METABOLIC ACIDOSIS IN PAEDIATRIC PATIENTS WITH MALARIA: PATHOGENESIS AND THE EFFECT ON TREATMENT WITH ARTEMETHER-LUMEFANTRINE

By

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MD, MMed

A thesis submitted in fulfillment of the requirement for the degree of Doctor of Philosophy of the Muhimbili University of Health and Allied Sciences

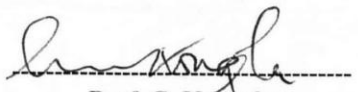
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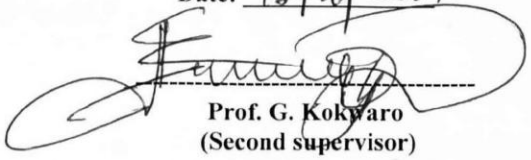
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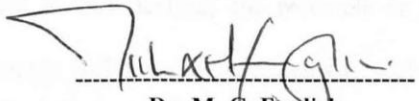
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Declaration and Copyright

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Dedication

To Fabiola, Kahabi, Felista, and Our Lady of Consolata

"The object of learning is not to fill a vessel but rather to ignite a fire"

-Socrates

Abstract

Although there have been considerable advances in the understanding of the pathogenesis of severe malaria in African children in recent years, case fatality remains high even when intravenous quinine and standard supportive care is given. Metabolic acidosis is now recognized as an important component of severe malaria and is the single best independent predictor of mortality. Most malaria deaths occur within 24 h after hospital admission, a short period for the full effect of treatment with quinine. These observations indicate that acidosis may be an important cause of death. This suggests that reduction of malaria mortality will depend on increased understanding and development of effective treatment of acidosis. However, few studies have looked into the aetiology or nature of metabolic acidosis in paediatric malaria patients. In addition, the consequences of metabolic acidosis that may be linked with mortality are still unknown. Possible consequences include the effect of acidosis on cell function and on malaria treatment. A possible interaction between *P. falciparum* malaria infection and non-antimalarial drugs used to treat malaria fever has been implicated. Based on small studies, it has been suggested that the intake of aspirin or other salicylate-containing antipyretics before hospitalization may play a role in the development of acidosis or may exacerbate *P. falciparum* infection.

In this thesis, metabolic acidosis in hospitalized paediatric patients with malaria was characterized using the Stewart's physicochemical approach (determining the strong ion difference, SID, and exploring for the presence of unidentified strong anions) as

well as the traditional approach (determining the albumin-corrected anion gap). The role of the intake of salicylates before hospitalization; the relationship between the intake of salicylates and development of severe malaria and mortality; and the consequence of acidosis on malaria treatment were also investigated.

In acidotic patients (base excess ≤ -10 mmol/l), the mean strong ion gap and mean albumin-corrected anion gap were high (15 mmol/l; 95 % CI = 14 –17; and 22 mmol/l; 95 % CI = 20 – 23, respectively. The mean SID was 38mmol/l (95 % CI = 37 – 39; normal = 48). Unidentified anions (> 5mmol/l) were present in 63 % of patients. In admission plasma samples, salicylates were detectable (≥ 5mg/l) in 14 % of acidotic patients and 17 % of non acidotic patients (P = 0.46). In a multivariable analysis, the odds ratio of exposure to salicylates among severe malaria cases was 2.8 (95 % CI; = 1.4 – 5.4). However, the odds ratio of exposure in those dying of malaria, acute gastroenteritis, pneumonia or acute bacterial meningitis was 1.2 (95 % CI; = 0.7 – 2.2). There was no significant difference in mean Day 7 lumefantrine concentration (surrogate of lumefantrine total AUC, and determinant of artemether-lumefantrine efficacy) between acidotic and non-acidotic patients (266 ng/ml; 95 % CI = 107 – 425 versus 279 ng/ml; 95 % CI = 243 – 316). Likewise, acidosis had no effect on lumefantrine bioavailability during the first 24 h of treatment with artemether-lumefantrine when acidosis is still present in most cases. Mean dose-corrected lumefantrine area under the plasma concentration-time curve in acidotic patients was 17.2 μg hr/ml versus 18 .1 μg hr/ml in non-acidotic patients (95 % CI for the difference between means = -13.9 – 15.8). Lumefantrine concentration on Day 7, was below the reported minimum (280 ng/ml) for complete cure in most

patients (median concentration 237 ng/ml; IQR = 130 – 345). However, in patients with moderate malaria, PCR-adjusted adequate clinical and parasitological response (ACPR) to artemether-lumefantrine by Day 28 was 94 %, a cure rate comparable to that in childhood uncomplicated malaria.

From the results presented in this thesis, it can be concluded that metabolic acidosis associated with childhood *P. falciparum* infection is characterized by a decrease in SID associated with the presence of strong anions in plasma. These strong anions include lactate, β-hydroxybutyrate and currently unidentified anions. The identity of unidentified anions will have major therapeutic implications and the future acidosis treatment should be with interventions that increase the SID. The intake of salicylate before hospitalization does not appear to be associated with development of acidosis. However, salicylate intake may be associated with an increased risk for malaria severity although is not associated with mortality. Acidosis in paediatric patients with moderate malaria has no significant effect on lumefantrine bioavailability and thus, no effect on treatment response to artemether-lumefantrine. Therefore, the use of this drug for the treatment of uncomplicated malaria can be extended to moderate malaria with satisfactory results.

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List of Abbreviations

A_{TOT}	Total plasma weak acid
ACPR	Adequate clinical and parasitological response
ACT	Artemensinin based combination therapy
AG	Anion gap
AG_c	Albumin-corrected anion gap
ATP	Adenosine triphosphate
AUC	Area under the plasma drug concentration-time curve
AUFS	Absorbance unit full scale
BCS	Blantyre coma score
BCG	Bromocresol green
BFM	Beflumetol (lumefantrine)
CSA	Chondroitin sulfate A
CSF	Cerebral spinal fluid
C_{max}	Maximum plasma concentration
CV	Coefficient of variance
dBFM	desbutyl-beflumetol
2,3-DPG	2,3-diphosphoglycerate
dl	Decilitre
DNA	Deoxyribonucleic acid
EC_{99}	99 % effective concentration
EIR	Entomological Inoculation Rate
EMP	Embden-Meyerhofs-Parnas pathway

ETF	Early treatment failure
ELISA	Enzyme-linked immunosorbent assay
FPIA	Fluorescence polarization immunoassay
GE	Gastroenteritis
G6PDH	Glucose-6-phosphate dehydrogenase
GIS	Geographic information system
GFR	Glomerular filtration rate
h	Hour
HFN	Halofantrine
HIV	Human immunodeficiency virus
HMPS	Hexose monophosphate shunt
HPLC	High performance liquid chromatography
HRP2	Histidine rich protein 2
ICAM1	Intercellular adhesion molecule 1
IFγ	Interferon gamma
IL	Interleukin
i. m.	Intramuscular
IMCI	Integrated management of childhood illnesses
iNOS	nitric oxide synthase
IQR	Inter quartile range
ITN	Insecticide treated nets
ITP	Intermittent treatment during pregnancy
ITT	Intention-to-treat
i. v.	Intravenous

KDH	Kilifi District Hospital
KEMRI	Kenya Medical Research Institute
l	Litre
LRTI	Lower respiratory tract infection
LDH	Lactate dehydrogenase
MARA	Mapping malaria risk in Africa
MIC	Minimum inhibitory concentration
Min	Minute
MIM	Multilateral initiative on malaria
MoA	Mechanism of action
MoH	Ministry of health
MSP	Merozoite surface protein
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide dinucleotide
NADPH	Reduced nicotinamide dinucleotide phosphate
ng	nanogram
NO	Nitric oxide
βOHB	D-3-hydroxybutyrate
OPD	Out patient department
OR	Odds ratio
OTC	Over-the-counter
PAR	Peak area ratio
pCO_2	Partial pressure of carbon dioxide
PCM	Severe malnutrition

PCR	Polymerase chain reaction
PfEMP1	Plasmodium falciparum erythrocyte membrane protein 1
PK	Pharmacokinetics
pK	Dissociation constant
PP	Per protocol
PPP	Pentose phosphate pathway
PRBC	Parasitised red blood cells
PRR	Number of parasites killed per cycle
QC	Quality control
QR	Respiratory quotient
RBM	Roll Back Malaria
ROS	Reactive oxygen species
R.S.D	Relative standard deviation
SID	Strong ion difference
SIG	Strong ion gap
SIRS	Systemic inflammatory response syndrome-like illness
SP	Pyrimethamine-sulphadoxine
TDR	Special Programme for Research and Training in Tropical Diseases
TGF	Tumour growth factor
TNF	Tumour necrosis factor
UNICEF	United Nations Children's Fund
UV	Ultra violet

VO2	Total oxygen consumption
WAZ	Weight for age Z score
WHO	World Health Organization

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CHAPTER ONE

GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

Although malaria has been successfully eradicated or controlled in countries with temperate climate and in some countries with moderate or low malaria transmission, eradication has failed in the tropical world including Africa. During the malaria eradication campaign of the 1960s, eradication was attempted in only a few African countries, and for various reasons including concerns about the feasibility and sustainability of malaria control measures in countries with poor infrastructure and high malaria transmission, eradication effort was frustrated in many sub-Saharan African countries (Kager 2002). As the result, global malaria eradication was declared impossible. However, some malaria control measures employing mainly vector control and effective case management continued in these countries. High malaria awareness and the widespread deployment of chloroquine, a cheap and effective drug at the time, ensured reasonable malaria control in many malaria endemic countries. The emergence of chloroquine resistance in *P. falciparum*, the most pernicious species of the four human malaria parasites and the commonest in many parts of Africa had adverse consequences to malaria control (Trape, Pison et al. 2002). Today, malaria remains one of the three major infectious diseases globally.

The emergence of drug resistance in *P. falciparum* is considered to be responsible for the global resurgence of malaria observed in the early 1990s (Marsh, 1998). It is estimated that sub-Saharan Africa bears 90 % of the world's burden for *Plasmodium falciparum* infection (Bank 1993), and 90 % of the world's severe malarial morbidity load is borne by African children where between one and two million children die of falciparum malaria each year (Greenwood, Bradley et al. 1987).

In eastern Africa, malaria attributable child mortality had doubled compared to 1980s (Korenromp, Williams et al. 2003). In Kenya, approximately 3.5 million children under the age of five years are exposed to stable malaria transmission (regular parasite exposure every year), and about 26,000 children die each year from the direct consequences of malarial infection (Snow, Gouws et al. 1998). In Tanzania, the minimum annual rate for severe malaria in children below five years of age is as high as 51 per 1000 children (Snow, Bastos de Azevedo et al. 1994).

There has been renewed international interest in malaria control in recent years, and considerable effort is being made by WHO and UNICEF through the roll back malaria initiative (RBM) to improve control measures including vector control, provision of insecticide-treated nets (ITN), introduction of intermittent treatment during pregnancy (ITP) and effective case management. Vector control and prompt effective case management have traditionally characterized malaria control in Africa and their

strengthening is expected to improve control. For instance, the use of ITNs is capable of reducing the incidence of clinical attacks of malaria in users (ter Kuile, F. O., Terlouw et al. 2003) and can substantially reduce child mortality (Lengeler 2000). However, ITNs and other control measures to reduce mosquito contact with humans do not completely eliminate mosquito bites, and will not totally prevent malaria. An effective vaccine is most desirable but remains unrealised.

In 2002 there were about 515 million clinical attacks of *P. falciparum* malaria in the world with 71 % of these attacks occurring in Africa, most of them in children under the age of five years(Snow, Guerra et al. 2005). In malaria-endemic areas with high transmission intensity, which includes East Africa, children between the age of six months and five years have the highest risk for clinical attacks and severe malaria. During the first five months of life children are protected by maternal antibodies acquired before birth from semi-immune mothers (Deloron, Dubois et al. 1997). However, these antibodies wane and by the age of five months children become non-immune. Clinical immunity to severe disease develops after repeated clinical attacks but is not fully acquired before the age of five years.

If a clinical attack of *P. falciparum* malaria in a non-immune patient is not promptly and effectively treated, unrestricted parasite multiplication may occur and lead to heavy parasite burdens, which cause vital organ dysfunction (severe disease) with high risk of death. This can occur within hours of the onset of symptoms (Greenwood, Bradley et al. 1987). *P. falciparum* parasite sequestration in the cerebral vasculature (cerebral malaria), was previously considered the major complication of severe malaria in African children

(Greenwood, Bradley et al. 1987; Molyneux, Taylor et al. 1989) and it was widely accepted as the commonest cause of severe malaria mortality. A small number of severe malaria mortality was attributed to severe malarial anaemia (Abdalla, Weatherall et al. 1980)

In recent years, there have been considerable advances in the understanding of the pathogenesis of severe malaria in African children (Marsh, Forster et al. 1995; Waller, Krishna et al. 1995; Allen, O'Donnell et al. 1996). It is now recognised that severe malaria in children is a complex syndrome affecting many organs. Metabolic acidosis, clinically presenting as respiratory distress is now recognised as an important component of severe malaria in children, and its presence is strongly associated with mortality (Taylor, Borgstein et al. 1993; Marsh, Forster et al. 1995; Allen, O'Donnell et al. 1996; English, Sauerwein et al. 1997). In addition, most of the deaths occurring in children with severe malaria occur within 24 h after hospital admission (Krishna, Waller et al. 1994; Marsh, Forster et al. 1995; Allen, O'Donnell et al. 1996). This means that most children die before getting the benefit from the full effect of antimalarial treatment. Therefore, mortality reduction will require increased understanding of complications notably (metabolic acidosis) and subsequent development and implementation of supportive treatments directed at these complications.

Metabolic acidosis occurring in severely ill patients is not a disease, but only a manifestation of severe underlying process and can result from several processes or aetiology. Understanding the underlying aetiology or pathophysiologic mechanism is critical for development of interventions directed at treating acidosis. Few studies have

characterized metabolic acidosis in African children with severe malaria (English, Sauerwein et al. 1997) or investigated aetiology (English, Marsh et al. 1996), and underlying mechanisms remain poorly understood.

In this thesis, metabolic acidosis in Kenyan children with malaria was characterised using both the traditional approach (based on the Henderson-Hasselbalch equation) and the more recent Stewart's physical-chemical approach, with the aim of identifying underlying pathophysiological mechanisms. Children with non-severe falciparum malaria and those with a range of other severe childhood illnesses were included for characterization of acidosis to extend the scope of previous studies, and investigate which characteristics of malarial acidosis are specific or more pronounced.

In parts of rural Africa, use of salicylate-containing drugs in febrile children is still common. Therefore, the role of salicylates as an aetiological factor for metabolic acidosis was also investigated. In addition, the intake of salicylate as a risk factor for development of severe malaria and its influence on outcome of hospital admission for malaria and other common infections was examined.

The relationship between the presence of metabolic acidosis and drug disposition in children with malaria is poorly understood. However, hypovolemia (English, Waruiru et al. 1996b) and sequestration (Planche, Onanga et al. 2004) are the two pathophysiological mechanisms currently thought to play a major role in the pathogenesis of acidosis in severe

malaria. Both hypovolemia and sequestration in malaria may be associated with reduced peripheral perfusion, which may affect the absorption of i. m. or orally administered drugs. Therefore, this thesis also focused on the effect of acidosis on the oral bioavailability of lumefantrine, the partner drug in artemether-lumefantrine fixed dose combination (Coartem®), and its effect on the efficacy of this combination, which is currently recommended first line treatment for uncomplicated malaria in many African countries.

1.2 LITERATURE REVIEW

1.2.1 Malaria

1.2.1.1 Historical Perspective

Malaria has been described since ancient times as a disease characterised by seasonal periodic fevers. In Europe, the disease was observed to be common in marshy areas and as the result, was originally known as ague. The name malaria came from Italian writers who thought that the disease was caused by foul smelling vapours coming from the Tiberian marshes. The word "malaria" literally means "bad air" in the Italian language. Therefore, the association between the bad air of the marshes (during the rainy season) and the occurrence of periodic fevers formed the basis for the name malaria.

Hippocrates, a Greek physician, associated malaria with standing water and is credited for providing the first clear description of the disease(Roddis 1964). However, the causal factor for this association remained a subject of debate until late 19th century (White 2003) when it was established that the pigment observed in the blood of some patients with periodic fever resulted from the destruction of red blood cells. The same pigment was thought to be responsible for the characteristic gray discoloration of internal organs of patients dying from the disease. Charles Louis Alphonse Laveran, a French surgeon observed moving bodies on a fresh blood sample from a patient with malaria in 1880, and he hypothesiesd that malaria was caused by a red blood cell parasite (White 2003). In 1897, Ronald Ross reported the presence of pigmented bodies in the gut of certain species of brown (dapple winged) mosquitoes (White 2003) and subsequently proved the existence of a complete *Plasmodium* life cycle involving a mosquito in avian malaria (White 2003)). He also identified anopheline mosquitoes as the vector for human malaria. However, before Ross demonstrated *Plasmodium falciparum* sporogony in anopheline mosquitoes, Bignami and colleagues, had successfully infected a healthy volunteer with *Plasmodium falciparum* from mosquito bites (White 2003). Therefore, a complete malaria parasite life cycle involving man and mosquitoes was established.

1.2.1.2 Malaria vectors

As demonstrated by Ronald Ross, female Anopheles mosquito species are the vectors for malaria transmission in man. There are several species complexes with varying ability to

transmit malaria to humans (vectorial capacity) (White 2003). This is because of the differences between species complexes in their natural abundance, breeding sites, flight ranges, choice of the source of a blood meal, and the vulnerability to environmental conditions and insecticides. *Anopheles gambiae* complex naturally occurs in high densities, is capable of enduring harsh conditions and bites humans frequently. As the result, *Anopheles gambiae* complex is the most effective malaria vector.

The abundance and distribution of human malaria vectors vary considerably even within relatively small geographical areas (Minakawa, Mutero et al. 1999). *Anopheles gambiae* complex and *Anopheles funestus* are the predominant malaria vectors in East Africa (Mbogo, C. N., Snow et al. 1995)). Anopheles gambiae complex is the most abundant and *Anopheles gambiae sensu stricto* is the most abundant species. Other Anopheles gambiae complex species found in east Africa include *Anopheles arabiensis* and *Anopheles merus* (Mbogo, Mwangangi et al. 2003). Therefore, *Anopheles gambiae sensu stricto* is associated with most of malaria transmission on the Kenyan coast where studies reported in this thesis were carried out.

1.2.1.3 Conditions for Malaria Transmission

Ambient temperature and altitude are the two most important factors for sporogony to occur in Anopheline mosquitoes. Generally, sporogony occurs in temperatures ranging between 16 ^{0}C and 33 ^{0}C, and an altitude of not more than 2000m above sea level.

However, the optimum conditions for malaria transmission includes high humidity, ambient temperature between 20 and 30 ^{0}C, and rainfall, which provide breeding sites (White 2003). As the result, favorable temperature, humidity and availability of suitable breeding sites during the rainy season are associated with increased mosquito populations with subsequent increase in malaria transmission several weeks after the start of the rains. However, excess rainfall washes away mosquito larvae and pupae and may be associated with a decline in mosquito populations (Gillies 1988).

Recent observations have demonstrated a complex relationship between human malaria vectors and the environment. Great variation between mosquito populations and rainfall has been observed (Mbogo, Mwangangi et al. 2003). Environmental heterogeneity in mosquito habitats in different areas may explain this variation. In addition, previously unrecognized survival mechanisms for Anopheline mosquitoes have been reported. *Anopheles gambiae sensu stricto* mosquitoes continuously reproduce throughout the year and their eggs seem to have the ability to remain dormant in moist soil to avoid desiccation at least for several days (Minakawa, Githure et al. 2001). Thus, Anopheline mosquitoes do not necessarily suffer severe population decline during the dry season and are able to maintain a large enough population size to cause malaria transmission.

Therefore, many factors including the availability and distribution of larval habitats, the nature of substrate (water) in the habitat, and the ecology of larval predators determine the relative mosquito abundance for a given locality (Minakawa, Githure et al. 2001).

1.2.1.4 Human Malaria Parasites

Malaria parasites are protozoan blood parasites of the genus *Plasmodium*. There are four species that are responsible for human disease: *falciparum, vivax, ovale* and *malariae*. *Plasmodium falciparum* and *Plasmodium vivax* are responsible for most of infections whereas *Plasmodium malariae* and *Plasmodium ovale* rarely cause infection(White 2003). *Plasmodium falciparum* causes almost all severe disease and deaths. It is not clear why this is the case but there is a suggestion that *Plasmodium falciparum* is a recent evolutionary acquisition of man (Conway, Fanello et al. 2000) and has improved ability to parasitize the human host. However, malaria in pregnancy caused by *Plasmodium vivax, ovale* or *malariae* may be associated with reduced birth weight predisposing babies to neonatal deaths (Nosten, McGready et al. 1999).

In terms of geographical distribution, *Plasmodium falciparum* is the principal human malaria parasite in all parts of Africa. *Plasmodium vivax* is common in North Africa but rare in the sub-Saharan region and *Plasmodium ovale* is uncommon outside West Africa. *Plasmodium malariae* is found in most areas of Africa (White 2003).

1.2.1.5 The life Cycle of human malaria parasites

The life cycle of human malaria parasites is essentially the same in all four species and is presented diagrammatically on (figure 1.1).

The life cycle of the parasite in the mosquito (exogenous sexual phase or sporogony), begins when a female Anopheline mosquito picks the parasite's mature sexual cells (gametocytes) during a blood meal from an infected individual. The male and female mature gametocytes undergo further maturation in mid gut of the mosquito. The male gametocyte divides, elongates and develops flagella (microgamete) making it motile and able to find the mature female gamete. The female gametocyte undergoes maturation to develop into a mature female gamete (macrogamete). When a microgamete finds a macrogamete, fertilization takes place and the processes of fusion and meiosis lead to the formation of a zygote.

The zygote develops a cytoskeleton and becomes a mobile worm-like structure of 18 – 24μm in diameter (the ookinete) within 18 – 24 h after formation. It then penetrates the mosquito stomach wall and settles on the outer surface where it changes into a small sphere with an elastic membrane (the oocyst). The oocyst expands by asexual division of its nucleus and cytoplasm to form motile, elongated sporozoites (10 – 15μm) increasing the size of the oocyst and weakening its membrane. Eventually, the sporozoites burst through the oocyst wall and invade the body cavity of the mosquito. From here, they migrate to the salivary glands and the mosquito becomes infective. Depending on the ambient temperature, parasite species, and the mosquito, parasite development in the mosquito takes between 8 and 35 days (White 2003). Therefore, mosquito longevity is a critical factor in determining the mosquito's capacity to transmit malaria (vectorial capacity).

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The parasite cycle in the human host (infection) begins when an infected female Anopheline mosquito inoculates plasmodium sporozoites at the time of feeding. This happens when the mosquito is searching for a vascular space before blood sucking (the probing phase of a blood meal). A brief parasitaemia then follows but this ends within about half an hour when all the sporozoites disappear from the blood stream by either penetrating into parenchymal cells of the liver (hepatocytes) or being phagocytosed. In an infection, only a few liver cells are infected and this stage (pre-erythrocytic schizogony) is asymptomatic (White 2003). In *Plasmodium vivax* and probably *Plasmodium ovale* infections a few of the sporozoites penetrate liver cells and develop into tiny single nucleated parasites (hyponozoites), which remain dormant in liver cells until weeks or months later. This characterises the relapses of the infections with these two species. There is no evidence for this kind of development in *Plasmodium falciparum* or *Plasmodium malariae*. Otherwise, sporozoites penetrating hepatocytes multiply and develop into mature tissue schizoints, which in turn further develop into merozoites enlarging to approximately 30 – 70μm in diameter before bursting and releasing the merozoites into the blood stream. The released merozoites invade red blood cells in liver sinusoids but some are phagocytosed.

