	1.04E-10
Beta-Alanine	1.95	1.31E-04	0.54	1.69E-01	2.33	3.91E-09
L-Malic acid	0.89	1.12E-01	1.39	3.93E-03	2.32	2.70E-07
Indoxyl sulfate	-0.29	4.36E-01	2.47	1.13E-04	2.27	6.28E-04
(S)-3,4-Dihydroxybutyric acid	0.89	1.15E-07	1.34	5.70E-10	2.23	2.05E-21
Aminoadipic acid	0.40	3.48E-01	1.82	1.14E-06	2.22	7.80E-08
Inodxyl glucuronide	0.15	3.48E-01	2.01	3.53E-07	2.12	9.75E-07
Tuftsin	1.25	1.71E-05	0.70	4.06E-02	1.98	4.80E-10
L-Glutamine	1.07	4.86E-04	0.77	1.11E-02	1.81	3.08E-09
Methionine sulfoxide	0.38	1.65E-01	1.26	3.01E-06	1.68	2.63E-12
Cinnamic acid	1.36	2.13E-04	0.28	4.78E-01	1.68	3.06E-06
L-Proline	-0.17	6.58E-01	1.84	2.91E-06	1.67	4.92E-09
Urocanic acid	0.18	8.43E-01	1.30	1.05E-03	1.53	2.19E-04
L-Phenylalanine	1.18	2.16E-09	0.31	1.47E-01	1.49	1.26E-11
Sarcosine	-0.25	2.90E-01	1.67	3.31E-07	1.45	4.27E-07
Pyroglutamic acid	0.81	1.29E-02	0.64	1.68E-02	1.45	1.06E-05
Citramalic acid	1.01	7.06E-09	0.33	3.51E-03	1.34	1.50E-13
L-Cystathionine	0.37	3.44E-01	0.99	4.09E-04	1.27	1.12E-05
2-Ketobutyric acid	1.15	3.19E-04	0.09	7.07E-01	1.24	2.00E-04
Gluconic acid	0.35	6.48E-01	0.84	9.99E-02	1.19	2.91E-02
Salicyluric acid	-0.13	3.27E-01	1.24	2.93E-04	1.06	1.79E-03
4-Pyridoxic acid	-0.01	9.91E-01	1.01	1.21E-02	1.04	7.93E-03
Alpha-ketoisovaleric acid	-0.47	2.22E-01	1.48	4.84E-06	1.03	6.28E-04
L-Homoserine	-0.12	4.44E-01	1.09	9.55E-10	0.98	3.91E-09

Table S2. **Differential abundance analysis of metabolites detected in plasma from patients with uncomplicated malaria (n=47), severe malaria (n=60), and healthy controls (n=45) in a metabolomic analysis (UHPLC-Orbitrap-MS) of plasma.**