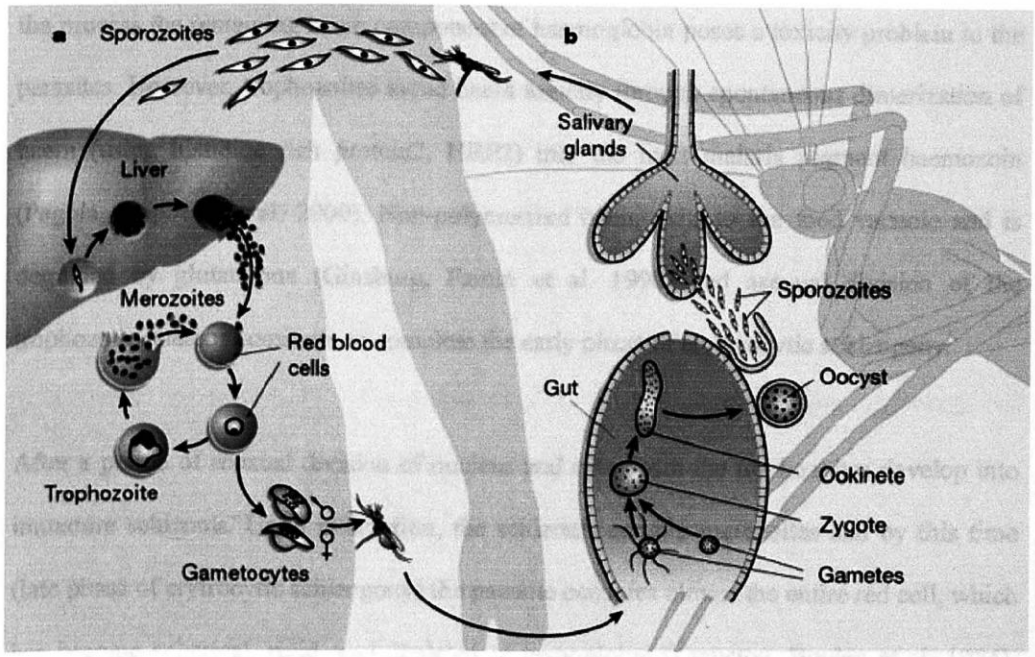


Figure 1.1: The life cycle of human malaria parasites

Adapted from Wirth, D. F.(Wirth 2002)

The process of merozoites red cell invasion involves first, attachment to the red cell surface, second, orientation so that the apical complex (containing rhoptrines and acronemes) abuts the cell, and then finally entry into the red cell. Once inside the red cell, the merozoite assumes a spherical form and develops into early erythrocytic stages of the parasite (trophozoites). These are the ring forms, young developing parasites, and are similar in all four plasmodium species. They are freely mobile within the red cell and grow logarithmically in size while consuming the erythrocyte content particularly haemoglobin. Developing trophozoites utilize amino acids from haemoglobin for protein synthesis and in

the process the remaining haem component of haemoglobin poses a toxicity problem to the parasites. However, trophozoites avoid haem toxicity through spontaneous dimerization of haem (using histidine rich protein2, HRP2) into the inert malaria pigment haemozoin (Pagola, Stephens et al. 2000). Non-polymerized haem exits to the food vacuole and is degraded by glutathione (Ginsburg, Famin et al. 1998) and asexual division of the trophozoite nucleus continues to complete the early phase of erythrocytic schizogony.

After a period of asexual division of nucleus and cytoplasm the trophozoites develop into immature schizonts. Upon maturation, the schizonts contain merozoites and by this time (late phase of erytrocytic schizogony) the parasite occupies almost the entire red cell, which has become spherical, rigid, and depleted of hemoglobin (Cranston, Boylan et al. 1984). This then ruptures releasing the merozoites, which rapidly re-invade new red cells. The infection expands logarithmically at around ten fold per cycle (Simpson, Aarons et al. 2002). The asexual erythrocytic cycle is approximately 48 hours for *P. falciparum*, *P. vivax*, and *P. ovale*, and 72 hours for *P. malariae*.

During the late erythrocytic schizogony phase of parasite development, *Plasmodium falciparum* infection is uniquely associated with expression of a high molecular weight strain-specific variant antigen, Plasmodium falciparum erythrocyte membrane protein 1 (PfEMP1) on the surface of infected red cells. This happens at approximately 12 – 14 hours of development of the trophozoites, and the process mediates cytoadherence, attachment of the infected red cell to vascular endothelium, (Leech, Barnwell et al. 1984). PfEMP1

expression increases when the asexual erythrocytic cycle approaches the mid point (24 h) and the infected red cells progressively disappear from the peripheral circulation by attaching to the endothelial walls of venules and capillaries in vital organs. Thus, at this stage, infected red cells are sequestered away from the peripheral circulation (sequestration).

Infection by *vivax, ovale* and *malariae* species, early and late erythrocytic stages of development are seen in the peripheral blood and cytoadherence is probably not present to a significant scale. However, in all four species of human malaria parasites, the erythrocytic cycle of schizogony is repeated over and over again in the course of infection with progressive increase of parasitaemia until controlled by the hosts' immunity, death of the host, or action of antimalarial drugs. After several cycles of erythrocytic schizogony, some merozoites differentiate into the sexual forms (gametocytes) ready to be picked by a female Anophelene mosquito during a blood meal to start the parasite life cycle all over again.

1.2.1.6 Parasite Virulence

Plasmodium falciparum is the most virulent of the four human malaria parasites. The reason for this is not clearly understood. However, its capacity to multiply fast, and the ability to sequester in deep vascular beds of vital organs are thought to be important virulence factors. Interestingly, virulence appears to be limited to some and not all of the

many *Plasmodium falciparum* strains found in nature (Robert, Ntoumi et al. 1996; Kun, Schmidt-Ott et al. 1998; Smith, Felger et al. 1999).

1.2.1.7 Parasite metabolism

By and large, parasite carbohydrate metabolism has been more extensively studied than parasite lipid and protein metabolism, and this section briefly describes parasite carbohydrate metabolism.

Unlike host cells, intraerythrocytic stages of *Plasmodium* species do not store energy reserves in the form of glycogen or lipids, and glucose in the host plasma is the main source of energy (Sherman 1979). Glucose in plasma is taken up by infected red cells and directly utilized by parasites through the process of glycolysis. This process takes place in the cytoplasm of a cell where glucose is oxidized to pyruvate with the generation of ATP. In anaerobic conditions pyruvate is converted to lactate whereas in aerobic conditions pyruvate is taken up in the mitochondria and converted to oxaloacetate, which participates in the citric acid cycle. Both in the host cell and plasmodium, glycolysis is accomplished through a series of ezymatic reactions constituting the Embden-Meyerhof-Parnas (EMP) pathway and almost all the glucose taken up by infected red cells pass through the anaerobic EMP pathway with the production of $_{D}$-lactate.

The development of molecular biology tools has made it possible to clone, sequence, and express the plasmodium genes for carbohydrate metabolizing enzymes, and all the enzymes of the EMP have been identified in *P. falciparum* infected red cell extracts (Sherman 1979).

In addition, the presence of the two enzymes constituting the pentose phosphate pathway (PPP) or hexose monophosphate shunt (HMPS) glucose-6-phosphate-dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase have been demonstrated in *P. falciparum* (Hempelmann and Wilson 1981; Roth, Calvin et al. 1988). This raises the possibility for glucose metabolism through HMPS in *Plasmodium* species. The main functions of pentose phosphate pathway in the mammalian cells include provision of reducing equivalents, such as reduced nicotinamide dinucleotide phosphate (NADPH) used for the reductive synthesis of fatty acids, cholesterol and other steroids and for maintenance of glutathione in a reduced form inside red cells. The pathway is also a source of pentoses such as ribose for nucleotide and nucleic acid synthesis. Interestingly, G6PDH deficiency in host red cells is associated with protection against malaria. The reason for this is unclear. However, deficiency of pentoses for the parasites, rather than oxidative stress, has been proposed as the explanation for the protection against *Plasmodium falciparum* in individuals deficient in G6PDH (Roth, Raventos-Suarez et al. 1982).

Whether or not *Plasmodium* species possess a functional citric acid cycle is a subject for debate (Fitch, Yunis et al. 1974). However, inhibitors of electron transport and mitochondrial protein synthesis have been shown to act as antimalarial drugs. Atovaquone

for example, is known to interfere with the function of the cytochrome bc complex in *Plasmodium falciparum* (Fry and Pudney 1992). Therefore, it is widely accepted that although the intraerythrocytic stages of *Plasmodium* species mainly depend on glycolysis for energy production and lack any oxidative energy-yielding pathway, some aspects of mitochondrial functions are essential for the survival of the malaria parasites.

1.2.1.8 Malaria epidemiology

A reservoir of viable gametocytes in humans, and the contact between humans and infected mosquito vectors are the two necessary conditions for malaria transmission to humans to continue. Any infected individual can be a source of viable gametocytes but in high transmission areas children are a more likely source of viable gametocytes. They are more susceptible to malaria disease and parasite densities are usually high, and gametocytaemia is detected more frequently in children (Boudin, Lyannaz et al. 1991; Bousema, Gouagna et al. 2004). However, the relative contribution of children to overall malaria transmission in a given population is unclear.

The average number of infective mosquito bites an individual gets per year (the entomological inoculation rate or EIR), allows direct estimation of transmission, which is easy to understand and compare. In theory, EIR is one of the best ways of defining malaria endemicity. A high EIR is associated with intense transmission, and the prevalence of clinical disease increases with increased transmission intensity (Trape, Lefebvre-Zante et

al. 1993). However, parasite prevalence does not seem to change with the seasonal change in transmission intensity (Trape, Lefebvre-Zante et al. 1993; Mbogo, Mwangangi et al. 2003).

The intensity of transmission influences the rate and pattern of severe disease in children. In areas of high transmission, severe disease is rare in older children, and severe anaemia is the commonest presentation of severe disease and affects younger children (1 to 3 years). Whereas in areas of low or moderate transmission, cerebral malaria is the commonest presentation of severe disease and affects older children (above 3 years of age) (Snow, Omumbo et al. 1997). However, the risk for severe disease in children is not directly related to transmission intensity. Disease and mortality risk 'saturate' such that the cumulative risk in low-moderate transmission settings approximates that in high transmission settings (Snow, Omumbo et al. 1997).

Environmental factors that determine distribution of malaria disease are diverse and complex. As a result, falciparum malaria exhibits a patchy non-uniform distribution within an endemic zone. However, climate can be considered the major determinant of malaria disease distribution with temperature and rainfall as the major factors. In Africa, temperature and rainfall limit malaria to the warm, humid regions where mosquitoes and parasites can breed and develop, and transmission can occur.

Temperature affects the transmission cycle in many different ways, but of particular importance is the effect on the duration of the parasite sporogonic cycle and vector survival. The proportion of mosquitoes surviving the parasite's incubation period determines the lower temperature limit of transmission. Rainfall provides mosquito-breeding sites and increases humidity, which enhances mosquito survival. There is a relationship, though not direct, between rainfall and mosquito abundance and an average of 80mm of rainfall per month for at least three to five months is a reasonable requirement for annual malaria transmission (Craig, Snow et al. 1999).

Therefore, a critical level of Anopheline mosquito population, determined by climatic suitability (temperature and rainfall), is necessary for malaria transmission to occur. The African continent can be divided into areas where there is great variation in temperature and rainfall within the year and thus, highly variable and seasonal transmission (unstable transmission), and areas where there is minimal variation in temperature and rainfall (stable transmission). In the highly seasonal areas, where the annual rainfall and temperature ranges are great, mosquito populations annually drop to levels where transmission cannot be sustained. As rain sets in and temperature rises, the vector populations must recover from their annual out-of-season lows to levels where transmission can again take place (Craig, Snow et al. 1999). This requires a period of highly suitable climate, especially if the season lasts only for a few months. On the other hand, where climate is stable all year, mosquito populations do not go through the same marked annual growth and decline cycles, but persist at more stable levels.

However, fluctuations in temperature and rainfall annually is very variable at continental, regional or even village level, and the transmission pattern can only be considered as a spectrum with areas of highly stable and highly unstable transmission at the extreme ends of this spectrum (Craig, Snow et al. 1999). Therefore, although malaria is endemic in sub-Saharan Africa, the level of malaria endemicity varies from one place to the other. However, because of uneven distribution of available malaria data within and between countries, and the absence, in many countries, of reliable disease surveillance systems ("World malaria situation in 1991. Part I" 1993) and the paucity of long-term surveillance studies for accurate estimation of EIRs, the picture of malaria endemicity in Africa is incomplete.

The situation has improved in recent years through the mapping malaria risk in Africa (MARA) initiative. MARA is combining empirical data collection and modeling to map out the malaria risk in Africa, and in 1990 it was estimated that about 66 million children (0 – 4 years) living in areas 50 % suitable for stable malaria transmission were exposed to malaria mortality risk, whereas about 54 million living in areas 90 % suitable for stable malaria transmission were exposed to mortality risk (Snow, Craig et al. 1999b).

The ability to develop immunity to malaria early in life is the single most important human factor which determines the pattern of disease (Snow, Omumbo et al. 1997). Unfortunately, this immunity is imperfectly acquired and does not give total protection against disease,

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rather, it is a clinical immunity associated with a reduced risk for severe disease. This immunity is acquired during childhood through repeated attacks of clinical disease and persists through adolescence to adulthood (Hogh 1996; Snow and Marsh 1998). In high transmission areas, most mothers are semi-immune and babies born to these mothers acquire maternal antibodies, before birth, notably *P. falciparum* specific IgG (Sehgal, Siddjiqui et al. 1989; Deloron, Dubois et al. 1997). In general, these antibodies wane by the time the baby is five months of age. Therefore, babies below six months of age rarely present with clinical or severe disease. On the other hand, children between six months and five years have not yet acquired clinical immunity and suffer repeated infections and are at highest risk for severe disease.

The pattern of malaria disease in African children is such that most children get repeated infections and most of these infections progress to clinical disease and a minority of clinical disease progress to severe disease. After five years of age, children still get repeated infections but a minority of these infections progress to clinical disease and severe disease is rare. The pattern of severe disease has been shown to vary according to the intensity of malaria transmission: in areas of low malaria endemicity, severe disease is dominated by cerebral malaria in children over two years of age, whereas in areas of intense transmission, severe malaria is dominated by severe malarial anaemia in young children and infants (Snow, Omumbo et al. 1997). Malaria is governed by a large number of environmental factors, which affect the distribution, transmission intensity, disease outcome, small-scale variation and seasonality. Therefore, the pattern of disease is a result of the complex

interaction between the parasite, the vector, the environment (risk of exposure) and the human host (ability to develop clinical immunity early in life).

1.2.1.9 Malaria disease burden in African children

In 1994, WHO reported that malaria was causing 1-2 million deaths and 300-500 million clinical episodes annually ("World malaria situation in 1991. Part I" 1993) and of the global deaths due to malaria about 90% occurred in Africa most of them in children under five years of age. These estimates were derived mainly from proxy parameters such as the number of childhood febrile episodes reported by parents during field surveys and there have been concerns that these figures may have been an underestimation of the true malaria burden in African children.

More recent estimates using data from carefully defined studies and population estimates using Geographic Information Systems (GIS) (Snow, Craig et al. 1999a), and accounting for children not seeking health care have shown that between 890,000 and 2.3 million children die from all forms of malaria annually in sub-Saharan Africa (Murphy, and Breman 2001). In this meta-analysis of malaria deaths attributable to the different forms of severe malaria, metabolic acidosis (the focus of this thesis) was associated with 6 % of childhood malaria deaths, the same proportion as cerebral malaria (figure 1.2). Severe anaemia had the largest contribution, but metabolic acidosis (respiratory distress) is a recently described form of severe malaria and few studies included in this meta-analysis

may have reported respiratory distress. In addition, the overlap between respiratory distress and severe anaemia is still poorly described and mortality in children with severe anaemia increases greatly with the presence of respiratory distress (figure 1.3). Therefore, it is likely that respiratory distress (acidosis) had a significant contribution to the 53 % of malaria deaths attributed to severe anaemia reported in this meta-analysis.

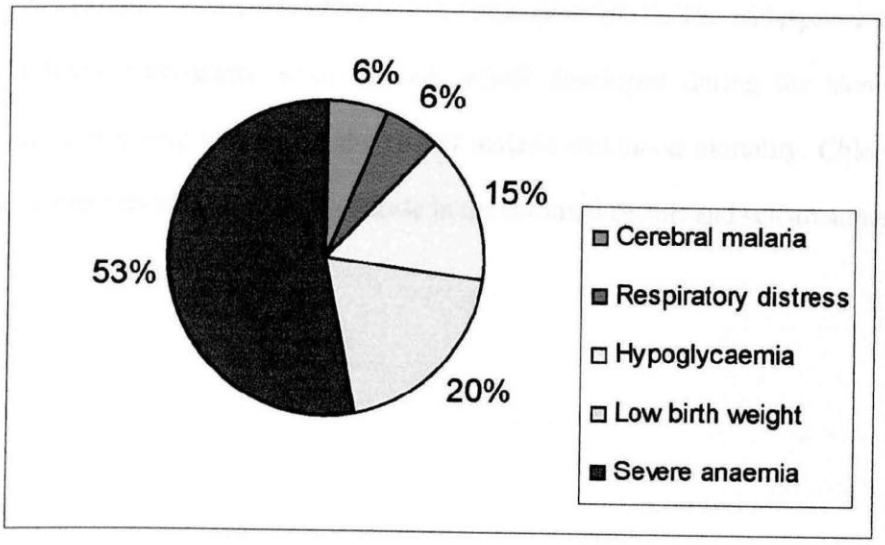


Figure 1.2: Contribution of specific forms of severe malaria to African childhood malaria mortality

Modified from Breman J. G. (Breman 2001)

Although genetic factors and individual and population immunity to malaria are the most important human host determinants of the malaria burden, parasite drug resistance appears to be a very significant determinant of malaria burden in Africa. Against a consistent downward trend in overall childhood mortality in endemic areas with stable malaria transmission in Africa, malaria mortality had risen proportionately as an attributable cause of death in childhood since 1990 compared with the periods before 1960 and between 1960 and 1989 (Trape, Pison et al. 1998; Snow, Trape et al. 2001). The widespread development of chloroquine resistance across Africa, which developed during the same period, is considered one single factor for the rise of malaria childhood mortality. Chloroquine was cheap, extremely effective, and available in the informal sector, and self-treatment was

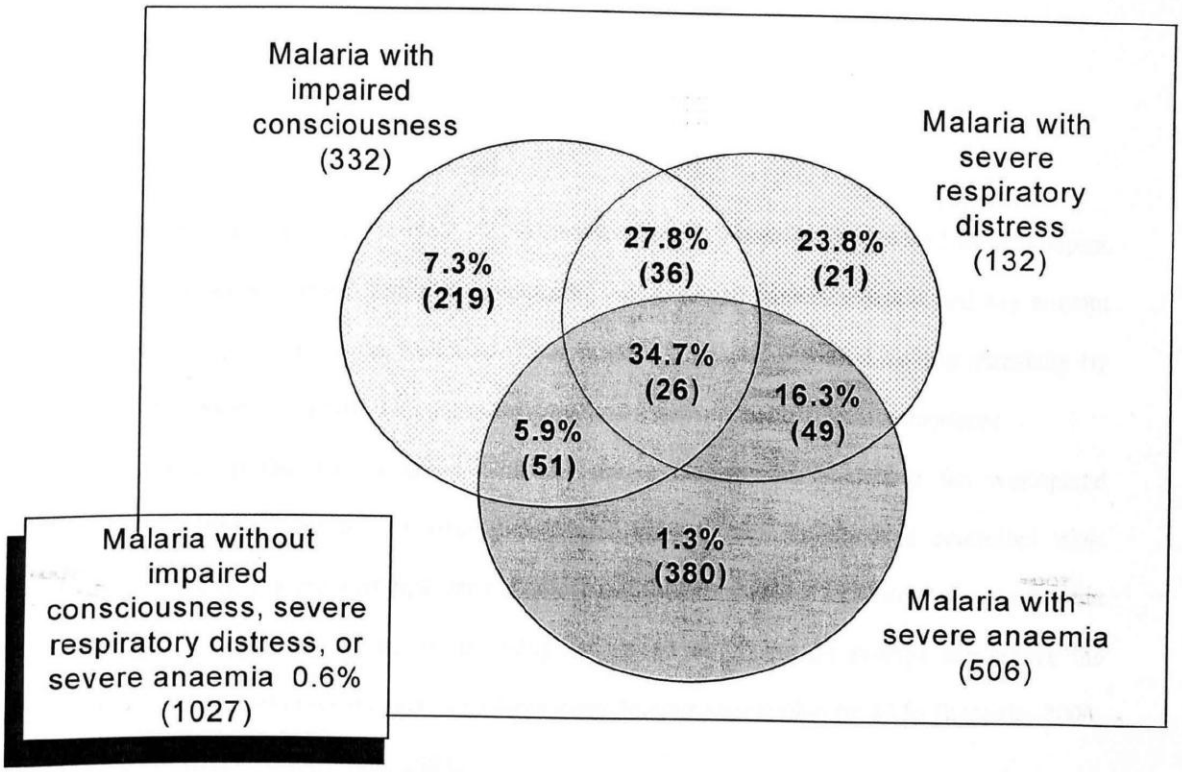


Figure 1.3: Clinical features on presentation and outcome of children with malaria to Kilifi District Hospital showing a significant increase in mortality when respiratory distress is present. **Adapted from (Marsh, Forster et al. 1995)**

popular before the 90s. Increased childhood malaria mortality associated with development of chloroquine resistance reflects the reliance almost entirely on the treatment of clinical episodes of malaria for malaria control in Africa before the Roll Back Malaria (RBM) initiative.

As described under section 1.2.1.9 above, malaria burden is determined by a complex interaction between human host, parasite, mosquito and the environment and any attempt towards achieving the Roll Back Malaria (RBM) goal of reducing malaria morbidity by half by the year 2010 will depend on new and improved malaria control measures targeting the various determinants of the malaria burden including the widespread distribution and use of insecticide treated nets (ITNs). Randomised controlled trials conducted in areas of different malaria transmission intensities in Africa have shown that ITNs can reduce childhood mortality by around 20 % and on average can reduce the incidence of clinical attacks of *Plasmodium falciparum* infection by 50 % (Lengeler 2000; ter Kuile, Terlouw et al. 2003).

However, prompt and effective treatment of clinical attacks of malaria is still a critical element of malaria control ("Global malaria control strategy" 1993). It is widely recognised that untreated falciparum malaria contributes directly and indirectly to death of non-immune individuals, sometimes within hours after the onset of symptoms (Greenwood, Bradley et al. 1987). In sub-Saharan Africa, where most malaria is due to *Plasmodium falciparum* and thus, potentially fatal, it is critical that children presenting with malaria start treatment within 24 hours of onset of symptoms to prevent progression to severe disease, and the management of those who develop severe disease is improved.

Certainly, prompt and effective treatment of childhood malaria together with improved management of severe disease will save many lives. Improved management of severe

disease may be achieved through improved understanding of pathophysiological mechanisms underlying the syndrome of severe childhood malaria. Although it is now established that metabolic acidosis is common and a strong predictor of mortality in severe childhood malaria (Taylor, Borgstein et al. 1993; Marsh, Forster et al. 1995; Allen, O'Donnell et al. 1996; English, Sauerwein et al. 1997), its underlying pathophysiologic mechanism remain poorly understood and no specific intervention (s) have been developed. Since most childhood malaria deaths occur within 12 to 24 h of admission (Marsh, Forster et al. 1995; Waller, Krishna et al. 1995; Allen, O'Donnell et al. 1996), it is argued that even modest improvements in management during the first 24 h such as introducing specific treatment for acidosis may improve survival (Newton, Taylor et al. 1998). In critically ill patients, the adverse effects of acidaemia (low blood pH) especially those on the cardiovascular system are considered to be the likely cause of death associated with acidosis (Swenson 2001), and the reversal of such effects in children with severe malaria and acidosis may be beneficial.

Therefore, the major focus of this thesis was to characterise metabolic acidosis in children with malaria in view of identifying key underlying mechanism(s) so that potential beneficial clinical interventions could be identified and proposed for testing in clinical trials.

1.2.1.10 Pathogenesis of severe childhood malaria

Understanding the pathogenesis of severe malaria is essential for improving its management. However, the pathophysiological aspects of severe malaria are complex and remain poorly understood. Nevertheless, the known possible mechanisms can be grouped into two main general mechanisms. First, are mechanisms associated with impaired micro vascular circulation that include sequestration, rosetting, aggregation, and reduced red cell deformability. Second, are those associated with the host pro-inflammatory response to *Plasmodium falciparum* antigens and other mediators of malaria severity i. e. cytokines, nitric oxide (NO), and reactive oxygen species (ROS).

I. Mechanisms associated with impaired micro vascular circulation

Sequestration

This is a phenomenon where parasitised red blood cells (PRBC) sequester into deep micro vascular beds of vital organs especially the brain. It is mediated through a process known as cytoadherence. As stated in the discussion on parasite life cycle in section 1.2.1.6, during the second half of the erythrocytic stage, *Plasmodium falciparum* synthesises proteins, which the parasite is able to insert into the surface of the red cell (van Schravendijk, Rock et al. 1991). The most studied of these proteins is the PfEMP1 family (Flick and Chen 2004). Red blood cells with PfEMP1 on their surface stick to the surface of vascular

endothelial cells by binding to host receptors present on these endothelial cells. The currently identified host receptors include intercellular adhesion molecule 1 (ICAM1) found mainly in the brain, CD36, found mainly in other tissues, and chondroitin sulfate A (CSA) found mainly on the placenta (Sherman, Eda et al. 2003). Red cells containing mature forms of *Plasmodium falciparum* (trophozoites and schizonts) that are stuck to endothelial cells are removed from the circulation and are hidden in these deep vascular beds evading the immune system (Sherwood, Roberts et al. 1986) until the schizonts rupture to release merozoites, which invade new circulating cells to start another cycle.

Sequestration occurs in the microvasculature (capillaries and venules) reducing lumen diameter and causing mechanical obstruction to blood flow. It has been recently reported that PRBC containing immature forms of the parasite (ring stages) can also sequester but at a much lesser degree than cells containing mature parasites (Pouvelle, Buffet et al. 2000). Since sequestration has been observed (*in vivo, ex vivo* and postmortem studies) only with *Plasmodium falciparum* and not with the other human malaria parasites which cause only mild disease, it is considered to be a feature of severity. Therefore, sequestration is considered to be central to the pathophysiology of falciparum malaria, particularly cerebral malaria, where mechanical obstruction is hypothesised to cause local ischemia leading to anaerobic glycolysis, production of lactic acid, coma, and death (Clark, Cowden et al. 1994).

However, regaining full normal neurological function after cerebral malaria in the majority of patients with coma (Molyneux 1990) is against this hypothesis (Clark, Rockett et al. 1992). Ischemic brain damage from other causes such as cerebral vascular accident is frequently associated with severe neurological deficits. However, the few published postmortem series done on patients dying of cerebral malaria, have demonstrated evidence for sequestered parasites in the brain. In addition, a recent large postmortem series of studies using electron microscopy, and applying quantitative as well as qualitative measures of pathological changes, provided evidence for a correlation between endothelial activation as well as sequestration and clinical disease (coma score) (Pongponratn, Turner et al. 2003). The authors concluded that sequestration is necessary but on its own is not sufficient to cause cerebral malaria. The inconsistent findings of studies of the relationship between cytoadherence measured *ex vivo* and severity of infection or clinical disease (Ho and White 1999) supports this hypothesis. Therefore, it is very likely that some other subtle metabolic or immunological processes such as the host inflammatory response to the parasites may have a role to play in the pathogenesis of cerebral malaria (see also section 1.2.2.1).

Rosetting

This is a phenomenon observed in *in vitro* experiments where two or more non-parasitised red cells adhere to a parasitised red cell forming rosettes. These can be viewed under the microscope when a suspension of parasitized erythrocytes is mounted (David, Handunnetti et al. 1988). Rosetting is mediated by the attachment of specific domains of PfEMP1 on

non- parasitised red cell surface molecules such as the compliment receptor CR1(Krych-Goldberg, Moulds et al. 2002). Unlike cytoadherence, rosetting has been demonstrated in all four human malaria parasite species (Udomsanpetch, Thanikkul et al. 1995; Angus, Thanikkul et al. 1996). However, like cytoadherence, in *Plasmodium falciparum*, rosetting starts during the second half of the blood development stage of the parasites slightly after cytoadherence has started (Treutiger, Carlson et al. 1998). Although all fresh isolates of *Plasmodium falciparum* do cytoadhere, not all isolates form rosettes. An association between malaria disease severity and rosetting has been reported in some studies but not in others (Carlson, Helmby et al. 1990). In addition, the parasite's ability to rosette was associated with severe disease in Kenyan children (Rowe, Obeiro et al. 1995) whereas no such association was observed in one study in Papua New Guinea (al-Yaman, Genton et al. 1995).

Aggregation

This is a cytoadherence property that has recently been reported amongst Kenyan children, where an association between aggregation and severe disease has been demonstrated (Pain, Ferguson et al. 2001). Aggregation is a phenomenon where parasitised red cells clump together in *ex vivo* cultures. It is mediated by platelets via the platelet surface molecule CD36, and is thought to contribute to vascular occlusion.

Reduced red cell deformability

Reduced red cell deformability is a phenomenon where red cells become less flexible and unable to pass through the microvasculature with luminal diameters less than 7.5 μm, the diameter of normal red blood cells. In 1983, Cranston and colleagues and later Nash and others reported that *Plasmodium falciparum* parasitized red blood cells become progressively less deformable as the intraerythrocytic parasite matures (Nash, O'Brien et al. 1989). Associated changes in red cells include change in shape from the normal biconcave disc to a more spherical shape and the increase in red cell membrane rigidity. In fact, all parameters determining deformability under normal circumstances seem to be affected by *Plasmodium falciparum* parasitisation (Paulitschke and Nash 1993).

Suggestions that reduced red cell deformability might also be present in the non-parasitized cells of an infected individual came from *ex vivo* studies. In these studies, it has been demonstrated that the filterability of red blood cells from patients with non-severe malaria is reduced (Areekul and Yamarat 1988). More recently, it has been shown in Thai adults that non-parasitized red blood cells become significantly more rigid with severe malaria and the changes in red cell membrane are the most likely cause of loss of deformability (Dondorp, Angus et al. 1997).

Anaemia and mortality are the two clinical correlates of reduced red cell deformability that have been reported in severe malaria. In one study, reduced red cell deformability was a

strong predictor of mortality in adults with severe malaria (Dondorp, Angus et al. 1997) whereas in another, reduced red cell deformability was shown to be closely correlated with anaemia (Dondorp, Angus et al. 1999). In addition, improvement in red cell deformability following blood transfusion has been reported amongst Kenyan children with severe malaria (Dondorp, Nyanoti et al. 2002). However, therapeutic interventions to improve red cell deformability are still in development (Singh and Kumaravel 1996).

In conclusion, impaired microcirculatory flow may be indeed central to the pathophysiology of severe malaria. Cytoadherence with subsequent sequestration; possible aggregation of other parasitized cells; and rosetting of the on coming non-parasitized cells with reduction in their deformability, all add up to substantially reduce luminal diameter of capillaries and venules. This may be particularly so in body organs where sequestration is intense such as the brain in patients with cerebral malaria.

II. Mechanisms associated with host pro-inflammatory response to *Plasmodium falciparum* antigens and other mediators of malaria severity

Cytokines

The malaria parasites release of toxins during schizont rupture has been hypothesized for many years to be the cause of the symptoms of malaria. For example, in 1981 Clark and others proposed that cytokines such as tumour necrosis factor (TNF) and interleukin 1 (IL-

1) are toxic when overproduced and could cause syndromes such as those seen in human malaria (Clark, Virelizier et al. 1981). It has subsequently been suggested that malaria parasites induce release of cytokines from host immune cells in a mutually amplifying chain reaction (Silamut and White 1993). TNF, which plays a pivotal role, is released initially and induces the release of IL-1 as well as interferon gamma (IFγ). These in turn induce the release of other pro-inflammatory cytokines, IL-8, IL-12, and IL-18. This release of pro-inflammatory cytokines is balanced by the release of anti-inflammatory cytokines, notably IL-6 and IL-10.

Although no malaria toxin has so far been identified, a candidate toxin of parasite origin, glycosylphosphatidylinositol (GPI), has recently been suggested, and is under investigation (Clark, and Schofield 2000). Clinical studies on the role of TNF in disease severity or outcome have been focused on cerebral malaria, and a positive correlation between circulating TNF levels and disease severity and outcome has been demonstrated in African children with cerebral malaria (Kwiatkowski, Hill et al. 1990). However, causality has not been established, and circulating TNF levels are high in both falciparum and vivax infections. In fact, higher TNF levels have been reported in vivax than falciparum infections (Karunaweera, Grau et al. 1992), yet vivax infections are not associated with severe disease. Through induction of increased vascular nitric oxide (NO) production, TNF has been hypothesized to cause coma in cerebral malaria (Clark, Rockett et al. 1992), but there is no direct evidence for this mechanism in humans despite direct evidence in murine

models (Grau, Piguet et al. 1989). Indeed, the use of anti-TNF antibodies in children with cerebral malaria did not improve clinical outcome (Luty, Perkins et al. 2000).

Host genetic factors may play a role in the interaction between malaria parasites and cytokines. For example, Gambian children with the (308 A) TNF2 allele, a polymorphism of the TNF promoter region, had a higher relative risk for death or neurological sequelae from cerebral malaria (McGuire, Hill et al. 1994) than children without this polymorphism. In addition, severe malaria anaemia has been associated with chronic low level of cytokines in repeated malaria attacks, and children with another polymorphism, allele (238 A) had increased risk for severe anaemia (McGuire, Knight et al. 1999).

All these findings suggest a role for TNF and other cytokines in the pathogenesis of severe malaria. However, a causal relationship between increased cytokine levels and severe malaria remains undetermined. It is likely that the complexity of the cytokine parasite interaction is responsible for the difficulty in interpreting circulating cytokine levels. The current methods for measuring cytokine levels do not allow for the determination of the bioactive fractions and it is possible that what matters is the local tissue cytokine production rather than the level of systemically circulating cytokines.

Nitric Oxide

The return to full neurological function after cerebral malaria in most cases prompted Clark and others to theoretically compare coma in cerebral malaria with coma in non-infectious clinical conditions (heat stroke, general anaesthesia, ethanol intoxication and hepatic encephalopathy) in which, coma is completely reversible, and hypothesise on the eatiology of coma. They hypothesized that nitric oxide (NO) has a central role in the causation of coma in these clinically similar conditions (Clark, Rockett et al. 1992). High levels of circulating cytokines could induce an increase in the production of inducible nitric oxide synthase (iNOS), an isoform of nitric oxide synthesizing enzymes, and this would lead to an increase in nitric oxide production from cerebral vascular endothelial cells. Nitric oxide could then diffuse into brain cells disrupting neuronal NO dependent excitatory neurotransmission causing coma.

However, NO is a short-lived molecule and freely diffuses across membranes, and direct measurement of its levels in body fluids is difficult. As a result, testing this hypothesis has proved challenging. However, measurement of products of NO metabolism has been used. For example, workers in Papua New Guinea described elevated nitrogen intermediates in children with cerebral malaria compared with conscious children, and higher levels in fatal cases than survivors (Al Yaman, Mokela et al. 1996). Interestingly, no such association has been reported in African children (Agbenyega, Angus et al. 1997; Dobbie, Crawley et al. 2000). Another study in Tanzanian children, demonstrated low levels of nitrate and nitrite

(more stable end products of NO metabolism) in children with cerebral malaria compared with uncomplicated malaria or sub-clinical infections (Anstey, Weinberg et al. 1996).

From these studies, it can be concluded that despite scientifically plausible hypothesis, evidence for the role of NO in the pathogenesis of severe malaria is still conflicting.

Reactive Oxygen Species

Reactive oxygen species (ROS) appear to be generated in acute malaria infections in response to oxidative stress. Increased monocyte generation of ROS has been associated with severe malaria in adults (Descamps-Latscha, Lunel-Fabiani et al. 1987). In addition, ROS have been associated with severe malarial anaemia in children (Greve, Kremsner et al. 2000; Griffiths, Ndungu et al. 2001). However, the relationship between severe malaria and ROS has not been extensively studied, and the role of ROS in the pathophysiology of severe malaria remains unclear.

In summary, the available literature on pathopysiology of severe childhood malaria suggests a central role for cytokines. First, cytokines have been shown to up-regulate the expression of host endothelial receptors (Malhotra, Salmon et al. 1988), notably ICAM1 in cerebral microvasculature, promoting cytoadherence and sequestration. This in turn is responsible for reduced tissue oxygenation, anaerobic glycolysis, and lactic acidosis. Second, TNF is hypothesized to induce increased expression of iNOS leading to increased

NO in brain cells and interfering with normal excitatory neurotransmission. And thirdly, cytokines activate leukocytes and possibly other cells to release ROS (Malhotra, Salmon et al. 1988). However, NO together with ROS mediate parasite killing (Wei, Charles et al. 1995) and this is beneficial to the host. It would appear that in childhood falciparum malaria infections, moderate production of inflammatory cytokines might be beneficial whereas overproduction may be harmful, and severe childhood malaria may be a result of an imbalance in production between pro-inflammatory and anti-inflammatory cytokine. However, varied functions of cytokines, NO and ROS have been reported, and recent reports suggest that NO is the most versatile player in the immune system (Bogdan 2001) and is involved in the pathogenesis and control of infectious and non-infectious diseases. Thus, parasite-cytokine interaction may be more complex than is currently known.

1.2.1.11 Clinical features of severe childhood malaria

Before 1990 the WHO defining criteria for severe malaria in children were based on descriptions of severe disease in adults, mostly from studies in South-East Asia (Warrell, Molyneux et al. 1990). However, there have been several studies in African children since then (Marsh, Forster et al. 1995; Waller, Krishna et al. 1995; Allen, O'Donnell et al. 1996) that have provided a comprehensive clinical description including prognostic features such as metabolic acidosis (Molyneux, Taylor et al. 1989; Taylor,., Borgstein et al. 1993; Marsh, Forster et al. 1995; Waller, Krishna et al. 1995) . As a result, the WHO defining criteria

have been modified with clear differences between severe malaria in children and adults (Table 1.1).

Based on these studies in African children, it is now widely accepted that severe malaria in children is a multi-system disease with three overlapping syndromes: impaired consciousness, severe respiratory distress (metabolic acidosis), and severe anaemia (Marsh, Forster et al. 1995). Neurological manifestations of severe malaria in children, which was formerly described generally as cerebral malaria, is now known to represent different distinct entities including prostration (too weak to sit up unaided or breast-feed for infants less than nine months), prolonged postictal stage of malaria associated seizures, covert status epilepticus, and true encephalopathy (Marsh, Forster et al. 1995).

Table 1.1: Differences between severe malaria in adults and children
(Adapted from Management of severe malaria: a practical handbook) (WHO 2000)

Sign or symptom	Adults	Children
History of cough	Uncommon	Common
Convulsions	Common	Very common
Duration of illness	5 – 7 days	1 – 2 days
Resolution of coma	2 – 4 days	1 – 2 days
Neurological sequelae	< 5 %	> 10 %
Jaundice	Common	Uncommon
Pretreatment hypoglycaemia	Uncommon	Common
Pulmonary oedema	Uncommon	Rare
Renal failure	Common	Uncommon
CSF opening pressure	usually normal	usually raised
Respiratory distress (Acidosis)	Sometimes	Common
Bleeding/clotting disturbances	Up to 10 %	Rare
Abnormality of brain stem Reflexes (e.g. oculovestibular, Oculocervical)	Rare	More common

Cerebral malaria

Until recently cerebral malaria was the best recognized and the most studied complication of severe malaria. However, the definition varied considerably between studies, and did not take into account any differences in presentation between adults and children. The recent comprehensive clinical description of severe malaria in children has lead to the development of a more agreeable definition so that results from different studies are comparable. Thus, cerebral malaria is now defined as the presence of unarousable coma in a child with fever (or recent history of fever) and presence of asexual forms of *P. falciparum* in the peripheral blood, after correction of hypoglycaemia, and exclusion of bacterial meningitis (Warrell, Looareesuwan et al. 1982; Warrell, Molyneux et al. 1990) and other locally common viral encephalopathies. Fever, coma, and parasitaemia are the key features, although the depth of coma has been variously defined.

However, Molyneux and colleagues (Molyneux, Taylor et al. 1989) modified the Glasgow coma-scoring scale into a suitable scale for children - the Blantyre coma score, to promote standardisation. This coma score (Table 1.2) is currently widely accepted and used for assessing children with coma particularly cerebral malaria. A summated score of ≤ 2 on the Blantyre coma scale in the presence of the other cerebral malaria defining criteria is consistent with a clinical diagnosis of cerebral malaria. Although considerable disagreement while assessing the scoring scale has been reported (Newton, Chokwe et al. 1997), the scoring system is based on simple bed side signs, and is reasonably reproducible

and sufficient to identify children with cerebral malaria. However, two shortcomings make this scoring scale prone to over diagnosis of cerebral malaria. First, children less than 9 months of age may not have developed the ability to localize a painful stimulus (Newton, Chokwe et al. 1997). And second, the scoring needs to be done at least one hour after a convulsion to avoid including children in a postictal state, (Molyneux, Taylor et al. 1989). Such children will score poorly initially but will regain consciousness quickly.

The cause of coma in childhood cerebral malaria is a subject for debate. Whereas systemic conditions like hypoglycaemia, seizures, hyperpyrexia, anemia and metabolic acidosis would not represent a direct effect of the parasite on the brain, they are potential explanatory factors (Newton, Taylor et al. 1998). Raised intracranial pressure has been observed(Newton, Kirkham et al. 1991; Waller, Crawley et al. 1991) and brain swelling has been demonstrated on CT scanning (Newton, Peshu et al. 1994) in African children with cerebral malaria. However, it remains unclear whether intracranial hypertension is a primary event in the pathogenesis of malarial coma. Using direct ophthalmoscopy, retinal haemorrages may be seen in up to 28 % of the cases (Molyneux, Taylor et al. 1989; Newton, Kirkham et al. 1991). Although findings from some studies have suggested an association between fatal outcome and retinal haemorrages (Looareesuwan, Warrell et al. 1983; Olumese, Adeyemo et al. 1997), studies in Malawi suggested that papilloedema and extra-macular retinal oedema, were the two retinal findings of prognostic significance in childhood cerebral malaria (Lewallen, Bakker et al. 1996).

In general, it appears that any neurological impairment, not necessarily deep coma, is associated with poor outcome (Molyneux, Taylor et al. 1989; Marsh, Forster et al. 1995; Waller, Krishna et al. 1995) and it has been argued that in clinical practice, any neurological impairment, defined as prostration or a Blantyre coma score of <5 (Table 1.2), in a child with malaria is an indication for intensive care, and parenteral antimalarial treatment (Newton, Hien et al. 2000).

Classically, a child with cerebral malaria presents with a history of fever of one to two days duration, which is associated with multiple convulsions that usually precipitate coma and hospital admission (Molyneux, Taylor et al. 1989). Acute bacterial meningitis is a common differential diagnosis of cerebral malaria in malaria endemic areas, and must be actively ruled out. For example, in Kenyan children, 4 % of children with impaired consciousness and a positive slide for malaria parasites also had pathogenic bacteria identified in CSF whereas 14 % of children less than one year of age with impaired consciousness and a positive slide for malaria parasites also had CSF culture-confirmed meningitis (Berkley, Mwangi et al. 1999). On the other hand, lack of diagnostic facilities in many African hospitals limit the exclusion of viral causes of neurological impairment.

Table 1.2: Blantyre coma scale[c]

Best motor response		Best verbal response		Eye movements	
Localizes painful stimulus[d]	2	Normal cry	2		
Withdraws from a painful stimulus	1	Abnormal cry	1	Follows a moving object	1
Extends, or has no response to a painful stimulus	0	No verbal response	0	Does not follow a moving object	0

From: Molyneux, Taylor et al. 1989

[c] The total score is the sum of the individual scores from the three categories
[d] The standard painful stimulus is firm pressure on a nail bed, the sternum, or the supra-orbital ridge.

Other clinical features in childhood cerebral malaria, include hypertonic posturing (decerebrate and decorticate rigidity, opisthotonus), and occulocephalic brain stem signs. Children with cerebral malaria are not spared from other features of severe malaria, and features of shock such as cold extremities and severe respiratory distress are particularly common (Marsh, Forster et al. 1995). In some studies, severe anaemia occurring with cerebral malaria has been shown to be associated with poor outcome (Genton, al-Yaman et al. 1997). For unknown reasons, African children with cerebral malaria are older compared to those with other syndromes of severe malaria (severe malarial anaemia or respiratory distress) (Marsh, Forster et al. 1995).

Despite appropriate parenteral antimalarial treatment and supportive care, cerebral malaria mortality remains high (Marsh, Forster et al. 1995). The cause of death is usually not clear, but progressive deterioration in brain stem function leads to cardiac or respiratory arrest in some children with cerebral malaria. Up to 10 % of children with cerebral malaria are discharged home with neurological sequelae and in at least half of them neurological sequelae resolve in 4 – 6 weeks (Brewster, Kwiatkowski et al. 1990). Neurological sequelae in cerebral malaria include cellebelar ataxia, hemiparesis, speech disorders, cortical blindness, hypotonia, behavioural disturbances and generalized spasticity.

Seizures

Seizures commonly accompany cerebral and non-cerebral childhood falciparum malaria. Up to 31 % of Kenyan children admitted to hospital with malaria have seizures as part of the presenting symptoms (Waruiru, Newton et al. 1996). However, seizures are more common in children with strictly defined cerebral malaria where up to 60% may have seizures (Crawley, Smith et al. 1996). Seizures in childhood malaria are of various types: partial motor, generalized tonic clonic, partial with secondary generalization, and subtle motor seizures (Crawley, Smith et al. 1996). They may be prolonged or repetitive, and up to 54 % may occur while the core body temperature is below 38 ^{0}C (Waruiru, Newton et al. 1996). For these reasons, seizures in malaria are less likely to be simple febrile seizures although a small proportion may be. In childhood cerebral malaria, recurrent or prolonged seizures are associated with death and neurological sequelae (Brewster, Kwiatkowski et al. 1990; Genton, al-Yaman et al. 1997; Carter, Ross et al. 2005). Seizures associated with a long postictal phase, and seizures too subtle to be clinically detectable are responsible for coma observed in some children with cerebral malaria and in this group, seizure treatment with anticonvulsant is associated with rapid regain of consciousness (Crawley, Smith et al. 2001).