	Uncomplicated Malaria (n=47) *vs* Healthy Controls (n=45)		Severe Malaria (n=60) *vs* Uncomplicated Malaria (n=47)		Severe Malaria (n=60) *vs* Healthy Controls (n=45)	
Name	Log2(FC)	Benjamini-Hochberg Adjusted P value	Log2(FC)	Benjamini-Hochberg Adjusted P value	Log2(FC)	Benjamini-Hochberg Adjusted P value
Sulfolithocholylglycine	0.87	3.29E-02	0.10	8.08E-01	0.97	3.67E-03
DL-2-Aminooctanoic acid	0.59	7.14E-03	0.36	1.59E-02	0.95	2.19E-06
L-Tyrosine	0.25	2.06E-01	0.67	3.26E-03	0.93	1.11E-04
L-Lactic acid	0.00	9.91E-01	0.88	5.82E-03	0.89	7.67E-03
Ketoleucine	0.33	6.06E-01	0.55	2.02E-01	0.89	5.90E-02
Azelaic acid	0.50	2.55E-02	0.28	1.88E-01	0.78	6.99E-04
Pantothenic acid	0.64	1.68E-07	0.13	1.24E-01	0.77	1.87E-10
2-Hydroxyadipic acid	0.56	3.06E-05	0.20	1.47E-01	0.76	5.48E-08
Hexanoylglycine	0.64	5.68E-03	0.12	4.85E-01	0.75	6.49E-04
5-Aminopentanoic acid	-0.15	5.46E-01	0.90	2.05E-05	0.75	6.71E-05
Shikimic acid	0.39	1.02E-01	0.33	9.25E-02	0.72	1.19E-03
2-trans,4-cis-Decadienoylcarnitine	0.57	1.76E-04	0.13	1.47E-01	0.70	9.80E-07
Hippuric acid	-1.48	5.31E-05	2.14	2.65E-04	0.63	2.91E-01
Glyceric acid	0.08	7.87E-01	0.49	1.05E-01	0.57	4.77E-02
L-Histidine	-0.47	2.04E-02	0.99	2.00E-05	0.52	1.23E-02
Glyoxylic acid	0.05	7.32E-01	0.47	2.93E-04	0.52	7.83E-05
5-Aminolevulinic acid	0.04	6.89E-01	0.45	2.83E-04	0.50	1.67E-05
L-Methionine	-0.19	6.06E-01	0.63	7.29E-02	0.45	2.72E-02
Pyruvic acid	-0.01	9.91E-01	0.42	1.15E-01	0.41	1.35E-01
L-Lysine	-0.29	3.47E-02	0.69	3.71E-07	0.41	1.13E-03
Glycolic acid	0.44	1.13E-01	-0.03	9.60E-01	0.40	1.20E-01
L-Serine	-0.21	3.44E-01	0.63	1.89E-02	0.40	4.11E-02
Bilirubin	-0.13	7.43E-01	0.45	1.69E-01	0.35	2.08E-01
Guanidoacetic acid	-0.49	1.93E-01	0.77	7.70E-02	0.30	3.36E-01
Phosphoric acid	0.42	6.02E-01	-0.15	8.08E-01	0.27	6.77E-01
Succinylacetone	-0.30	2.95E-01	0.53	4.48E-02	0.23	4.20E-01
Nicotinamide ribotide	-0.01	6.89E-01	0.18	7.70E-02	0.17	6.28E-02
Propionic acid	-0.01	9.85E-01	0.15	5.62E-01	0.14	6.41E-01
Vanillylmandelic acid	0.20	6.64E-01	-0.15	6.75E-01	0.02	9.48E-01
3-Hydroxybutyric acid	-0.05	7.94E-01	-0.01	9.66E-01	-0.06	6.78E-01
Uridine 5'-diphosphate	0.06	7.44E-01	-0.12	4.32E-01	-0.07	6.22E-01
L-Dopa	0.38	5.76E-04	-0.46	3.76E-02	-0.09	6.71E-01
2,5-Furandicarboxylic acid	-0.08	6.89E-01	-0.05	7.71E-01	-0.13	4.31E-01
trans-trans-Muconic acid	0.54	1.99E-01	-0.68	1.55E-02	-0.14	4.89E-01
Glycerophosphocholine	-0.76	2.63E-08	0.60	1.16E-04	-0.18	2.01E-01
Taurine	-0.84	2.23E-02	0.42	7.51E-02	-0.42	2.54E-01
Pelargonidin	0.04	7.42E-01	-0.50	2.26E-04	-0.46	9.05E-04
Citrulline	-1.27	4.32E-16	0.79	1.20E-06	-0.49	6.99E-04
L-Glutamic acid	-0.82	5.68E-03	0.00	9.87E-01	-0.81	5.14E-03
Alanyltryptophan	-1.45	1.11E-04	0.55	9.75E-02	-0.88	6.03E-03
Inosine	-1.46	8.04E-06	0.64	4.53E-02	-0.89	2.91E-03
Dehydroepiandrosterone sulfate	-1.59	5.68E-05	0.47	2.21E-01	-1.15	1.23E-03
L-Aspartic acid	-1.66	3.83E-04	0.49	1.36E-01	-1.15	4.11E-03

Table S2 (Continued). **Differential abundance analysis of metabolites detected in plasma from patients with uncomplicated malaria (n=47), severe malaria (n=60), and healthy controls (n=45) in a metabolomic analysis (UHPLC-Orbitrap-MS) of plasma (Continued).**

Table S3. **Plasma γ-aminobutyric acid (GABA) measured in patients (n=10) from Bangladesh.**

Patient identifier	Condition	Age (years)	Sex	GABA (μmol/L)	Coma depth (GCS)	L-lactate (mmol/L)	Creatinine (μmol/L)	Outcome
CW009	Severe Malaria	23	Female	249	9	4.37	591	Died
CW038	Severe Malaria	55	Male	70.6	14	1.34	636	Survived
15-1-032	Severe Malaria	25	Male	53.7	3	4.05	520	Died
CW010	Severe Malaria	35	Female	26.1	6	13.76	137	Died
CW001	Severe Malaria	10	Female	24.7	7	1.76	304	Died
CW005	Severe Malaria	18	Male	17.1	10	5.07	305	Survived
15-1-045	Severe Malaria	60	Female	15.8	4	3.86	403	Died
16-1-029	Severe Malaria	13	Female	4.74	15	3.18	289	Survived
17-1-032	Bacterial meningitis*	50	Male	3.59	7	0.95	262	Died
15-1-029	Severe Malaria	32	Male	2.11	15	3.3	114	Survived

Plasma free amino acids were quantified in 295 patients using HPLC-MRM-mass spectrometry, γ-aminobutyric acid (GABA) was elevated and quantified within the limit of detection in 10 patients; their clinical and laboratory observations are described above.

*Patient included in the SIRS/sepsis study group.