The cause of seizures in falciparum malaria remains unclear, but as noted above, fever may account for a minority of them. Metabolic and electrolyte derangements such as

hyponatremia and hypoglycaemia do not seem to be associated with seizures(Newton, Taylor et al. 1998). In Thai children, *Plasmodium vivax* infection was found to be less associated with seizures (Wattanagoon, Srivilairit et al. 1994), and it is thought that *Plasmodium falciparum* may be epileptogenic in its own right. Since seizures are common in childhood falciparum malaria, and are associated with neurological sequelae, they are an important cause of mental handicap in endemic areas. Therefore, treatments aimed at controlling or preventing seizures in falciparum malaria are of public health importance. An assessment of the use of 20mg per kg, phenobarbitone for seizure prophylaxis in childhood falciparum malaria with impaired consciousness, was associated with reduction in seizure frequency (Crawley, Waruiru et al. 2000). However, there was increased mortality in the treatment group and further studies have been done to look into ways of optimizing the use of anticonvulsants in seizures associated with childhood falciparum malaria (Kokwaro, Ogutu et al. 2003).

Impaired consciousness and prostration

Not all children with falciparum malaria presenting with neurological manifestations will fit the strict definition of cerebral malaria. Two groups of other neurological manifestation apart from cerebral malaria can be identified. First, children with altered consciousness who do not score maximum on the Blantyre coma score, but with depth of coma which is not enough for them to be classified as cerebral malaria patients. Second, are children who are fully conscious but hypotonic and too weak to sit up unaided or

breast-feed (younger infants), i. e. the group of children with prostration. For example, in a study of Gambian children with severe malaria and neurological manifestation, 32 % of patients in this study had impaired consciousness, whereas 26 % had prostration (Waller, Krishna et al. 1995). Although most children with prostration or impaired consciousness will improve in level of consciousness upon initiation of appropriate treatment, deterioration occurs in some cases (Waller, Krishna et al. 1995). In Kenyan children with severe malaria, mortality was 5 % in those with impaired consciousness or prostration (Marsh, Forster et al. 1995). Therefore, although cerebral malaria carries the highest risk of dying, other neurological manifestations of severe malaria are associated with sequelae and poor outcome.

Prostration can easily be assessed at the bedside by trying to get the child to sit up unaided or breast feed, and where health workers are trained, prostration has proved to be a simple and reproducible clinical test. However, neurological manifestations in childhood falciparum malaria do not often occur in isolation, but in association with other features of severe disease, and the prognosis seems to be influenced by the presence of these other features of severe disease (figure 1.3).

Respiratory distress

One important contribution of studies comprehensively describing severe malaria in African children has been the recognition of respiratory distress as a separate entity in

the syndrome of severe malaria. Traditionally, breathlessness and respiratory signs were associated with heart failure secondary to severe anaemia, or pulmonary oedema complicating severe malaria mainly in adults. None of the studies on severe childhood malaria in Africa or Papua New Guinea has unequivocally documented signs of heart failure or pulmonary oedema (Marsh, Forster et al. 1995; Waller, Krishna et al. 1995; Allen, O'Donnell et al. 1996). By contrast, respiratory distress was observed in children without severe anaemia, and instead, a severe metabolic acidosis was found to commonly underlie respiratory distress in children with severe malaria. More importantly, metabolic acidosis was found to be a strong independent predictor of death in these patients (Marsh, Forster et al. 1995; Waller, Krishna et al. 1995; Allen, O'Donnell et al. 1996).

Signs of respiratory distress in severe childhood malaria may include increased rate of breathing, nasal flaring, subcostal-indrawing and deep (Kussmaul's) breathing. However, in Kenyan children with severe malaria subcostal indrawing and deep breathing were most reliably associated with metabolic acidosis (Marsh, Forster et al. 1995).

Besides, deep breathing has been shown to have good inter-observer agreement and high sensitivity and specificity for detecting severe metabolic acidosis (English, Murphy et al. 1995; English, New et al. 1996). Therefore, with trained health workers, deep breathing can be used as a bedside sign to detect and appropriately treat (if treatment is widely available) children with acidosis in many hospitals in Africa where assessment of acid-

base status by a blood test may not be possible. Metabolic acidosis in malaria is the focus of this thesis, and it is further described in section 1.2.2.3.

Severe malarial anaemia

Severe malarial anaemia in children has long been recognized and is defined by a hemoglobin concentration below 5g per dl occurring with peripheral parasitaemia. Loss of red cells and marrow suppression due to cytokine imbalance (Perkins, Weinberg et al. 2000) are the two main mechanisms implicated for its development. The causes of red cell loss in childhood falciparum malaria include the loss of red blood cells resulting from rupture of PRBC during schizogony, splenic clearance of PRBC, and shortened lifespan of non-parasitised red cells. The shortened life span of non-parasitized red cells is thought to result from immune mediated mechanisms such as reduced red cell deformability and the destruction of the red cell membrane by reactive oxygen species (Greve, Kremsner et al. 2000). Some data have suggested that severe malaria suppresses the protective effect of TGF-β1 and IL-12 and overproduction of TNF-α promotes the development of severe anaemia (Perkins, Weinberg et al. 2000).

In severe malaria with hyperparasitaemia (more than 20 % of red cell infected), the rate at which anaemia develops may be in a matter of hours. In most fatal cases, other signs accompany severe anaemia and when respiratory distress is present, the risk of death is increases considerably (figure 1.3).

Hypoglycemia

Characteristic clinical signs of hypoglycaemia cannot be distinguished from those of severe malaria, and hypoglycaemia is present when the blood glucose is 2.2mmol/l or less. Hypoglycaemia is a common complication of severe malaria in children and affects up to 32 % of these patients (White, Miller et al. 1987). It commonly occurs in association with acidosis and coma (White, Miller et al. 1987) and carries prognostic significance (Genton, al-Yaman et al. 1997; Schellenberg, Menendez et al. 1999).

Fever, repeated convulsions, and parasite metabolic processes greatly increase glucose demand in these children (White, Miller et al. 1987). In addition, glycogen stores in children are limited, and they can only last for 12 hours without food intake (Wolfsdorf, Sadeghi-Nejad et al. 1982). Studies have shown that insulin secretion is appropriately suppressed and lactate and ketone levels are appropriately raised (White, Miller et al. 1987; Taylor, Molyneux et al. 1988) in African children with severe malaria and hypoglycaemia. Post-admission hypoglycaemia commonly occurs in children with hypoglycaemia on admission (Krishna, Waller et al. 1994; English, Wale et al. 1998). Therefore, post-admission hypoglycaemia is often not a new development during treatment. In contrast to adults and pregnant women (White, Warrell et al. 1983), quinine-induced hyperinsulinaemia and subsequent hypoglycaemia is considered not to be a major problem in African children with severe malaria on quinine treatment (White, Miller et al. 1987; Taylor, Molyneux et al. 1988).

At present, much evidence suggests that impaired hepatic gluconeogenesis may be the main pathogenic mechanism for hypoglycaemia in severe childhood malaria (Taylor, Molyneux et al. 1988; English, Wale et al. 1998). However, other mechanisms cannot completely be discounted.

1.2.1.12 Management of severe malaria in children

Malaria parasite eradication or development of an effective malaria vaccine would prevent many deaths, but both of these have not been achieved in Africa. The widespread development of chloroquine resistance in *Plasmodium falciparum,* and later resistance to antifolates in malaria endemic areas, meant that prompt and effective treatment of clinical attacks would not prevent the progression to severe disease in most children. However, the current deployment of artemensinin-based combination therapy (ACT) in many countries is expected to reverse the situation and a significant reduction in the incidence of severe childhood malaria may be expected. Nevertheless, improved severe malaria magement is still needed to reduce mortality in those who develop severe disease.

With the comprehensive description of the clinical phenotype of severe malaria in African children, the WHO has now provided guidelines for the management of severe malaria in adults and children (WHO 2000). Children with severe malaria are very sick,

and some of them are critically ill. The management guidelines are based on the principles of management of critically ill patients as highlighted below.

Resuscitation

As outlined in section 1.2.2.2 above, the usual clinical presentation of childhood severe malaria includes coma, prolonged partial or generalized seizures, severe respiratory distress, severe anaemia, and sometimes features of circulatory collapse or shock. Therefore, the required resuscitation, like in any other critically ill patient, should be based on principles of basic life support, i. e. ensuring a clear airway, adequate and effective breathing, and restoration of adequate circulating volume (ABC) in that order.

Monitoring and supportive care

Essential elements of monitoring in the management of severe childhood malaria include monitoring for level of consciousness, overt or subtle seizures, hypoglycaemia, hyperpyrexia and breathing abnormalities. Careful monitoring of fluid balance by maintaining an input output chart at all times when the child is unable to take orally is essential. Adequate fluid resuscitation in those with features of acidosis or shock, and rapid blood transfusion in those with severe anaemia should be part of improved supportive care. Nursing care of the unconscious child includes insertion of a nasogastric tube to prevent aspiration pneumonia and frequent turning (every 2 hours) to prevent

bedsores is also required. Hypoglycaemia on admission or post-admission should be monitored and corrected with dextrose infusions.

The indication for blood transfusion in children with severe malaria needs a careful assessment on a case-by-case basis, and consideration should not be limited to the level of hemoglobin or haematocrit. However, the presence of respiratory distress, impaired consciousness or hyperparasitaemia (more than 20 % of red cell parasitized) in a patient with haemoglobin between 4 and 6g/dl are indications for blood transfusion(Newton, Taylor et al. 1998).

It is now widely accepted that most children with severe malaria have some degree of volume depletion (English, Waruiru et al. 1997; Maitland, Levin et al. 2003; Planche, Onanga et al. 2004), and rapid blood transfusion plus fluid resuscitation result in rapid reductions in blood lactate and clinical improvement (English, Waruiru et al. 1996b). Contrary to the traditional thinking of cautious fluid restriction and slow blood transfusion in children with severe malaria anaemia, there is now argument for aggressive blood transfusion as soon as is available for those requiring it unless there is definitive clinical evidence for heart failure or pulmonary oedema (English 2000).

However, blood transfusion in resource poor setting in malaria endemic areas is associated with risks of HIV or hepatitis B infection. HIV prevalence among potential blood donors is high, and screening for HIV and hepatitis B do not completely eliminate

the risk of transmitting these infections to children receiving blood. In addition, the presence of other causes of anaemia such as iron deficiency, complicate the diagnosis of severe malarial anaemia. Therefore, treatment guidelines for the appropriate use of blood in the treatment of severe malarial anaemia need further refinement.

The current recommendation for the treatment of acidosis in severe childhood malaria is limited to the correction of any reversible putative cause of acidosis: intravenous fluid administration for dehydration or shock, blood transfusion for severe anaemia, and seizure prophylaxis to prevent further seizures and lactic acid generation. Specific treatments for acidosis in intensive care units around the world include direct alkalinisation, renal replacement therapy and use of dichloacetate (a molecule, which stimulates pyruvate dehydrogenase, a rate-limiting enzyme for lactate metabolism) (Levraut and Grimaud 2003). In general, the choice of treatment is based on the possible underlying mechanism and the nature of acidosis as some treatments may be of benefit in some but of no benefit or hazardous in other types of acidosis (Hale, Crase et al. 1984; Cooper, Walley et al. 1990; Rehm and Finsterer 2003). The underlying pathophysiologic mechanisms for metabolic acidosis in children with severe malaria remain poorly understood and supportive care, specifically targeting acidosis, has not been developed. *Therefore, the main focus of the first study reported in this thesis was a detailed characterization of metabolic acidosis in children with malaria and had a broad objective of contributing to the choice of potentially beneficial treatment for development.*

Definitive treatment

Quinine is still effective and remains the drug of first choice in the treatment of African children with severe malaria. Parenteral or rectal formulations of arteminsinin derivatives can also be used (McIntosh and Olliaro 2000). It is also important to treat concurrent conditions such as meningitis and bacterial sepsis. Initial presumptive treatment for meningitis is essential in those with neurological manifestation. In order to exclude meningitis, a lumbar puncture is performed where appropriate (Berkley, Mwangi et al. 1999) and if facilities are available, blood culture to exclude bacterial sepsis is indicated(Berkley, Mwarumba et al. 1999).

1.2.2 Metabolic acidosis in childhood malaria

1.2.2.1 Normal acid-base physiology

Body fluids including blood plasma are aqueous (water-containing) solutions and acid-base physiology is about the balancing of hydrogen ions in these body fluids compartments. In aqueous solutions, water is not only the solvent, but also takes place in chemical reactions because of water molecules' weak tendency to dissociate into hydrogen (H^+) and hydroxyl (OH^-) ions. Pure water has equal concentration of these ions and is a neutral solution as far as acid-base status is concerned. However, when water contains other chemicals there is a differential concentration of hydrogen and hydroxyl ions and aqueous solutions are either acidic (excess hydrogen ions) or alkaline (excess hydroxyl ions) (Holmes 1993).

Carbon dioxide produced from carbohydrate aerobic metabolism forms the greatest amount of acid the body generates daily (approximately 25M) (Holmes 1993). Being a volatile acid, it is blown off through the lungs. Protein metabolism is the other significant source of acid and is the principal source of non-volatile or fixed acids generating about 70 mmol of acid per day (Holmes 1993). In normal healthy subjects

non-volatile acids are produced in excess of bases, and excess acid is excreted through the kidneys (Holmes 1993).

In plasma, hydrogen ions are present in very low concentration (nano moles) and direct measurements were associated with practical difficulties. As a result, the pH scale - the non-linear transformation of hydrogen ion concentration (-log 1/ $[H^+]$) was developed (Holmes 1993) and is used to quantify acid-base balance. The pH is on a negative logarithmic scale and the lower the pH the higher the hydrogen ion concentration. A small change in the pH scale represents a substantial increase or decrease in hydrogen ion concentration.

The structure of proteins and the integrity of enzyme systems are exquisitely sensitive to changes in pH and disturbances of hydrogen ion concentration have profound effects on normal physiology. In normal healthy subjects the blood pH is kept within a narrow range of between 7.35 and 7.45 (Holmes 1993). This is achieved through simple chemical buffering in body fluids, cells, and through respiratory and renal compensatory mechanisms when simple chemical buffering is overwhelmed. Buffer systems in human blood are shown in table 1.3 whereas the daily buffering capacity of each system is shown in table 1.4. Practically, these buffer systems can be divided into two: first, the carbon dioxide bicarbonate buffer pair, and second, are the remaining buffers (the non-bicarbonate buffer pair), which includes quantitatively mainly hemoglobin and plasma proteins, notably albumin.

Table 1.3: Blood buffers

(Modified from (Holmes 1993)

Bicarbonate buffer pair					
CO_2	+	H_2O	H^+	+	HCO_3^-
CO_2	corresponds to buffer acid				
HCO_3^-	Corresponds to buffer base				
Non-bicarbonate buffers					
HPr	H+	+	$^bPr^-$		
HPr	Corresponds to non-bicarbonate buffer acid				
Pr^-	Corresponds to non-bicarbonate buffer base				

Henderson L. J. was the first to describe the relationship between the concentration of carbonic acid, hydrogen ions, and bicarbonate (Fencl and Leith 1993). He applied the law of mass action to the bicarbonate buffer system. Since carbonic acid cannot be directly measured because of instantaneous dissociation, Henderson used the concentration of dissolved molecular carbon dioxide ($[CO_2]$) to represent carbonic acid concentration as illustrated below.

$$H_2CO_3 \leftrightarrow H^+ + HCO_3^- \qquad \text{Equation 1.1}$$

[b] Stands for "protein"

By law of mass action:

$[H^+]\,[HCO_3^-] / [CO_2] = K$ (dissociation constant) Equation 1.2

$[H^+] = K\,([CO_2] / [HCO_3^-])$ Equation 1.3, is the Henderson equation

The introduction of the pH notation and the development of tests to measure blood pH led to an increasing recognition of the influence of the arterial partial pressure of carbon dioxide ($PaCO_2$) on acid-base balance. Hasselbalch introduced the pH notation and the arterial partial pressure of carbon dioxide into the Henderson equation to produce the Henderson-Hasselbalch equation. However, using Henry's law of gases, he expressed the concentration of dissolved molecular carbon dioxide in terms of the product of partial pressure of carbon dioxide and its solubility coefficient as shown in the equation below.

$pH = pK + \log_{10} [HCO_3^-] / 0.03\,([PaCO_2])$ Equation 1.4, is the Henderson-Hasselbalch equation

In a buffer pair at equilibrium, as shown in Equation 1.1, the concentration of the different reacting species in the equation is the same. From equation 1.4, and since $\log_{10}$ of 1 is equal to zero, the pH at this point is equal to the dissociation constant (pK_a). Therefore, the pK_a is the pH at which the concentrations of the conjugate acid and

conjugate base are the same. As a general rule, a buffer pair has the greatest buffering power when the pH equals the pK_a, and in addition, a buffer pair usually provides useful buffering within one pH unit on either side of the pK_a (Holmes 1993). For example, the bicarbonate buffer system in blood has a pK_a value of 6.1 and is a useful buffer within a narrow pH range of 5.1 and 7.1, which is outside the physiological pH. Thus, in a closed system (e. g. a blood sample in a tube), the carbon dioxide bicarbonate buffer pair is a less useful buffer system. However, *in vivo*, the concentration of carbon dioxide and bicarbonate can be altered by the action of the lungs and kidneys respectively, and this buffer pair operates in an open system. This makes the bicarbonate buffer system very efficient and crucial for acid-base balance in humans.

Table 1.4: Buffering capacity of whole blood buffers

Adapted from (Holmes 1993)

Nature of acid load	Buffering system	% of buffering
CO_2	Non-bicarbonate	100
Fixed acid	Bicarbonate	
	• Plasma	35
	• Erythrocytes	18
	Hemoglobin	35
	Plasma proteins	7
	Phosphate	5

In man, simple chemical buffering from extra-cellular and intra-cellular buffers form the first line of defense against acid-base disturbance (Holmes 1993). Extra-cellular buffers are immediately available whereas intra-cellular buffers take a matter of minutes to be effective. Bone, is an organ with immense intra-cellular buffering power, but its blood supply is low and its buffering requires hours or days to become available. When simple chemical buffering is overwhelmed, disorders of acid-balance develop. An increase in hydrogen ions concentration in blood stimulates peripheral chemoreceptors leading to hyperventilation (Respiratory compensation) (Holmes 1993). The onset of this compensation is immediate and carbon dioxide is blown off causing the arterial partial pressure of carbon dioxide to fall below normal. Renal compensation for acid-base disorders takes days to be effective and is through the kidneys ability to excrete excess acid. Although respiratory and renal compensatory mechanisms may achieve complete compensation with normalisation of blood pH, in clinical practice the compensation is often partial.

Acid-base disorders can either be associated with excess in blood of acid (acidaemia) or base (alkalaemia). However, reference is often made to the acid-base status of the body as a whole where the process leading to acidaemia is known as acidosis, and the process leading to alkalaemia, alkalosis (Holmes 1993). Therefore, acid-base disorders may be present even when the blood pH has been driven back to normal or close to normal by compensatory mechanisms. However, it is important to remember that acidosis and alkalosis are not mutually exclusive and acid-base disorders may be of a mixed picture.

1.2.2.2 Approach to the classification and quantification of the degree of metabolic acidosis

The traditional approach

The development of the Henderson-Hasselbalch equation set the stage for the basis of classifying acid-base disorders according to the nature of change in the variables on the equation leading to the disorder. If the primary change is in the partial pressure of carbon dioxide, the aid-base disorder is described as respiratory whereas, if the primary change is in bicarbonate concentration the disorder is metabolic (Fencl and Leith 1993). Therefore, acidosis or alkalosis can be respiratory or metabolic or mixed in nature. A comprehensive review of all acid-base disorders is beyond the scope of this thesis, and the brief description in the paragraphs below is on the clinical approach to metabolic acidosis.

The traditional approach to metabolic acidosis is based on the Henderson-Hasselbelch equation. In this approach, a low blood pH associated with low bicarbonate concentration defines metabolic acidosis. However, established metabolic acidosis is always accompanied by hyperventilation (respiratory compensation), which is mainly associated with an increase in tidal volume. This leads to a reduction in pCO_2 and the resulting blood pH will depend on this respiratory response. When the respiratory

response is not affected by an impairment of respiratory function, the respiratory response can be predicted from the blood bicarbonate concentration and simple formulae (e. g. $pCO_2 = 1.5\ [HCO_3^-] + 8$) (Albert, Dell et al. 1967) are used for this purpose. If pCO_2 is higher than expected for the bicarbonate concentration then there is a respiratory component to the acidosis. On the other hand, if pCO_2 is appropriate for the bicarbonate concentration a pure metabolic acidosis is diagnosed.

According to the traditional approach, a decrease in bicarbonate concentration is taken to be a consequence of metabolic acidosis, and two mechanisms can lead to a decrease in plasma bicarbonate concentration. First, is the presence in plasma of excess non-volatile or fixed acids, which are otherwise absent in healthy subjects. These acids, consume bicarbonate during simple chemical buffering. The second mechanism is the loss of bicarbonate from plasma, which can result from conditions associated with loss of body fluids in excess of bicarbonate. The commonest clinical conditions associated with bicarbonate loss include severe diarrhea, renal tubular acidosis and conditions associated with loss of alkaline fluid such as fistulae or instrumentation e. g. a stoma.

Excess non-volatile acids in plasma may be organic, inorganic, endogenous or exogenous. Accumulation of endogenous organic acids (e.g. lactate, ketones) can be due to increased generation or decreased utilisation (hepatic clearance) of these acids or a combination of the two. Plasma accumulation of endogenous inorganic acids is mainly due to decreased renal clearance whereas accumulation of exogenous acids is a result of

addition to plasma of acids from exogenous sources for example, in acidosis associated with drug poisoning.

Whereas with respiratory acidosis the level of carbon dioxide tension directly reflects the severity of acidosis, the level of bicarbonate as a parameter in the clinical assessment of the severity or degree of metabolic acidosis is limited. The Henderson-Hasselbelch equation assumes that bicarbonate concentration varies independent of PCO_2, but in reality, this is not the case. Plasma bicarbonate concentration varies with PCO_2 to maintain chemical equilibrium such that whenever PCO_2 increases, plasma bicarbonate concentration increases (metabolic alkalosis) to compensate for respiratory acidosis. In addition, in physicochemical systems with significant concentrations of fixed weak acid buffers (e. g. proteins and phosphates) such as plasma, a change in carbon dioxide tension causes a change in bicarbonate concentration (Fencl and Leith 1993). Therefore, defining the degree or severity of metabolic acidosis using the level of decrease of plasma bicarbonate concentration is inaccurate. For this reason, the standard bicarbonate and later, the total buffer base and base excess were developed as PCO_2-independent parameters for the assessment of the metabolic component of acid-base disorders.

The standard bicarbonate is the plasma bicarbonate concentration measured at standard or physiological PCO_2 (40 mmHg) and temperature (37°C). The normal range for standard bicarbonate in healthy subjects is 22 – 26 mmo/l, and a value below 22mmol/l defines metabolic acidosis. However, the standard bicarbonate reflects a change in the

carbon dioxide bicarbonate buffer alone, and does not incorporate the non-bicarbonate buffers. This is a major limitation, which lead to the development of the measurement of the total buffer base and the base excess (Holmes 1993).

The total buffer base is the summed concentration of plasma bicarbonate and that of non-bicarbonate buffers measured at physiological pCO_2 and temperature. In normal healthy subjects the concentrations of bicarbonate and the non- bicarbonate buffer base measured at these physiological conditions are approximately equal, and the value of total buffer base is around 48mmol/l. The base excess is the measure of the degree of change of the normal total buffer base and reasonably reflects the degree of metabolic acidosis. However, haemoglobin is a major contributor to non-bicarbonate buffers and a significant change in haeoglobin such as anaemia may affect the value of the total buffer base (Holmes 1993). This limitation led Siggaard-Andersen to develop normograms incorporating haemoglobin where total buffer base and base excess can be read off a given haemoglobin value (Siggaard-Anderson 1963).

At present, normograms and equations are built in blood gas analysers and assessment of acid-base status of a patient can be determined using a small venous or arterial blood sample. The pCO_2, dissolved carbon dioxide, and pH of the sample are directly measured and other parameter such as bicarbonate, standard bicarbonate, and base excess are calculated using inbuilt equations and normograms. Results adjusted for the patient's temperature and haemoglobin can be obtained by keying in these values before

sample analysis. However, the principle for determination of base excess (the commonest parameter used in clinical practice) involves first, equilibrating the blood sample with a gas mixture containing pCO_2 of 40 mmHg at 37°C. Then, measuring the sample pH at this equilibrium, and finally back titrating the same. That is, base is added to bring the pH back to the normal value of 7.4. The amount of base added to bring the sample pH back to normal (in mmol per liter), is the base deficit equivalent to the decrease in the total buffer base. This value is the base deficit or the negative base excess if given as a negative value as it is often expressed. In a population of normal healthy individuals the base excess is normally in the range of ± 2.5 mmol/l (Holmes 1993). In all the studies involving acid-base measurement in this thesis, the degree of metabolic acidosis was assessed using the base excess.

The principle of elactroneutrality dictates that the sum of all the positive charges in extra-cellular fluid is exactly neutralized by an equal number of negative charges. Na^+ and K^+ account for approximately 96 % of cations in extra-cellular fluid whereas Cl^- and HCO_3^- account for 83 % of extracellular anions (Holmes 1993). As the results, in clinical practice, the sum of measured anions does not fully equal the sum of cations, and the difference is equal to the anion gap (equation 1.5) (Emmett and Narins 1977).

Although clinically useful for assessment of severity of metabolic acidosis, the total buffer base or base excess does not give an indication of whether acidosis is due to excess non-volatile acids or bicarbonate loss. Yet this is important clinical information

that is needed for decision on treatment. To solve this problem, plasma electrolytes including chloride are routinely measured and the anion gap (see equation 3) is calculated to determine the source of acidosis.

$$AG\ (\text{anion gap}) = [Na^+] + [K^+] - ([HCO_3^-] + [Cl^-]) \qquad \text{Equation 1.5}$$

In non-acidotic patients, the anion gap is due to anions present in normal concentration but not measured notably albumin and phosphate. When ion selective electrodes, which measure free electrolytes in plasma, are used to determine plasma electrolytes, the cut-off value for normal anion gap is 13mmol/l (Winter, Pearson et al. 1990; Lolekha, Vanavanan et al. 2001; Lolekha, Vanavanan et al. 2003). In a patient with metabolic acidosis, a high anion gap suggests the presence of non-volatile acids whereas a normal anion gap suggests loss of bicarbonate, which is usually accompanied by chloride shift from inside cells to plasma to maintain electro neutrality (hyperchloraemic acidosis). However, since the normal anion gap is largely due to the negative charge on albumin, significant changes in plasma albumin may affect its usefulness (Fencl and Rossing 1989; Fencl, Jabor et al. 2000), and a correction factor for albumin concentration has been derived to improve the accuracy of the anion gap in patients with abnormal plasma albumin levels (Figge, Jabor et al. 1998).

However, as described above, the traditional approach where the degree of metabolic acidosis is measured using base excess and the possible cause of acidosis determined

using the anion gap has several shortcomings. The Henderson-Hasselbalch equation does not allow for an accurate assessment of the degree of the metabolic component of acid-base disorders. Parameters developed to improve the accuracy of the tradition approach also have shortcomings. The base excess does not tell the clinician about the underlying mechanism for metabolic acidosis, and although the albumin-corrected-anion gap can accurately detect excess non-volatile acids, it does not tell about the nature of the excess acids (e. g. organic versus inorganic).

Against a background of widely accepted shortcomings with the traditional approach and the arrival of computers, P. A. Stewart, applied basic physical-chemical principles of aqueous solutions (the principles of electro neutrality and conservation of mass) to blood plasma and developed quantitative analysis of acid-base chemistry (Stewart 1983).

This approach presents a way for complete quantitative treatment of body fluids as physico-chemical systems. The method analyses acid-base equilibriums in individual fluid compartments such as in blood, interstitial fluid compartment, or intracellular fluid compartment and incorporates the detailed composition of the system under study.

The physico-chemical approach

According to the Stewart's theory, plasma pH and bicarbonate concentration are dependent variables and blood pH can only be affected by three independent parameters: the carbon dioxide tension (pCO_2), the strong ion difference (SID), and the total plasma weak acid (A_{TOT}) notably albumin and phosphorus. The SID is the difference between the sum of all the strong cations (Na^+, K^+, Ca^{2+}, Mg^{2+}) in the physico-chemical system (e. g. plasma) and the sum of all strong anions (Cl^-, other strong ions that may be present and measured e.g. lactate), and it reflects the net charge of all the strong ions in the system. Strong ions are those, which are completely dissociated in water, and in plasma, exist only as ions. Through their influence on water dissociation, these parameters independently determine plasma pH. The principles of this quantitative analysis of acid-base balance (physico-chemical approach) include the following (Fencl and Leith 1993):

- Acid-base homeostasis in a given body fluid compartment is established by regulation of the independent variables
- Acid-base disorders are primarily caused by changes in one or more of the independent variable
- Assessment of acid-base status is best done by measuring and analyzing the independent variables
- The essence of all therapeutic interventions is adjustment of the independent variables.

Metabolic acidosis is produced by a decrease in SID, which generates an electrochemical force that results in an increase in water dissociation and a consequent increase in free hydrogen ion concentration. The primary mechanism behind a decrease in SID is the presence of strong anions in excess of cations. Excess strong anions commonly encountered in clinical practice include chloride, lactate and ketones. However, an increase in the total plasma weak acid (A_{TOT}) in its own right can lead to a decrease in SID and cause metabolic acidosis. Furthermore, a decrease in SID and metabolic acidosis may result from the addition to plasma of exogenous acids or compounds that can be metabolised to acids such as with salicylate, ethanol, or methanol poisoning.

In normal blood plasma however, strong cations (mainly Na^+) outnumber strong anions (mainly Cl^-) and the normal SID is between 40 and 42mmol/L(Kellum 2000). Yet according to the principle of electrical neutrality, plasma cannot be charged. Therefore, the negative charge from CO_2 (HCO_3^-) and the total weak acids must balance this positive SID, and SID – (HCO_3^- + A_{TOT}) must be equal to zero, to maintain electro neutrality. This value of negative charge (from CO_2 and A_{TOT}) balancing the SID of normal plasma is called the effective SID. Therefore, using the physico-chemical approach, the presence of excess strong anions can be estimated by calculating the strong ion gap (SIG), which is the difference between the SID from the patient's sample (apparent SID) and the effective SID (see appendix I). However, the strong ion gap is

different from the anion gap in that the value of the SIG for a normal plasma sample is equal or close to zero whereas, the value of anion gap is 8 – 13mmol/L. The SIG does not suffer the limitation of the anion gap as albumin concentration is incorporated in the calculation of the SIG.

In general, the traditional and the physico-chemical approach to acid-base balance are similar. The SID is the mirror image of the total buffer base and the base excess defines the change in SID from the equilibrium point (pH = 7.4 and pCO_2 = 40mmHg) (Schlichtig 1997). The main difference in the two approaches is in how they approach the understanding of mechanisms underlying metabolic acidosis.

The proponents of the physico-chemical approach argue that in the traditional approach, a decrease in bicarbonate concentration is erroneously taken as the cause of metabolic acidosis. There is now evidence to show that bicarbonate concentration is just a correlate of acidosis and the values of bicarbonate and pH are dependent on pCO_2. They further argue that mechanisms for acidosis can only be understood when independent variables (causal determinants) are considered (Kellum 2000), and thus, understanding how the body regulates pH, is only through understanding how it regulates the independent variables: pCO2, SID and A_{TOT}.

According to the Stewart's approach, water is the major source of hydrogen ions in plasma and the kidneys regulate acid-base balance through the handling of chloride ions

rather than hydrogen ion excretion as considered in the traditional approach. Chloride influences SID which in turn influences water dissociation and hence hydrogen ions.

Both approaches were used in this thesis to characterize metabolic acidosis in children with malaria.

A logical and clinically useful classification of metabolic acidosis (based on the nature of the excess strong anions) has recently been suggested (Levraut and Grimaud 2003) (Table 1.5). Toxic metabolic acidosis arises when exogenous toxins such as salicylates, ethylene glycol, methanol, or ethanol are involved. Organic metabolic acidosis, lactic or ketoacidosis reflects the presence of a metabolic distress
that indicates the failure of energy metabolic pathways, and is associated with increased production of organic acids or a decrease in their clearance. By contrast, mineral metabolic acidosis simply indicates that protons normally eliminated by the kidneys are in excess.

Table 1.5: Main causes of acute metabolic acidosis in ICU settings

(Adapted from Levraunt J and D. Grimaud(Levraut and Grimaud 2003)

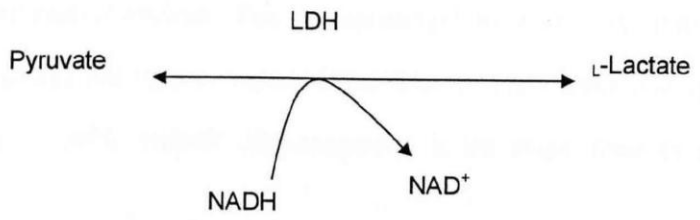
Mineral	Organic	Toxic
Excess chloride: • Massive sodium chloride resuscitation • Ammonium or arginine chloride • Total parenteral nutrition • Diarrhoea, high colostomy output • Biliary or pancreatic fistula • Renal tubular acidosis Other mineral acids (renal failure): • Phosphoric acids • Sulfuric acids • Hippuric acid	$_{L}$-Lactic acid Hypoxia: • Circulatory failure • Severe anaemia • Cardiac arrest • Seizures • Severe asthma • Toxic (cyanide, CO, biguanides) Sepsis Severe hepatic failure Thiamine deficiency $_{D}$-Lactic acid: • Short bowel syndrome Ketoacidosis: • Diabetes mellitus • Ethanol intoxication • Prolonged fast	Toxic agents causing specific metabolic acidosises: • Salicylates • Ethylene glycol

1.2.2.3 Putative aetiological factors for metabolic acidosis in children with malaria

Hyperlactataemia

Lactic acid almost completely dissociates into lactate and hydrogen ions at physiological blood pH (strong anion) and the excess lactate generated is associated with acidosis. Lactate is an end product of anaerobic glucose metabolism and is formed by reduction of Pyruvate as shown in equation 1.6. However, during glycolysis, more hydrogen ions are produced on hydrolysis of ATP independently of lactate production (Zilva 1978). When this hydrolysis occurs aerobically protons are consumed within mitochondria (electron transport), but under anaerobic conditions protons are not recycled and accumulate reducing the pH. Acidosis occurs when the ratio of glycolytic to mitochondrial ATP hydrolysis increases above a certain threshold irrespective of how much lactate is produced (Fink 1996). Therefore, it is possible to have acidosis without hyperlactatemia and vice versa.

Equation 1.6



Hyperlactataemia (blood lactate ≥ 5mmol/l) commonly accompanies metabolic acidosis associated with severe malaria in children (Krishna, Waller et al. 1994). However, normal blood lactate concentration is observed in a considerable proportion of children with severe malaria and acidosis. In a series of children with severe malaria in Papua New Guinea, several children with hyperlactataemia had normal acid-base status and several with acidosis had normal lactate levels (Allen, O'Donnell et al. 1996). Likewise, in a study of acidosis in Kenyan children with severe malaria, English and others observed hyperlactataemia in 17 % of children without acidosis (English, Sauerwein et al. 1997). The authors concluded that the presence of an elevated blood lactate level needed not to be an essential or major component of any metabolic acidosis in African children. It is not clear whether acidosis in children with normal blood lactate is due to a different aetiological mechanism or the independence of acidosis development on amount of lactate produced. In disease however, lactate is mainly produced during anaerobic glycolysis resulting from reduced tissue oxygen delivery. In severe malaria, there are several pathogenetic factors including repeated generalized seizures, reduced tissues perfusion due to sequestration or volume depletion; and severe anaemia that may be associated with reduced tissue oxygen delivery.

Repeated generalized seizures are associated with lactic acid production as a result of the intense activity of skeletal muscles. This is compounded by respiratory impairment during seizure episodes and reduced hepatic blood flow in severe malaria(Molyneux, Looareesuwan et al. 1989). Hepatic gluconeogenesis is the major route of lactate

clearance in healthy individuals (Stallknecht, Vissing et al. 1998), thus if hepatic blood flow is not significantly impaired the lactate produced is readily metabolised to glucose and becomes a source of energy again.

In Malawian children, a recent history of convulsions was a more common finding in acidaemic children (Taylor, Borgstein et al. 1993) and a study in Papua New Guinea, lactate levels were higher in children who had convulsions before admission compared to those without such a history (Allen, O'Donnell et al. 1996). Despite this association, seizures as a significant contributor to acidosis in severe childhood malaria have not been supported by data from other studies. For example, Krishna and colleagues, observed no relationship between admission lactate level and time of convulsions before admission (Krishna, Waller et al. 1994). These authors also observed a greater fall in the percentage of lactate four hours after admission, in children with recent history of convulsions than other survivors. The presence of sustained hyperlactataemia was the most powerful prognostic indicator of a fatal outcome. These findings reflect the transient nature of hyperlactataemia from seizures where lactate is readily converted to glucose in the post seizure period.

Circulatory insufficiency associated with hypoxia is by far the commonest cause of lactic acidosis globally. Reduced blood volume or hypovolemia has been demonstrated in severe childhood malaria. In one study, about 30 % of children with severe malaria were dehydrated on admission (English, Waruiru et al. 1997) and more recently,

Maitland and colleagues have shown that features of shock are common amongst children admitted with severe malaria (Maitland, Levin et al. 2003). Furthermore, in children with severe malaria, normal-saline bolus infusions or blood transfusion (in those with severe anaemia) is associated with significant reduction in blood lactate and clinical improvement (English, Waruiru et al. 1996b). Therefore, it is very likely that hypovolemia and severe anaemia are important aetiological factors for acidosis in severe childhood malaria.

The reduction in hemoglobin concentration or red cell mass (anaemia) is associated with reduced blood oxygen carrying capacity leading to hypoxiaemia and anaerobic glycolysis, which in turn lead to an increase in lactate production. Theoretically therefore, severe anaemia may be an important contributing factor to lactic acidosis in severe childhood malaria. However, hyperlactataemia with normal acid-base status was associated with lower hemoglobin in Kenyan children with severe malaria (English, Sauerwein et al. 1997) suggesting that an accumulation of an oxygen debt resulting from reduced tissue oxygenation or unmet increased oxygen demand contributed to the generation of lactic acidosis. This was supported by findings of a small study of children with severe malarial anaemia and respiratory distress where there was an increase in total oxygen consumption (VO2), a fall in respiratory quotient (QR), and a resolution of lactic acidosis during blood transfusion (English, Muambi et al. 1997). The increase in VO2 during blood transfusion suggested that an inability to meet a higher oxygen

demand rather than a primary decrease in oxygen supply was the more likely mechanism for accumulation of an oxygen debt.

In addition, parasite sequestration in the microvasculature of vital organs is associated with local tissue ischemia due to mechanical blockage to blood supply. As a result, oxygen supply, especially to the brain in cerebral malaria, is reduced and lactate production is increased.

Theoretically, all these factors would promote anaerobic metabolism increasing lactate and proton production leading to metabolic acidosis. Lactate excess in severe childhood malaria suggests a net increase in lactate production from the brain, and skeletal muscles, which is not matched with lactate metabolism. A suggestion that increased lactate production rather than decreased disposal is the most important determinant of hyperlactataemia (Agbenyega, Angus et al. 2000) has recently been made from a study of lactate kinetics in African children with severe malaria. However, a role of reduced hepatic blood flow (Pukrittayakamee, White et al. 1992) had been suggested in previous reports. It has been argued that at such low liver blood flow rates as those observed in severe malaria, lactate delivery to hepatocytes may become the rate limiting step to lactate metabolism (Lupo, Cefalu et al. 1990). Specific defects in lactate handling by the liver have also been suggested (Krishna, Waller et al. 1994).

Inflammatory cytokines

Cytokines have been hypothesized to be responsible for increased lactate production in severe malaria, and there has been a considerable debate over whether increased anaerobic glycolysis or cytokine induced inhibition of oxidative metabolism is more important in causing lactic acidosis (Clark, Jacobson et al. 1997), Interferon-gamma and Tumour necrosis factor (TNF) have been shown to synergise in the induction of nitric oxide production, and nitric oxide has been observed to inhibit mitochondrial respiration in vascular smooth muscle cells (Geng, Hansson et al. 1992). TNF administered to normal volunteers has been found to increase resting lactate levels without producing hypotension (Van der Poll, Romijn et al. 1991) suggesting an effect not mediated through circulatory insufficiency. Therefore, Clark and others have hypothesized that inflammatory cytokines generated during severe malaria infection in children, may induce sufficient nitric oxide (NO) to cause biochemical hypoxia by inhibiting aconitase and cytochrome oxidase enzymes, and hence inhibiting oxidative metabolism (Clark, Jacobson et al. 1997).

A contributory, or possibly a direct role for inflammatory cytokines in the pathogenesis of acidosis in severe childhood malaria, draws support from findings by Krishna and others in Gambian children with severe malaria. In these children, blood cytokine, and lactate levels on admission, were positively correlated, independent of blood pressure (Krishna, Waller et al. 1994). However, levels fluctuated substantially after admission,

and unlike lactate, TNF concentration 4 hours after admission was not an independent predictor of outcome. Furthermore, it is now widely accepted that cytokine levels in blood may not necessarily reflect local levels in tissues where production may be high hence it is hard to interpret cytokine blood concentration results. Hypotension is a late event in children with shock and is an unreliable marker of circulatory insufficiency. Therefore, correlation between lactate and cytokine levels independent of blood pressure does not entirely exclude the possibility of cytokines acting through enhancement of sequestration.

When there is increased glucose flux through the glycolytic pathway (hypermetabolism), hyperlactatemia can develop despite sufficient oxygen supply. The blood plasma lactate pyruvate ratio can be used to differentiate between hypermetabolism and anaerobic glycolysis secondary to hypoxia, and is a good measure of the Redox State (Day, Phu et al. 2000). When there is hypoxia, lactate production is in excess of pyruvate and the lactate pyruvate ratio increases. Generally, a lactate pyruvate ratio of 15 or more is considered to indicate hypoxia (Day, Phu et al. 2000). In a large series of Vietnamese adults with severe malaria, plasma lactate and creatinine were the two main contributors to acidosis (Day, Phu et al. 2000), and there was marked elevation of the lactate pyruvate ratio that was greatest in those with the most severe disease. The authors concluded that tissue hypoxia and renal insufficiency were the two common causes of acidosis in severe malaria. However, it is unlikely that one mechanism can explain the pathogenesis of acidosis in severe malaria. The role of inflammatory cytokines in the

pathophysiology of severe malaria is increasingly being recognized and their possible role in the development of acidosis cannot be completely discounted.

Parasite metabolism

Plasmodium falciparum produces the D isomer of lactic acid ($_D$-lactate) (Zolg, Macleod et al. 1984) during metabolism. *P. falciparum* infected red blood cells release both L-lactate and $_D$-lactate with particularly high rates trophozoites or schizont stage parasites (Vander Jagt, Hunsaker et al. 1990) due to high rate of glucose consumption. On the other hand, humans produce only $_L$-lactate as a product of glycolysis (Berg, Tymoczko et al. 2002) and not $_D$-lactate, though elevated concentrations of $_D$-lactate have been found in serum of patients after gastrointestinal surgery (Thurn, Pierpont et al. 1985) when it is produced by abnormal intestinal bacteria, as well as in patients with diabetes mellitus where it impacts on the rate of glycolysis (Christopher, Broussard et al. 1995). In *P. falciparum,* $_D$-lactate represents between 6 to 70 % of the total lactate released by parasites and would allow for differentiation between parasite and host metabolism.

However, the contribution of $_D$-lactate to hyperlactataemia associated with severe childhood malaria is difficult to quantify. There are currently no established reliable methods for estimating the total parasite biomass (Ochola, Marsh et al. 2005) as well as the concentration of $_D$-lactate in blood (Swenson 2001). Nevertheless, available data

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suggest that $_{D}$-lactate released by parasites may be quantitatively of minor importance (Krishna, Waller et al. 1994).

The available methods for measuring blood lactate can only measure $_{L}$-lactate (Swenson 2001), and some studies have reported lack of correlation between acidosis and peripheral parasitaemia (English, Sauerwein et al. 1997). The rate of $_{L}$-lactate export from infected red cells is much less (Pfaller, Krogstad et al. 1982; Vander Jagt, Hunsaker et al. 1990) than the rate of $_{L}$-lactate metabolism in a normal adult (Mordes, J. P., Tranquada, R.E. & Rossini, A. A., as cited in (Krishna, Waller et al. 1994)). Therefore, the quantity of lactate produced by infected erythrocytes is thought to be insufficient to cause sustained hyperlactataemia provided lactate clearance is not impaired. Host tissues metabolising anaerobically, are considered quantitatively the most important source of lactate.

Renal impairment

Falciparum malaria has a profound effect on renal function in adults with severe malaria, and acute tubular necrosis is the most important cause of malaria death in South-East Asian countries (Trang, Phu et al. 1992). However, studies in children have found a less deleterious effect of falciparum malaria on renal function. Incipient acute polyuric or anuric renal tubular dysfunction was observed in some Nigerian children during acute falciparum malaria illness (Sowunmi 1996). In this small study, creatinine clearance as

a measure of glomerular filtration rate (GFR) was significantly impaired in 45 % of the children during acute illness. This was consistent with earlier observations amongst Indian children (Ahmad, Danish et al. 1989). Hypovolemia and haemoglobinuria are the hypothesized causes of renal impairment in falciparum malaria, but sequestration of mature forms of plasmodium falciparum in the renal vascular bed has been presumed to play a substantial role in malaria associated renal impairment (Sitprija 1988; White and Ho 1992).

Although acute renal failure is uncommon in children with severe malaria, impaired renal function indicated by moderately raised plasma creatinine or urea is common, and is of prognostic significance. In Gambian children with severe malaria, evidence for renal impairment was an important prognostic indicator (Waller, Krishna et al. 1995). Similarly, raised plasma creatinine and urea were independent predictors of metabolic acidosis, together with blood lactate, explaining about 64 % of variability in base excess in Kenyan children with severe malaria (English, Sauerwein et al. 1997). This means that reduced renal acid excretion may be a contributor to metabolic acidosis in severe childhood malaria.

Use of salicylate-containing drugs for treatment of fever at home

Despite excruciating digestive tract side effects, the Willow bark (Salicylates) had been used for treatment of fever and inflammation for centuries. The discovery of a

modification of salicylic (the active ingredient of the Willow bark acid) in 1897 by Felix Hoffman at Bayer, that avoided common side effects led to the development of Aspirin (acetylsalicylic acid). Today, aspirin is widely used for its antipyresis, analgesic, anti-inflammatory and antiplatelet actions. However, the widespread use of aspirin has been associated with several problems. The readily availability of the drug in the home has been associated with prevalent accidental acute poisoning in children (Done 1978). In the United States, the incidence of acute salicylate poisoning amongst children markedly dropped (Remington, Rowley et al. 1986; Arrowsmith, Kennedy et al. 1987) after the introduction and enforcement of mandatory safety packaging for aspirin and all non-aspirin salicylates ("National surveillance for Reye syndrome, 1981: update, Reye syndrome and salicylate usage" 1982; Davis and Buffler 1992). However, treating pediatric fever episodes or painful childhood illnesses with Aspirin in the home continued and was associated with sporadic cases of chronic salicylate poisoning (Done 1978). In 1980's several studies demonstrated a strong epidemiological association between the ingestion of Aspirin during antecedent varicella or influenza like illness and the presence of Reye's syndrome (Halpin, Holtzhauer et al. 1982; Hall, Plaster et al. 1988; Hurwitz 1989). This is an acute illness almost exclusively seen in children, and is characterized by an encephalopathy associated with various hepatic metabolic derangements together with fatty degeneration of hepatocytes (Reye, Morgan et al. 1963).

Due to these safety concerns, the use of aspirin and non-aspirin salicylates in children is no longer recommended, and acetaminophen (paracetamol) is the recommended drug of choice for treating paediatric fever episodes in the home. Yet, contrary to WHO recommendation, Aspirin is still widely used in rural Africa for home-treatment of childhood fever episodes. The treatment of a childhood febrile episode starts in the home with a proprietary antipyretic bought over-the-counter at shops or other retail outlets (Igun 1979; van der Geest 1987; Snow, Peshu et al. 1992; Mwenesi, Harpham et al. 1995) where Aspirin and non-aspirin salicylates are still widely available as over-the-counter preparations.

In a study in Malawi, Slutsker and others found nearly 70 % of parents to have treated their children with an antipyretic (commonly containing Aspirin) at the first sign of fever (Slutsker, Chitsulo et al. 1994). Similarly, Marsh and others found Aspirin almost universally used for home-treatment of fever in children in Kilifi district on the Kenyan coast (Marsh, Mutemi et al. 1999). Aspirin may be preferred to acetaminophen because of its potent antipyretic effect and relatively low cost. In addition, the presence on the East African market of generic antipyretic and analgesics combining Aspirin and other drugs may lead to caretakers or parents giving such combination products to children without knowing that they contain aspirin.

The risk for toxicity associated with the use of salicylates in children is due to their unusual pharmacokinetic properties. The main metabolic pathway for salicylic acid in

the liver is saturable, and saturation occurs at a relatively low amount of the drug in the body (Levy 1978). At blood concentrations below 300μg/ml, the biotransformation of salicylic acid is by first order kinetics. This means that biotransformation appropriately increases with increasing blood concentration, and the rate of biotransformation is always greater than the rate of absorption. However, at blood concentrations above 300μg/ml, biotransformation changes to zero order kinetics i.e., the rate of biotransformation remains constant irrespective of increasing blood concentration and if absorption is still taking place, the rate of absorption may become greater than the rate of biotransformation leading to a disproportionate increase in blood salicylate concentration with increasing dose. This unusual metabolism is not only observed when a single large dose is taken but also with repeated therapeutic doses (Levy, Amsel et al. 1969). The initial appropriate paediatric dose of aspirin for antipyresis is usually not enough to clear the fever (Done 1983), and parents are likely to give a repeat dose too soon, or even double the dose out of fear of the effect of fever on their child. This way the risk of toxicity is increased.

Due to the chemical nature of salicylates, acidosis in children increases the risk for toxicity even when only therapeutic doses are used. Salicylic acid is a weak acid with a pK value of 3, and thus, further ionisation occurs with increasing pH. However, only unionised salicylic acid can cross the cell membrane to be bound in tissues, and ionisation is less with the low pH in acidotic patients. Therefore, in acidotic patients

salicylic acid tissue binding is increased and renal elimination is impaired thus increased risk for toxicity.

Chronic salicylate poisoning has been demonstrated in Kenyan children with severe malaria (English, Marsh et al. 1996) raising concerns that the antecedent use of aspirin in childhood malaria episodes may play a role in the pathogenesis of severe malaria in many parts of rural Africa where salicylates are still widely used in children. The clinical features of chronic salicylate poisoning in children cannot be distinguished from those of severe malaria. Both are characterised by fever, altered consciousness, seizures, and respiratory distress with underlying metabolic acidosis. In both, metabolic acidosis is often associated with hyperlactataemia (Kreisberg and Wood 1983). Given the absence of a simple clinical definition for malaria, it is important to determine what the role of chronic salicylate poisoning might be in malaria severity where salicylates are still used in children. Therefore, one of the focuses of this thesis was to examine the true extent of antecedent salicylate use amongst children admitted to hospital with common (in Africa) childhood illnesses including malaria. The association between the presence of metabolic acidosis and the antecedent use of salicylates was also investigated.

Under-nutrition and food deprivation

During starvation or food deprivation in man, energy metabolism switches from the usual predominantly glucose oxidation to using more energy from the abundant store of calories in adipose tissue(Cahill 1976) and this is true even within one day of starvation(Romijn, Godfried et al. 1990). Fat is broken down to fatty acids, which are transported to the liver where they are oxidized to Acetyl Coenzyme A (acetylCoA). Acetoacetate is then formed by condensation of two molecules of acetlyCoA, and in the mitochondial matrix, depending on the NADH/NAD^+ ratio; acetoacetate is reduced to D-3-hydroxybutyrate (βOHB). Some acetoacetate undergoes a slow spontaneous decarboxylation to acetone, which is volatile and is eliminated in the breath. Acetoacetate, 3-hydroxybutyrate and acetone are sometimes referred to as ketone bodies. Acetoacetate is taken up in the mitochondrial tricarboxylic cycle, to yield energy, and it is now known that acetoacetate and 3-hydroxybutyrate are normal products of respiration and are quantitatively important as sources of energy (Robinson and Williamson 1980).

Acetone measurement in serum requires special precautions and the assay needs to be done promptly. On the other hand, D-3-hydroxybutyrate (βOHB) is stable and can be measured at a convenient time in previously frozen plasma or serum samples. Therefore, measurement of βOHB alone, as undertaken in this thesis, will usually suffice for

quantitative measurement of ketones for clinical purposes (Wolfsdorf, Sadeghi-Nejad et al. 1982).

Ketonuria, the presence of ketone bodies in urine, is frequently observed in children after a brief period of food deprivation. However, a longer period of food deprivation is required for ketonuria to occur in adults. This suggests that the switch of metabolism from glucose to fatty acid oxidation comes sooner in children than adults. In children, the concentration of acetoacetate and βOHB may increase 7 to 14 times the basal level respectively after only 24 h of fasting (Wolfsdorf, Sadeghi-Nejad et al. 1982).Given the prevalent under-nutrition in African children and the reduced food intake during the period (usually two to three days) preceding hospital admission for severe malaria, high levels of ketone bodies may be attained in such children.

Ketone bodies are organic acids and although they are used up as alternative sources of energy during food deprivation, high levels in blood may significantly contribute to a metabolic acidosis. Ketonuria is a common finding in children with severe malaria. For example, in a study of children with severe malaria in Papua New Guinea, ketonuria was more common in children with acidosis (Allen, O'Donnell et al. 1996). In addition, there is a parallel between acidosis in severe childhood malaria and the increase in serum ketoacid level following food deprivation in children; both are more common in younger than older children(Wolfsdorf, Sadeghi-Nejad et al. 1982; English, Sauerwein et al. 1997).

1.2.2.4 Prognostic significance of acidosis in malaria

Almost all the descriptive studies of severe childhood malaria have consistently shown that acidosis (measured by any of bicarbonate, lactate, pH, base excess) (Taylor, Borgstein et al. 1993; Marsh, Forster et al. 1995; Waller, Krishna et al. 1995; Allen, O'Donnell et al. 1996; English, Sauerwein et al. 1997) or the clinical correlate respiratory distress or deep breathing (English, Waruiru et al. 1996a), is the single most important prognostic indicator in children with severe malaria. Similar findings have been reported in adults with severe malaria. For example, standard base deficit (positive base excess) was reported to be the single most powerful predictor of a fatal outcome in a large study of Vietnamese adults with severe malaria (Day, Phu et al. 2000). These findings strongly suggest that metabolic acidosis is in the causal pathway between severe malaria and death.

As stated under section 1.2.2.1, the structure of proteins and the integrity of enzyme systems are so sensitive to changes in pH that changes such as acidosis may have profound effects on normal physiology. Experimental studies have shown that acidosis depresses numerous cellular synthesis activities (Kashiwagura, Deutsch et al. 1984; England, Chastain et al. 1991). For example, when intracellular pH is less than 6.9, myocardial contractility as well as sympathetic activity of catecholamines is significantly depressed (Opie 1965). In addition, hyperkalaemia, a life threatening

electrolyte imbalance, is a classical side effect of metabolic acidosis. Thus, acidosis affects the transmembrane potential, which could promote re-entrant arrhythmia (Orchard and Cingolani 1994).

There are several consequences of severe acidosis that may cause death. However, most of the evidence for these consequences is based on experimental studies, and extrapolation of results to humans is difficult and sometimes flawed. A potent protective effect of extra cellular acidosis on anoxic cells has been observed in experimental studies (Morimoto, Kemmotsu et al. 1997; Qian, Nieminen et al. 1997) and some authors have argued that severe metabolic acidosis in critically ill patients is an epiphenomenon (Levraut and Grimaud 2003). In addition, it is very difficult to isolate the consequences of acidosis from those of the underlying pathologic process responsible for the acidosis. As the result, clinical studies evaluating consequences of acidosis with mortality as the primary end-point would require very large sample sizes, which may be impossible to achieve.

In malaria, the association between metabolic acidosis and mortality is consistent and strong. Any of the effects of acidosis on: cellular function including myocytes, sympathetic activity on catecholamines, transmembrane potential or electrolytes level may operate. In one study, acidosis was shown to enhance cytoadherence, a critical factor for sequestration (Marsh, Marsh et al. 1988). However, studies specifically looking into the consequences of metabolic acidosis in severe malaria are still lacking.

Metabolic acidosis is known to affect the oxygen carrying capacity of blood through its effect on erythrocytes 2, 3,-diphosphoglycerate (2, 3-DPG) concentration. For example, acidosis of more than short duration progressively lowers erythrocyte 2, 3-DPG, and in severe diabetic ketoacidosis the concentration of this molecule may decrease to less than 10% of its normal value (Bellingham, Detter et al. 1971; Alberti, Emerson et al. 1972; Beech, Williams et al. 1995). 2, 3-DPG is an important intermediate metabolite in the glycolytic pathway of erythrocytes, and has a significant effect on the oxygen affinity of hemoglobin. As a result, the intra erythrocytic concentration of 2, 3-DPG is generally elevated in anaemic patients (Torrance, Jacobs et al. 1970). However, a decrease in 2, 3-DPG has been described in an animal model of severe malaria (Krishna, Shoubridge et al. 1983) , and a gradual decrease in erythrocyte 2, 3-DPG activity as the parasite matures from ring stage to schizonts during *in vitro* cultures of *P. falciparum* infected red cells has recently been reported (Dubey, Hegde et al. 2003). Therefore, severe metabolic acidosis in malaria may enhance hypoxia.

The effect of the consequences of metabolic acidosis on the efficacy of antimalarial drugs is another possible link between acidosis and poor outcome in malaria. For example, acidosis has been shown to inhibit the uptake of antimalarial drugs by infected erythrocytes (Yayon, Cabantchik et al. 1985). In a randomized controlled trial comparing i. m. artemether and i. v. quinine in children with severe malaria, mortality was higher in the artemether compared to the quinine group, in children with respiratory

distress (Murphy, S., English et al. 1996). In addition, the maximum artemether concentration (C_{max}), and the area under the time plasma concentration curve (AUC) were lower in children with respiratory distress. Together, these results suggested that metabolic acidosis or its consequences affected the bioavailability of artemether from the i. m. injection site. However, i. m. artemether pharmacokinetics and acidosis was not the primary aim of this study and the number of children included for this analysis was small. Although, it has been subsequently shown that poor i. m. artemether bioavailability is more likely due to formulation factors rather than decreased peripheral perfusion associated with acidosis (Mithwani, Aarons et al. 2004), the effect of acidosis on the bioavailability of antimalarial drugs following oral or i. m. administration still needs further investigation. Studies investigating the effect of the consequences of acidosis on biomarkers that may determine outcome such as disease severity factors and drug efficacy are lacking. *Therefore, part of the work described in this thesis focused on the effect of acidosis on the disposition of antimalarial drugs in children with moderate malaria using oral bioavailability of lumefantrine as a prototype marker.*

In section 1.2.3 below, the rationale for arteminsinin-based combination therapy for the treatment of malaria in general, and the rationale for the use of artemether-lumefantrine in children with moderate malaria in particular are described.

1.2.3 Anti-malarial drug resistance and combination therapy

The global malaria eradication campaign of the 1960s was a failure in tropical countries with high malaria transmission including those in Africa. Concerns about the feasibility and sustainability of malaria control measures in countries with poor infrastructure and high malaria transmission frustrated international funding to these countries and global eradication was declared impossible. However, some level of malaria control employing mainly vector control and effective case management continued in these countries. High malaria awareness and the widespread deployment of chloroquine, a cheap and effective drug, lead to reasonable malaria control in many African countries. However, the emergence and spread of chloroquine resistance in *P. falciparum,* the most pernicious species of the four human malaria parasites and commonest in Africa, had adverse consequences to malaria control.

Unfortunately, the effective case management with chloroquine was a monotherapy and the drug has a long elimination half-life. As the result, there was a high rate of parasite exposure to sub therapeutic concentrations of the drug (see section 1.2.4.1 for details) and resistance developed (Watkins and Mosobo 1993). By early 1990s, malaria was exerting an immense disease burden in Africa, and there was clear evidence for a global resurgence of malaria (Marsh, 1998). In eastern Africa, the malaria attributable child mortality had doubled in the 1990s compared to the 1980s (Korenromp, Williams et al. 2003). Further still, the use of sulfadoxine-pyrimethamine as an alternative treatment

after widespread development of chloroquine resistance was also a monotherapy and the drug has a long elimination half-life; resistance to SP developed and spread rapidly. The mechanisms of development and spread of drug resistance are briefly described below

1.2.3.1 The de novo emergence and spread of drug resistance

The genetic event which produces a mutant resistant parasite is spontaneous, rare and considered to be independent of the drug used whereas the spread of resistance is the function of the ability of the mutant parasite to survive through many parasite generations producing gametocyte densities sufficient to be transmitted (White 2004). This ability is associated with fitness advantage to the resistant parasite conferred by the presence of the antimalarial drug to which resistance has developed. This is considered to be the explanation for the apparent stimulation of gametocytogenesis, which characterises poor therapeutic responses to SP (Bousema, Gouagna et al. 2003). There is a suggestion that it is the relative transmission advantage conferred by increased gametocyte carriage that has driven the spread of SP resistance (White and Pongtavornpinyo 2003).

Acquired immunity to malaria is an important determinant of the probability of selection of resistant parasites. Immune responses kill parasites irrespective of drug sensitivity and high parasite densities are usually not found in semi-immune individuals (e.g. adults and

older children in high transmission areas) in whom most parasitaemia is asymptomatic and not treated (White 2004). For this reason, it is estimated that a large proportion of the parasite population worldwide is in non-immune patients from law transmission areas and children and pregnant women in high transmission areas (White 2004). Hence, the *de novo* selection of resistance is more likely to occur in surviving hyperparasitaemic patients because of the following reasons (White 2004):

The probability of *de novo* selection of resistance depends on the parasite numbers; the more the numbers the higher the probability of resistance selection.

Parasites in surviving hyperparasitaemic patients are more likely to have been exposed not only to antimalarial drugs but also to sub therapeutic concentrations of these drugs as disease severity factors such as excessive vomiting and reduced peripheral perfusion impair drug absorption following oral or i. m. administration.

In subsequent infections, resistant parasites have a slow initial response to treatment as well as an increased recrudescence rate. In the absence of immunity, increased recrudescence rate is associated with an increased likelihood of generating sufficient viable gametocytes densities for mosquitoes to transmit and hence propagating drug resistance. The observations that chloroquine resistance emerged in low-transmission areas, and antifolate resistance has increased more rapidly in low-transmission than in high-transmission (White 2004) areas support this hypotheis.

1.2.3.2 Prevention of the spread of drug resistance by combination therapy

According to WHO, the concept of combination therapy is based on the synergistic or additive potential of two or more drugs, to improve therapeutic efficacy and also delay the development of resistance to the individual components of the combination. This concept has been used successfully for the treatment of tuberculosis, leprosy and now HIV.

Combination therapy (CT) with antimalarial drugs is the simultaneous use of two or more blood schizontocidal drugs with independent modes of action and different biochemical targets in the parasite. In the context of this definition, multiple-drug therapies that include a nonantimalarial drug to enhance the antimalarial effect of a blood schizontocidal drug are not considered combination therapy. Similarly, certain antimalarial drugs that fit the criteria of synergistic fixed-dose combinations are operationally considered as single products in that neither of the individual components would be given alone for anti-malarial therapy. It is argued that antimalarial combination therapy will be even more effective in preventing the emergence and spread of resistance for the following reasons (White and Pongtavornpinyo 2003; White 2004):

The genetic event that produces the resistant mutant is rare (White, Nosten et al. 1999; White and Pongtavornpinyo 2003).

The number of estimated parasites in the human population in the entire world is relatively small (White and Pongtavornpinyo 2003).

For the concept of antimalarial combination therapy to work, it is critical that the parasite encounters both drugs at all times. This calls for matched pharmacokinetic properties of the partner drugs in the combination. For example, the terminal elimination half-life of a drug is an extremely important pharmacokinetic property as it determines how long the drug stays in the blood, and to insure that the parasite encounters both drugs at all times, the terminal elimination half-life need to be matched in antimalarial combination therapies. A summary of key characteristics of an ideal antimalarial combination therapy recommended by a GSK Advisory Board (London, 2004) is shown in Table 1.6.

There is now a global consensus that artemisinin-based combination antimalarial therapy will prevent the emergence and spread of drug resistance in *P. falciparum.* Whereas drug resistance has developed to all antimalarial drug classes, no stable and therapeutically significant resistance to artemesinins has been reported and none has ever been induced in the laboratory (White 1997) despite their use for many years in China. In addition, artemensins have a very high parasite-killing rate, they are well tolerated, and no serious adverse effects have been associated with their use.

Artemesinins have a short elimination half life and the ideal partner drug in artemesinin-based antimalrial combination therapy would be one with a short elimination half-life. However, for complete parasite clearance for a *P. falciparum* malaria infection, therapeutic blood concentration need to be maintained for at least for 3 parasite life cycles (at least 6 days) (White and Krishna 1989)and combinations of an artemesinin-based with a similarly short elimination half-life partner drug would have to be administered for at least 7 days. Long treatment periods are associated with poor compliance and a 7-day course of antimalarial treatment, especially with artemesinins, which are associated with rapid parasite clearance and resolution of symptoms, would particularly be associated with poor compliance after the initial days of treatment. This would increase the likelihood of exposing the parasites to sub therapeutic levels of the drug, something not desired if the emergence of resistance is to be prevented. Indeed, short courses (3 – 4 days) of artemesinins derivative monotherapy are associated with high recrudescence rates (Bunnag, Viravan et al. 1991).

Table 1.6: Key characteristics of an ideal antimalarial combination therapy

Independent mechanism of action (MoA)	PRR $\geq 10^2$ (number of parasites killed/cycle)
No known resistance	Matching or complimentary pharmacokinetics (PK)
High therapeutic index (safe)	EC_{99} at 2–4 hours
Tolerable	Increased bioavailability (decreased variability)
Easy to produce (availability)	Minimum requirement of oral bid for ≤ 3 days
Cost of goods $\leq$\$3	Stability >3 years shelf-life
For use in all populations	Co-formulation required
Gametocytocidal (desired for both)	

It has been recommended that, the elimination half-life of the partner drug for an artemesinin-based combination therapy must exceed 24 h if the duration of treatment with the combination is three days (White 2004). Based on this concept, artemesinin derivatives have been combined with partner drugs with relatively longer elimination half-life such as mefloquine (2 weeks) and lumefantrine (6 days) with excellent clinical responses. For example, the systematic deployment of a 3-day course of artesunate and high dose mefloquine (25mg/kg) was associated with a marked decline in the incidence of clinical attacks of *P. falciparum* malaria and a reversal of an already increasing mefloquine resistance in Thailand (Brockman, Price et al. 2000; Nosten, van Vugt et al. 2000)..

1.2.4 Artemether-lumefantrine

Artemether-lumefantrine (Coartem®), produced by Norvatis Pharma, Basel Switzerland is currently the only fixed dose formulation artemesinin-based antimalarial combination therapy (ACT) on the WHO essential drug list. Coartem® contains a fixed-dose (20mg) of a marketed product, artemether (a semi-synthetic derivative of artemesinin) and 120mg, of lumefantrine, a synthetic racemic fluorene derivative developed by the Chinese Academy of Military Medical Science. In a single oral formulation, Coartem® combines the benefits of the rapid parasite-killing rate of artemether and the relatively longer elimination half-life of lumefantrine to prevent asexual parasite recrudescence

after a 3-day course of treatment (Ezzet, Mull et al. 1998). A 6-dose regimen is now the standard treatment with artemether-lumefantrine and the manufacturere's recommended dosage regimen according to body weight is summarized on table 1.7.

Table 1.7: Dosing regimens for artemether-lumefantrine

Dosage	Day 1	Day 2	Day 3
Weight			
5 – 14 kg	1 tab stat 1 tab after 8 h	1 tab bid	1 tab bid
15 – 24 kg	2 tabs stat 2 tabs after 8 h	2 tabs bid	2 tabs bid
25 – 34 kg	3 tabs stat 3 tabs after 8 h	3 tabs bid	3 tabs bid
35 kg and adults	4 tabs stat 4 tabs after 8 h	4 tabs bid	4 tabs bid

Clinical trials conducted in close to 2,000 patients including children conducted in areas with multi-drug resistant malaria around the world have shown that Coartem® is highly efficacious and well tolerated (Bakshi, Hermeling-Fritz et al. 2000). The 3-day 6-dose regimen has consistently produced Day 28 PCR-corrected cure rates, a highly sensitive marker of complete parasitological cure, of more than 95 % (Vugt, Wilairatana et al. 1999; van Vugt, Looareesuwan et al. 2000; Falade, Makanga et al. 2005; Piola, Fogg et al. 2005). In addition, the six-dose regimen of Coartem® (has produced better efficacy and tolerability results than artesunate-amodiaquine (Mutabingwa, Anthony et al. 2005) and Coartem® is better tolerated than artesunate-mefloquine (van Vugt, Brockman et al. 1998; Mayxay, Khanthavong et al. 2004).

To combat drug resistant malaria in Africa, WHO advocates the widespread use of ACTs in national malaria control programmes as first-line treatment of uncomplicated falciparum malaria(Taylor, Rigal et al. 2003). By June 2006, 21 African countries had adopted artemether-lumefantrine as first-line treatment for uncomplicated *P. falciparum* malaria (Global antimalarial drug policies database-AFRO; http://www.who.int/malaria//amdp/amdp_afro.htm). In 2004, Kenya adopted aretemether-lumefantrine as first-line treatment of uncomplicated falciparum malaria and this policy is currently being implemented.

1.2.4.1 Artemether-lumefantrine for the treatment of children with moderate malaria

A strict definition of severe malaria has been developed based on studies looking into the clinical features of severe malaria. Simple bed side signs which define those children more likely to die have formed the defining criteria for severe malaria (prostration, coma, respiratory distress (Marsh, Forster et al. 1995). As the result, clinical malaria is commonly described as uncomplicated (mild) or severe with uncomplicated malaria being treated with oral medication on an out patient basis whereas severe malaria requiring critical care management in hospital with parenteral antimalarial treatment.

The strict definition of severe malaria based on prognostic significance is not in agreement with the broader definition recommended by the WHO, which includes

multiple convulsions, severe anaemia without respiratory distress and hyperparasitaemia (WHO/HTM/MAL/2006.1108; http://www.who.int/malaria/includes_en/whomalariapublications19982004.htm).

However, in reality, clinical malaria is a spectrum with different severity levels from uncomplicated to severe and a classification including mild (uncomplicated), moderate and severe malaria is proposed in this thesis.

Children with malaria and sick enough to require hospitalization but who show none of the features of the strict definition of severe disease can be described as having moderate malaria. This group of children includes those with a haemoglobin level of < 5g/dl or a haematrocrit < 15 % without respiratory distress, those with two or more convulsions within a 24-h period and those who have vomited more than twice within the previous 24 h before admission. These are danger signs according to the IMCI guidelines and patients with this history have an increased risk of clinical deterioration and require supervised management in hospital.

Treatment guidelines have not been developed for children with moderate malaria, and in practice, parenteral quinine is the usual treatment at many district hospitals in Africa. Oral antimalarial treatment according to the prevailing national guideline for the treatment of uncomplicated malaria has been standard treatment of children with moderate malaria at Kilifi district hospital for many years and our experience suggest that whereas few of them have persistent vomiting precluding oral antimalarial treatment, most of them do not vomit everything and oral medication can be given.

Since treatement is supervised in hospital, the few who do not tolerate oral medication are put on parenteral medication.

Potential advantages of artemether-lumefantrine for moderate malaria

In view of the available efficacy and tolerability data for artemether-lumefantrine, its use in patients with moderate malaria is an attractive alternative. Its rapid parasite-killing rate may prevent progression to severe disease and like in uncomplicated malaria the drug is more likely to be well tolerated. In hospitalized children, the problem of a complex dosing schedule will not arise since treatment will be supervised. Compliance will not be a problem; malaria symptoms and signs will be rapidly resolved by the effect of artemether; and hospital stay will be shortened with considerable cost savings.

The practical advantages of treating many admitted malaria patients with oral therapy can only safely be gained if the oral therapy is highly efficacious. However, the efficacy of artemether-lumefantrine in paediatric patients with moderate malaria has not been evaluated. Lumefantrine is highly lipophilic and its oral absorption highly variable between patients (White, van Vugt et al. 1999). There are possibilities that disease related physiological changes such as acidosis (associated with hypovolemia and hence reduced splanchinic perfusion) might affect the disposition lumefantrine and hence, the efficacy of artemether-lumefantrine.

Possible disadvantages

According to the WHO antimalarial drug resistance is defined as the ability of a parasite strain to survive and multiply despite the administration and adequate bioavailability of a drug given in doses equal to or higher than those usually recommended, but within the limits of tolerance of the subject. Several behavioural, pharmaceutical and pharmacokinetic factors may affect the probability of parasites encountering sub therapeutic levels of antimalarial drugs increasing the chances of emergency of drug resistance. The magnitude of the terminal elimination half-life and bioavailbility are the two important pharmacokinetic factors.

In high transmission areas, asymptomatic parasitaemia is common and many people presenting with clinical disease harbour different parasite genotypes together with the one causing symptoms. Antimalarial treatment in these patients leads to exposure to antimalarial drugs of the parasites causing clinical disease and any other parasites that emerge from the liver during the drug's elimination phase. Therefore, the longer the elimination half-life of the drug the greater the probability that any newly acquired parasite will encounter a partially effective drug concentration (Hastings, Watkins et al. 2002). If the drug concentration in the blood during the terminal elimination phase is below the minimum inhibitory concentration (MIC) for the sensitive parasites, the number of parasites will increase, increasing the probability of spontaneous mutation and survival of resistant gametocytes to transmissible densities. In addition, resistant

parasites acquired from elsewhere will develop and then spread while suppressing sensitive parasites.

Infective parasites never encounter intermediate selective concentrations of artemisinin derivatives because these compounds are eliminated completely within the 2-day life cycle of the asexual parasite. The elimination half-life of dihydroartemesinin the active metabolite of artemensinin derivatives, is only 45 minutes (Teja-Isavadharm, Nosten et al. 1996). On the other hand, the relatively long elimination half-life of 3 to 4 days (Ezzet, van Vugt et al. 2000) for lumefantrine might still be a selective filter for newly acquired infections during the elimination phase of the drug, if the concentrations are below the MIC for sensitive parasites. Lumefantrine absorption is greatly increased when taken with food especially fatty meals. Blood concentrations are low after the initial doses when patients are typically vomiting and anorexic and increase in parallel with subsequent clinical improvement (Ezzet, Mull et al. 1998). Since doses are chosen based on the therapeutic ratio, which defines the difference between a therapeutically effective dose and a dose capable of inducing adverse effects, poor oral bioavailability may impair efficacy and increases the probability of parasites encountering sub therapeutic drug levels favouring the spread of resistance.

Metabolic acidosis in paediatric patients with severe malaria has been associated with poor artemether bioavailability following i. m. administration (Murphy, Mberu et al. 1997). However, it has been subsequently shown that poor i. m. artemether

bioavailability is more likely due to formulation factors rather than decreased peripheral perfusion associated with acidosis (Mithwani, Aarons et al. 2004). Nevertheless, the effect of acidosis on the bioavailability of antimalarial drugs following oral or i. m. administration still needs further investigation. In particular, the oral bioavailability of antimalarial drugs with established poor aqueous solubility is of concern in children with acidosis; since it is possible that reduced perfusion of the gut occurs as part of systemically reduced perfusion (Fiddian-Green 1993).

1.2.5 Salicylates and malaria severity in children

Factors that may influence the outcome of a malaria infection in African children are complex. They may be related to the human host, parasite, vector, or the environment (Greenwood 1989). Treatment seeking practices or the response of parents at the first symptom of a malaria infection is one factor with major public health implications. It is widely accepted that prompt and effective treatment will prevent progression to severe disease ("Global malaria control strategy" 1993). On the other hand, delayed treatment gives ample time for the parasite to multiply, mature, and sequester. Indeed, some indication that early treatment may reduce severe malaria morbidity has recently been reported (Sirima, Konate et al. 2003). However, if the prompt treatment at the onset of fever includes the administration of preparations that enhance parasite virulence such as salicylates the risk of severe disease may be increased (Clark, Whitten et al. 2001).

In some parts of Africa it is common that some paediatric patients presenting with malaria to hospital have been treated with salicylate-containing drugs (Slutsker, Chitsulo et al. 1994; Marsh, Mutemi et al. 1999). Salicylates have been shown to enhance and prolong interferon gamma-induced inducible nitric oxide synthase (iNOS) expression (Schwenger, Bellosta et al. 1997), which leads to increased nitric oxide production. As described under pathogenesis of severe malaria in section 1.2.1.11, nitric oxide has been implicated in the development of coma and acidosis. Since falciparum malaria infection is associated with increased levels of circulating cytokines including interferon gamma (Silamut and White 1993), the presence of salicylate in the system (not necessarily in toxic concentration) may enhance nitric oxide production and progression to severe disease. Therefore, the use of aspirin (acetyl salicylate) or other salicylates for treating malaria fever episodes may influence the progression to severe disease.

Toxic plasma concentration of salicylates may trigger a systemic inflammatory response syndrome-like illness (SIRS) in adult patients with sepsis (Chalasani, Roman et al. 1996). This syndrome is characterised by two or more of the following singns: hyperthermia or hypothermia, tachycardia, hyperpoenia or hyperventilation, and leukocytosis or leukopaenia. However, the syndrome has also been reported in patients without an obvious infectious source and it can be activated by conditions such as tissue ischemia and haemorrhagic shock (Chalasani, Roman et al. 1996). SIRS syndrome is triggered by activation of immune cells leading to exaggerated production of proinflammatory cytokines (Dinarello, Gelfand et al. 1993). Administration of

cytokines, such as interleukin-1 can induce the syndrome in laboratory animals. In addition, anti-cytokine antibodies can prevent experimental SIRS (Okusawa, Gelfand et al. 1988). These data from experimental studies demonstrate parallels between the pathogenesis of SIRS and that of severe malaria in African children.

More recently, evidence for increased iNOS expression has been reported in tissues from fatal cases of cerebral malaria and it has been hypothesized that the intake of salicylate (not necessarily in toxic doses) during a malaria fever episode may exacerbate *P. falciparum* infections in children (Clark, Whitten et al. 2001). If this hypothesis is true, it may be possible to achieve a substantial reduction in childhood severe malaria morbidity and mortality through health education and implementation of appropriate drug policies without necessarily having to reduce the overall incidence of malaria infection. Taken together, the studies discussed above suggest a contribution by salicylates to malaria severity. *Therefore, another question tackled by this thesis was whether there is an association between the intake of salicylates and malaria severity and outcome of hospital admission for common infections including malaria.*

1.3 STATEMENT OF THE RESEARCH PROBLEM

Falciparum malaria remains the major cause of childhood mortality in sub-Saharan Africa, claiming about one million lives annually. Although there have been considerable advances in the understanding of severe malaria in African children in recent years, severe malaria case fatality remains high even when standard supportive care and definitive treatment is given during studies of severe childhood malaria at research centers of excellence. Metabolic acidosis, clinically presenting as respiratory distress, is now recognised as an important component of severe malaria in children, and it is the single best independent predictor of mortality.

Most of the deaths occurring in children with severe malaria occur within 24 hours after hospital admission. This is insufficient time for them to get the benefit from the full effect of treatment with quinine, which is still highly efficacious in Africa. Therefore, it appears that mortality reduction in children with severe malaria will depend on increased understanding of complications notably metabolic acidosis. Yet, few studies have looked into the nature of metabolic acidosis in detail or have investigated its aetiology. The underlying pathophysiologic mechanisms for acidosis in these children remain poorly understood and no specific interventions have been developed. Therefore, there is an urgent need for a detailed characterization of metabolic acidosis leading to identification

of underlying pathophysiologic mechanisms. This is critical for the development of potential specific treatment options that would form standard adjunct treatment for severe malaria in children.

In addition, the consequences of metabolic acidosis in severe childhood malaria that may be linked to mortality are still unknown. However, it is important to understand how acidosis might cause death in severe malaria, and if one predominant mechanism is identified, it could act as a drug target for new adjunct treatment in children with severe malaria. Equally important is the understanding of the effect of acidosis on the pharmacokinetics of some antimalarial drugs. For example, there are suggestions that acidosis may affect the bioavailability of i. m. and orally administered antimalarial drugs. Therefore, the understanding of the effect of acidosis on pharmacokinetics of antimalarial drugs may lead to an improvement in drug formulations and thus, improved efficacy.

One putative aetiological factor for metabolic acidosis in African children with severe malaria is the antecedent use of aspirin or other salicylates during the initial stage of a malaria fever episode. The suggestion, based on a small study is that the intake of salicylate is associated with severe malaria with or without acidosis. However, this hypothesis has not been tested in large clinical studies. Therefore, it is of public health importance to identify social factors such as the intake of salicylates that may determine

who develops severe malaria. Many lives could be saved by health education and appropriate drug policies.

The specific objectives and hypotheses for the studies undertaken are therefore presented in the following section.

1.4 OBJECTIVES AND HYPOTHESES

1.4.1 Objectives

To characterise the acidosis associated with severe childhood malaria

To investigate the association between the intake of salicylate and the presence of metabolic acidosis on admission in children with malaria or other common diseases in African children

To investigate the role of the intake of salicylate in the outcomes of *P. falciparum* malaria infection and other common serious childhood infections.

To examine the effect of acidosis on the therapeutic efficacy of artemether-lumefantrine and bioavailability of lumefantrine in children with moderate malaria

1.4.2 Null hypotheses

The intake of salicylate during a malaria episode does not exacerbates *P. falciparum* infection in children

Acidosis has no effect on lumefantrine oral bioavailability in artemether-lumefantrine-treated children with moderate malaria

1.5 STUDY AREA AND STUDY POPULATION

1.5.1 Study area and the community

The clinical studies reported in this thesis were conducted at the Kenya Medical Research Institute (KEMRI)'s Center for Geographic Medicine Research-Coast and Wellcome Trust Research Laboratories at Kilifi District Hospital (KDH). Drug assay, pharmacokinetic analysis, and parasite genotyping were performed at the Wellcome Trust Clinical Pharmacology and Molecular Parasitology Laboratories in Nairobi. KDH is located in Kilifi Township, which is the administrative center of Kilifi District and is located about 60km north of Mombasa on the Kenyan coast.

About 21% of the population served by the KDH comes from an urban community of civil servants and traders in Kilifi town, and 79% is from scattered rural homesteads along the coast and inland. The population in Kilifi district is predominantly Giryama, a sub-group of the Mijikenda ethnic group. The rural communities are mainly engaged in subsistence farming in which, maize and cassava are grown for home consumption, whereas coconut and cashew nuts are grown as cash crops. In general, the KDH serves the population of Kilifi district, in particular however, it serves a community from an area (approximately 700 km^2) in and around Kilifi township designated here as the study area (figure 1.4). This area has been under demographic and epidemiological surveillance for more than 10 years. The majority of the population lives within 2 km of a bus route providing regular transport to KDH. About 80 % of children attending the maternal and child health clinic, and those admitted to KDH reside in the study area whose total population is now about 200 000 people 18 % of whom are children under the age of five years.

The climate in the study area is generally hot and humid with mean daily minimum and maximum temperature of 22 ^{0}C and 30 ^{0}C respectively, and a mean relative humidity of 77 % (weather data from the Mtwapa weather station). Coastal Kenya has two distinct wet seasons, April to June and October to November. Precipitation varies from 75 to 1,200 mm per year throughout the coastal plain. The anopheline vectors of medical importance in this region are *An. gambiae, An. arabiensis*, and *An. Funestus* (Mbogo, Snow et al. 1995; Mbogo, Mwangangi et al. 2003). In the study area, malaria is hyper

endemic and the population receives between 1 and 60 infective bites per person per year. There is wide variation in the Entomological Inoculation Rate (EIR) within the area averaging about ten infective bites per person per year in the northern study area and about 35 infective bites per person per year in the southern study area (Mbogo, Mwangangi et al. 2003). From cross-sectional surveys in the area, approximately 50 % of the children are infected with *Plasmodium falciparum* and the risk of malaria disease requiring hospitalization is high (Snow, Molyneux et al. 1996).

1.5.2 Study population and routine paediatric care at the KDH

Paediatric patients recruited into studies reported in this thesis included children with severe and moderate malaria. In addition, children with acute gastro-enteritis, lower respiratory tract infection, malnutrition, and those with acute bacterial meningitis – the other common childhood severe illnesses in Africa, were enrolled for comparison. However, since the focus of this thesis was on acidosis in children with malaria, only the routine management of children with malaria is reported in this section. The management of other diseases was according to protocols in use at the center at the time.

Pediatric services at the KDH are run by the Kenyan Ministry of Health (MoH) in collaboration with the KEMRI/Wellcome Trust Programme. Hospital supplies as well as medical staff are jointly provided by the two institutions. The clinical services are provided by clinicians from the KEMRI/Wellcome Trust Research Programme on a 24

hour, 7 days a week basis. There are two paediatric wards at the KDH: a general ward (Ward 1) and a high dependence unit (KEMRI Ward). Ward 1 is general pediatric ward with 35 beds capacity catering for all paediatric (0 – 13 years) admissions coming through the KDH out patient department (OPD). At the OPD, children are seen by MoH clinicians where patients who are not too ill to require hospital admission are treated and allowed back home. Those patients ill enough to require hospitalization are admitted to Ward 1 and evaluated by KEMRI clinicians upon admission. Those fulfilling the criteria for admission to the high dependency unit (i. e. those with features of severe disease) are transferred to KEMRI ward, which is a 10-bed ward with equipement for monitoring severely ill paediatric patients. Patients admitted to this ward include those with severe malaria (Marsh, Forster et al. 1995) and those with other life threatening infections or conditions such as meningitis, tetanus, severe burns, and snake bite. Patients with moderate malaria are admitted and nursed in ward 1.

Nursing staff in Ward 1 are MoH employees whereas those in KEMRI Ward are employees of KEMRI/Wellcome Trust Research Programme and are specially trained in research and nursing care of the critically ill child. The programme also provides supportive staff (field workers) for routine medical care services and research activities on Ward 1 and KEMRI ward. In addition, the KEMRI/Wellcome Trust Programme runs a paediatric outpatient clinic where children recruited into studies during hospital admission can be followed up after discharge, and children attending the KDH Outpatient Clinic or Maternal and Child Health Clinic can be recruited into outpatient

studies. Out patient studies include studies looking into the management of uncomplicated malaria in out patient paediatric patients and epidemiological studies of malaria, RSV and Pneumococcal disease. The KDH is a typical busy African rural hospital where as many as 5000 paediatric patients are admitted per year.

Routine investigations

Routine investigation for all paediatric patients admitted to Ward1 and KEMRI Ward at the time of the studies reported in this thesis included a full blood count, blood slide for malaria parasites, electrolytes, creatinine, and blood culture. Full blood count including hemoglobin was done using a Coulter counter (Coulter micro diff. II, Coulter Corporation, Miami, Florida, USA). A thick and thin smear were made on a single glass slide, which was dried, fixed and stained (10 % Giemsa stain) for 10 minutes and read on a light microscope. Malaria parasites were counted and reported per 100 white blood cells from the thick smear, and if there were numerous parasites count per 500 red blood cells from the thin smear was done. A smear was declared negative if no parasite was seen, after examining at least 100 high-powered microscope fields.

Plasma electrolytes (sodium and potassium) were measured using a Corning 614 Na^+/K^+ autoanalyser (equipped with ion selective electrodes, CIBA-Corning Diagnostics Ltd, UK) whereas plasma creatinine was measured using a Beckman creatinine analyser 2 (Beckman, Fullerton, USA). Blood culture was done routinely using the Bactec technique (BACTEC 9050, Becton Dickinson) as part of a surveillance study of

bacteraemia. All laboratory measurements were subject to daily quality control according to standard quality control procedures.

Management of Moderate Malaria at the KDH

Upon admission to Ward1, a field-worker was the child's first point of contact with in-patient care. At this point, social and demographic information, as well as temperature, transcutaneous oxygen saturation (using a pulse oxymeter), and anthropometric measurements were taken. For children eligible for various studies, an informed consent process for enrollment into a particular study was also initiated. The child was then taken to the admission room where she/he was seen by a clinician. A detailed history and physical examination was done, and the findings were recorded on a standardized form (see Appendix II). Blood samples for routine investigation, as described above and any additional samples appropriate for the child's care were taken. Study samples were taken after obtaining an informed consent and were taken at the same time as the routine investigation samples. After completion of the admission procedure, the child was handed over to the nurse on duty at the nurse's station where appropriate supportive and definitive treatment and monitoring if any were started. However, a triage system was in place and the fieldworkers had been trained in identifying danger signs. Children with one or more danger signs were taken to the admission room immediately, seen by a clinician and appropriate resuscitation and supportive care was started before the child's

transfer to the high dependency unit. The admission procedure was completed afterwards.

Patients with moderate malaria i. e., sick enough to warrant hospitalization but who showed none of the signs of severe malaria (table 1.7) were nursed in Ward 1. Malaria treatment was with the first-line antimalarial drug for treatment of uncomplicated malaria according to prevailing national policy. In the period between the year 2000 and 2003, a single dose of sulphadoxine-pyrimethamine (SP) according to body weight as recommended by WHO (table 3.1) was used for treatment of moderate malaria. In early 2004 Kenya adopted artemether-lumefantrine as first-line drug for the treatment of uncomplicated malaria. However, this policy has not yet been implemented and as an interim measure, the division of malaria control recommends a combination of amodiaquine and SP. These paediatric patients with moderate malaria were carefully monitored for development of signs of severe disease and upon recognition of any such sign, which included development of prostration or coma or respiratory distress or a prolonged (more than 5 minutes) convulsion, the patient was immediately transferred to KEMRI Ward where treatment for severe malaria was started. Transfer to the high dependency unit was also indicated if the patient developed repeated short convulsions (at least 3 within a period of 2 h).

Table 1.8: Definitions of severe malaria in children

(Marsh, Forster et al. 1995)

Syndrome	Definition
Severe respiratory distress	Deep breathing (Kussmal's acidotic breathing), chest indrawing
Coma	Inability to localize a painful stimulus
Prostration	Inability to sit upright in a child normally able to do so, or inability to drink in the case of children too young to sit

Management of severe malaria at the KDH

Patients admitted with severe malaria were nursed on the high dependence ward. Upon arrival on the ward they were resuscitated as appropriate and immediately started on supportive care and intravenous quinine. A loading dose of 15 mg/kg quinine base was infused over 4 h followed by at least six doses of 10 mg/kg infusions given every 12 h (Winstanley, Newton et al. 1993). The loading dose was skipped if there was a clear history of intravenous or intra-muscular quinine administration within 24 h before admission. Resuscitation included a 20 ml/kg normal-saline bolus for children with acidosis (negative base excess of ≤ -10 mmol/L), and a 10ml/kg normal-saline bolus followed by blood transfusion (20ml/kg whole blood) for children with acidosis and severe anaemia (Hb ≤ 5g/dl). For patients with low oxygen saturation (< 90 % by pulse oxymetry) resuscitation included the administration of high-flow oxygen. Any convulsion lasting more than 5 min was treated with anticonvulsants - aiming at stopping the seizure – according to the following protocol: Seizures were first treated with short acting drugs: intravenous (0.3 mg/kg) or rectal (0.5mg/kg) diazepam, or intra-muscular (0.2 – 0.4ml/kg) or rectal (0.2 – 0.8ml/kg) paraldehyde. Refractory or recurrent seizures were treated with phenytoin - a loading dose of 18mg/kg infused in normal-saline over 20 min and maintenance dose of 2.5mg/kg every 12 h for at least 48 h, or phenobarbitone, 15mg/kg infused in normal saline over 20 min. Seizures not responding

to phenytoin and phenobarbitone were treated with thiopentone or chlormethiazole, administered in the presence of a consultant paeditrician.

A lumbar puncture was done on all children with neurological manifestations unless there were contraindications, for example, signs of raised intracranial pressure (posturing with brain stem signs, or papilloedema) (Newton, Kirkham et al. 1991) or a patient was in profound coma (BCS ≤ 2) in whom a lumbar puncture was done after the level of consciousness had improved to at least a BCS of > 2. All children with neurological manifestations were started on empirical bacterial meningitis treatment, i. e., benzylpenicillin (50mg/kg) every six hour and chloramphenicol, 25mg/kg until meningitis had been excluded. Any child from whom a pathogenic organism was isolated from CSF or blood was not considered to have severe malaria for the purposes of the studies reported in this thesis. In addition, children with an elevated CSF white blood cell count ($> 10/mm^3$) and a reduced blood: CSF ratio for glucose ($< 2:1$) in whom CSF culture was negative but whose primary diagnosis was considered to be meningitis was not included as severe malaria.

Supportive care for children with severe malaria included maintenance intravenous fluid (dextrose/saline, 4 ml/kg/hr) for those unable to take orally, and correction for hypoglycaemia. Hypoglycaemia (blood glucose $\leq$ 2.2mmol/l) was corrected with a 2ml/kg bolus of 25 % w/v dextrose, followed by maintenance of 4.3 % w/v dextrose and 0.18 % w/v saline solution. The blood glucose was measured again one hour after the

bolus, and if it was found to be ≤ 2.2mmol/l, another bolus dose was given and the maintenance fluid was changed from dextrose/saline to 10 % dextrose until the patient was able to take food orally.

Blood transfusion for those with rapidly falling hemoglobin levels associated with hyperparasitaemia was given at 20ml/kg given over 4h. However, frusemide was not routinely given with the blood transfusion as most of children with severe malaria were dehydrated.

Appropriate management of other complications such as shock and electrolyte derangement was done according to protocols in use at the center at the time.

Vital signs, neurological status, and blood glucose were monitored every day and a review by a clinician was done every 4 h during the first 24 h of admission. When these children were well enough to sit without support and able to take food and medication orally, they were discharged from the high dependency unit to Ward 1 for further care including a full course of first-line antimalarials as described above.

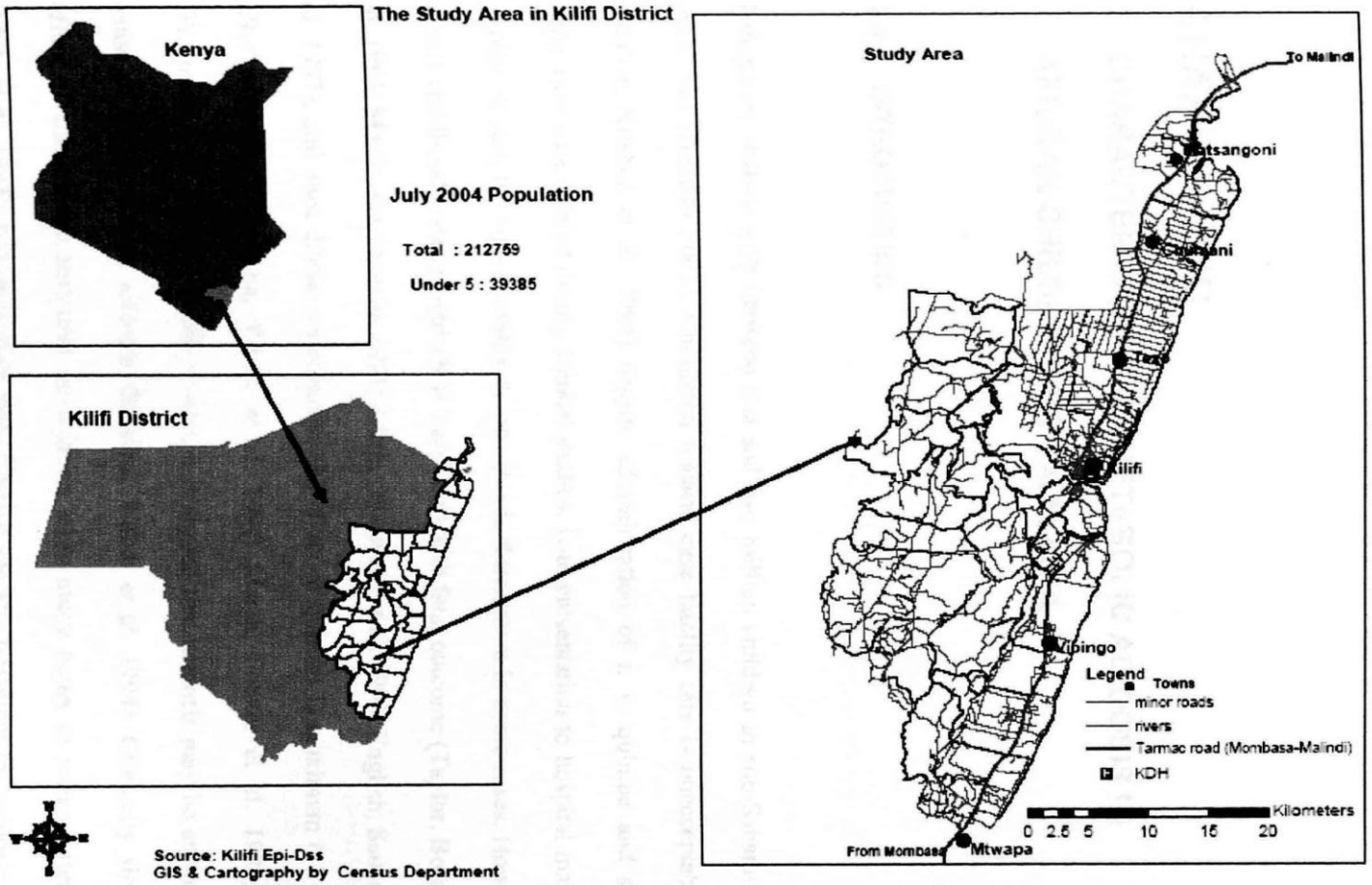


Figure 1.4: The study area: Kindly provided by the Kilifi EPi-Dss, Census Department

CHAPTER TWO

2 CHARACTERIZATION OF METABOLIC ACIDOSIS IN AFRICAN CHILDREN WITH MALARIA

2.1 INTRODUCTION

Falciparum malaria kills between one and two million children in sub-Saharan Africa each year (Breman 2001). Childhood malaria case fatality rate is unacceptably high (Waller, Krishna et al. 1995) despite administration of i. v. quinine and standard supportive care offered during clinical studies. Late presentation to hospital may partly explain in part, the high mortality despite standard treatment in some cases. However, in severe childhood malaria, acidosis is associated with fatal outcome (Taylor, Borgstein et al. 1993; Marsh, Forster et al. 1995; Allen, O'Donnell et al. 1996; English, Sauerwein et al. 1997), and most deaths occur before the effect of quinine is maximum (12 to 24 h after admission) (Krishna, Waller et al. 1994; Marsh, Forster et al. 1995; Allen, O'Donnell et al. 1996). For this reason, it is argued that acidosis may be an important cause of death in these children (Krishna, Waller et al. 1994). Clinically significant effect of anti-malaria treatment on acidosis takes many hours to achieve (ter Kuile, White et al. 1993), and immediate and effective specific treatment for acidosis may be an important adjunct treatment of severe childhood malaria.

Dichloroacetate (a lactate-lowering drug) is in phase II clinical development (Agbenyega, Planche et al. 2003). Its use is based on the fact that metabolic acidosis in children with severe malaria is associated with a raised blood lactate in most cases (Krishna, Waller et al. 1994; English, Sauerwein et al. 1997). However, lactate is not elevated in up to a third of children with severe malaria (English, Sauerwein et al. 1997). In addition, a large clinical trial has shown that use of dichloroacetate was not associated with reduced mortality in critically ill adults with severe lactic acidosis (Stacpoole, Wright et al. 1992). Therefore, other mechanisms may be important, and characterization to identify underlying pathophysiological mechanisms is urgently needed.

The observation of chronic salicylate poisoning in Kenyan children with severe malaria and acidosis (English, Marsh et al. 1996) suggested a role for salicylates. The use of aspirin and non-aspirin salicylates in children is no longer recommended in most developed countries due to safety concerns. In many African countries however, the treatment of a childhood febrile episode starts in the home with proprietary antipyretics bought over-the-counter at shops or other retail outlets (Snow, Peshu et al. 1992; Mwenesi, Harpham et al. 1995), and aspirin and non-aspirin salicylates are still widely available as OTC antipyretic preparations (Goodman, Kachur et al. 2004). As the result, such products are still used widely for the treatment of childhood fever episodes

including malaria fever in the home (van der Geest 1987; Slutsker, Chitsulo et al. 1994; Marsh, Mutemi et al. 1999).

The aims of the study in this chapter were: a) to characterise metabolic acidosis in children with malaria, b) to investigate the role of salicylates. Metabolic acidosis was charactrised using both the traditional and the Stewart's approach, and the scope of previous studies was extended by including children with moderate falciparum malaria and those with a range of severe childhood illnesses to investigate which characteristics of acidosis in malaria are specific or more pronounced.

2.2 METHODS

2.2.1 Study patients

Paediatric patients admitted to Ward 1 during the study period were eligible for inclusion if they fulfilled the following inclusion criteria:

- Patients aged three month to thirteen years.
- Written informed consent obtained (appendix III).

Patients with any of the following exclusion criteria were excluded

- Admitted primarily due to surgical conditions such as trauma, burns, or elective surgeries
- Absence of indication for routine blood sampling
- Being re-admitted with the same problem within two week after discharge
- Presence of known chronic diseases such as renal failure, sickle cell disease and malignancies
- Parent or accompanying guardian not giving written informed consent

The study protocol was reviewed and approved by the KEMRI Scientific Steering and National Ethical Review committees (appendix V).

2.2.2 Procedures

Children went through the routine admission procedure as described in chapter one. On admission, a research clinician reviewed the child and collected data on clinical symptoms and signs using a pre-coded questionnaire (see appendix II), and assessed disease severity according to the classification on table 1.8

During hospitalization, children were managed and treated according to KDH inpatient guidelines at the time (see chapter one for malaria) and their subsequent outcome and discharge diagnosis was recorded. An extra admission-venous-blood sample (2ml) for

purposes of this study was taken at the same time as the routine admission blood sample. Aliquots of this sample were used for determining blood gases and blood lactate. The remaining sample was centrifuged, plasma separated and analysed for sodium, potassium, creatinine, chloride, albumin, inorganic phosphorous, 3-hydroxybutyrate, and salicylate.

2.2.2.1 Blood gases, plasma electrolytes and creatinine

Aliquots (0.5ml) of whole blood for gases were collected in heparinised syringes and immediately analyzed (IL 1620 Blood Gas System, Instrumentation Laboratories, Milano Italy). An aliquot of plasma (60µl) was analysed for sodium and potassium (614 ISE Na^+/K^+ analyser, Chiron Diagnostics Limited, Essex, England) and creatinine (Creatinine analyser 2, Beckman Coulter Inc., Fullerton, CA, USA). The remaining plasma was stored at $-70\ ^0C$ until analysed for chloride, albumin, inorganic phosphorus, 3-hydroxybutyrate, and salicylate as described below.

2.2.2.2 Measuring chloride

Plasma chloride assay was performed daily on a Chloride Analyzer 925 (Ciba Corning Diagnostics Limited, Essex, England). First, the analyser was conditioned using a commercial combined acid buffer reagent (Bayer Corporation, MA USA) and then calibrated using a chloride meter standard (100 mmol/L) solution (Bayer Corporation,

MA USA). Quality control samples (Randox Laboratories Ltd, Crumlin Co. Antrim, UK) at three levels: low (86.1mmol/L), medium (101mmol/L) and high (120mmol/L), were analysed and the results recorded on a quality control chart before running test samples. Test samples were analysed when all controls were within the specified range. However, controls at the three levels were run again after every 10 test samples.

2.2.2.3 Measuring plasma albumin

Plasma albumin was determined photometrically (Photometer 5010, Boehringer Manaheim, Germany) using Bromocresol Green reagent (BCG) - Randox Laboratories Ltd, Crumlin Co. Antrim, UK). Each batch of tests included a reagent blank (containing only 1.5 ml of BCG), a standard (45 g/l) and low (25.6g/l), medium (35.7g/l), and high (46.0g/l) controls (Randox Laboratories Ltd, Crumlin Co. Antrim, UK). BCG (1.5 ml) and standard, controls, and test samples were added (5µl each) to appropriately labeled 3ml-Pyrex-tubes and mixed well (MS1 minishaker, IKA-Works, Inc. Wilmington NC, USA). The tubes were then incubated for 5 min at room temperature before presentation to the photometer's sampling probe. Absorbency for a test sample was read by subtracting blank absorbency form test sample absorbency at 578nm, and the photometer calculated the corresponding albumin concentration using a standard calibration curve.

2.2.2.4 Measuring inorganic phosphorus

A test procedure involving ready-to-use reagents, blank reagent (reagent 1) and Molybdate (reagent 2) (Randox Laboratories Ltd, Crumlin Co. Antrim, UK) was used for inorganic phosphorous assay. First, a working blank reagent was prepared by mixing the two reagents at reagent blank Molybdate ratio of 7:3. Second, measurement of inorganic phosphorus of the working blank reagent and test samples was done using a photometer with a reference standard calibration curve (Photometer 5010, Boehringer Manaheim, Germany). To ensure accuracy and validity of readings, each run of test samples included a standard (1.61 mmol/l) and quality control samples at three levels: low (0.95 mmol/l), medium (1.25 mmol/l), high (2.28 mmol/l) (Randox Laboratories Ltd, Crumlin Co. Antrim, UK). Blank working reagent (1 ml), and standard, quality control, and test samples (10µl each) were added to appropriately labeled 3 ml-Pyrex tubes and mixed well (MS1 minishaker, IKA-Works, Inc. Wilmington NC, USA). The tubes were then incubated for 10 min at room temperature before analysis. Absorbence for a test sample was read by subtracting blank absorbence at 340nm. The corresponding concentrations were calculated by the photometer using a standard calibration curve.

2.2.2.5 Lactate measurements

Aliquots of blood for lactate were collected in tubes containing heparin, sodium nitrite, and fluoride, and were stored at –20 ^{0}C before analysis within 24 h of collection (GM 7 analyser, Analox Instruments Ltd, Hammersmith, London, UK). The analyser was calibrated daily (lactate standard, Analox Instruments Ltd, Hammersmith, London, UK), and a quality control serum (Analox Instruments Ltd, Hammersmith, London, UK) analysed before test samples. In addition, the quality control serum was analysed after every 10 test samples.

2.2.2.6 Measuring 3-hydroxybutyrate

Plasma 3-hydroxybutyrate (β-OH) was determined using an enzymatic reaction (Procedure No. 310-UV, Sigma Diagnostics, St. Louis, Missouri USA) based on the method first described by Williamson and others (Williamson, Mellanby et al. 1962). The procedure is rapid and can be adapted for use in automated instruments without difficulty (Li, Lee et al. 1980; Moore, Marcus et al. 1982).

The enzymatic reaction in procedure No. 310-UV is as follows:

$$\beta\text{-OH} + \text{NAD} \xrightarrow{\beta\text{-HBDH}} \text{Acetoacetate} + \text{NADH}$$

The enzyme, β-hydroxybutyrate dehydrogenase (β-HBDH) acts as a catalyst in the oxidation of betahydroxybutyrate to acetoacetate. This oxidation results in an equimolar amount of nicotinamide adenine dinucleotide (NAD), which is being reduced to NADH. Reduced nicotinamide adenine dinucleotide (NADH) absorbs light at 340 nm, and the increase in absorbency at 340 nm is directly proportional to β-hydroxybutyrate concentration in the test sample.

The reagents for this procedure were purchased from the manufacturer (Sigma Diagnostics, St. Louis, Missouri USA) and included the following:

β-OH reagent solution lyophilized preparation containing (4.6mmol/l NAD, oxamic acid and buffer at pH 7.6)

β-HBDH Solution (β-hydroxybutyrate dehydrogenase enzyme, 50 units/ml with buffer and stabilizers at pH 7.6)

Calibrator solution (50mg/l D-β-hydroxybutyrate with preservative),

Quality control samples: normal (0.69 mmol/l); elevated (7.2 mmol/l)

Validation of the Sigma procedure No. 310-UV

Since this method was being used for the first time at the KEMRI/Wellcome Trust Research Laboratories, validation experiments were performed before using it on test samples.

A calibration curve for absorbency reading to determine the linearity of the assay in our laboratory was prepared. The calibrator solution (50mg/L D-β-Hydroxybutyrate with preservative) was serially diluted with deionised water to obtain concentrations of 10, 20, 30, and 40 mg/l and the absorbency of these working calibrator solutions was determine.

β-OH reagent solution (3 ml) was added to Pyrex-tubes labelled B (blank), 10, 20, 30, and 40 and warmed to 37°C. Blank (deionised water), calibrator solutions (50µl each) were then added to respective dilutions and the tubes mixed well (MS1 minishaker, IKA) after each addition, and the samples were then presented to a photometer sampling probe (Photometer 5010, Boehringer Manaheim, Germany).

The photometer sampled 50µl and read the initial absorbency (340nm). Fifty micro litre β-HBDH solution (enzyme) was then added (50µl) to each tube, vortexing after each addition, and the tubes were then incubated in a water bath at 37.0°C for 10 min before the final absorbence was read. The difference in the final and initial absorbence gave the

change in absorbance in the sample, and subtracting the change in absorbency for the blank solution from the change of absorbency for the calibrator sample gave the absorbency reading for the calibrator sample. A linear plot of absorbency against the known concentration was prepared, and a useful working linearity limit of 5mmol/l was obtained.

This calibration curve was checked against the calibrator and quality control samples from the manufacturer (Sigma Diagnostics, St. Louis, Missouri USA), and quality control samples from another company (Randox Laboratories Ltd, Crumlin Co. Antrim, UK) according to conditions for procedure No. 310-UV as described above. 3-hydroxybutyrate concentration was calculated as follows:

[β-HBA] mmol/l = (ΔA SAMPLE – ΔA BLANK) X 104 X 0.096

Where: ΔA SAMPLE = (Final – Initial absorbency reading of sample)

ΔA BLANK = (Final – Initial absorbency reading of blank)

104 the molecular weight of 3-hydroxybutyrate

0.096 Conversion factor

The results were within the specified range of concentration except for the elevated quality control from Sigma. This was repeatedly above the manufacturer's target value of 6.1 (range: 5.1 – 7.2 mmol/l). According to the manufacturer, the linearity limit for procedure No. 310-UV was 7.7mmol/l. However, in our laboratory, the values for elevated control fell above this limit.

To solve this problem, the elevated control from Sigma was diluted (one part control and one part deionised water) and a set of readings made on each sample ten times and the calculated concentration was multiplied by 2. Mean concentration and the standard deviation were 7.4mmol/L, and 0.032 respectively, and the coefficient of variance (CV) was 0.43 %. With this modification to the elevated control, the standard calibration curve gave acceptable accuracy and reliability even at half the recommended volume of test sample and reagents. Test samples were therefore, run at half volume (1.5ml instead of 3ml), and the target value for elevated control was set at 7.4mmol/L. Each run of test samples included quality control samples from Sigma and Randox, and any test sample reading above 5mmol/l was diluted (1:2) with deionised water and the calculate concentration multiplied by 2.

Assay procedure

To appropriately labelled Pyrex-tubes, 1.5ml of 3-hydroxybutyrate (β-OH) reagent solution was added and the tubes warmed to 37°C. Deionised water (blank,), plasma samples, and quality control samples (Sigma Diagnostics, St. Louis, Missouri USA and Randox Laboratories Ltd, Crumlin Co. Antrim, UK) were added (50µl, each) to the tubes and vortexed (MS1 minishaker, IKA) after each addition. The samples were then placed on the photometer-sampling probe (Photometer 5010, Boehringer Manaheim Germany) and the initial absorbence read at 340 nm. β-HBDH (50µl) was then added to each tube vortexing after each addition, and the tubes were incubated (water bath) at 37.0°C for 10 min before the final absorbance was read. The concentration of 3-hydroxybutyrate (mmol/l) was calculated as described above.

2.2.2.7 Measuring salicylate by fluorescence polarisation immunoassay

Salicylate in plasma was determined by fluorescence polarisation immunoassay (FPIA) using the Abbott TDx analyser (Abbott TDx analyzer, Abbott Diagnostics, Abbott Park, IL USA). This is a test system (TDxFLx system), which uses fluorescence polarisation, and competitive binding immunoassay technologies to quantitatively measure salicylates in human serum or plasma.

The following ready-to-use items were provided with TDxFLx system:

i) Reagent pack containing the following items preserved in Sodium Azide.

- Antibody (S), < 25 % salicylate sheep and rabbit antiserum in buffer with protein stabilizer

- Fluorescein labeled antigen (T), < 0.01 % salicylate Fluorescein Tracer in buffer with surfactant and protein stabilizer.

- Pretreatment solution (P), buffer with surfactant and protein stabilizer.

ii) Six calibrators with known concentration (mg/l) spanning the range of the calibration curve (0.0, 50, 100, 200, 400, 800).

iii) Three controls with target value (range) in mg/l, Low: 75 (63.80 – 86.30, Intermediate: 300 (270.00 – 330.00 and High: 600 (540.00 – 660.0).

iv) Cuvettes and sample cartridges

v) Dilution buffer

Principle of operation

The analyser has a tungsten halogen lump, which emits light of different wavelengths, colours, and random spatial orientation. An interference filter, located in front of the light source, filters the emitted light so that only blue light at 481 – 489nm passes through. The blue light passes through a liquid-crystal polarizer producing plane polarized blue light. When this plane polarized blue light passes through the cuvette, containing reagent and test sample, it hits the tracer or fluorophore raising it to an excited state. In the process of returning to a steady state after excitation, the fluorophore emits green light of between 525 and 550nm wavelength.

When the tracer is bound to a large antibody molecule, its freely rotational property in solution is decreased and the emitted green light remains in the same plane as the blue excitation light, thus polarization is retained. By contrast, when the tracer is not bound to an antibody, it is left to rotate freely and the green light emitted by the tracer as it comes to a steady state after excitation, is in a different plane with that of polarized blue light and polarization is lost. Therefore, the degree of polarization is directly proportional to the size of the molecule and polarization increases as the molecular size increases.

The analyser is programmed to allow a period of incubation for salicylate in the test sample and tracer-labeled antigen in the reagent compete for binding sites on the

antibody molecule, before the final reading of fluorescence polarization. When the test sample contains a low concentration of salicylate, more tracer-antigen complex binds to antibody and less tracer-antigen complex remains in solution. At steady state, there is a high concentration of bound tracer in the reaction mixture and polarization is high. Conversely, if the test sample contains a high concentration of salicylate, less tracer-antigen complex binds to antibody and at steady state, there is low bound tracer in the reaction mixture and polarization is low. However, the precise relationship between polarization and concentration of salicylate in a sample is established by measuring the polarization values of calibrators with known concentrations of the drug (plotting a standard calibration curve).

Method validation

Salicylate assay by fluorescence polarization immunoassay (FPIA) using the Abbott TDx analyzer has been validated against well-established HPLC methods (Karnes and Beightol 1985). The standard calibration curve is precise, accurate, and sufficiently stable to perform reproducible measurements up to 29 days after a single calibration. However, superior accuracy of the method is indicated for salicylate concentrations between 50 and 800mg/l whereas its performance for salicylate concentrations between 0 and 50mg/l is less favourable. Unlike HPLC methods, FPIA with the Abbot TDx analyzer does not require analyte extraction procedures, and is it more rapid and efficient.

Before the initial calibration and subsequent analysis with the salicylate assay, factory set parameters for this assay were verified according to the manufacturer's instruction. First, the sampling probe was washed with distilled water and dried. Second, the calibrators and all controls were loaded (see assay procedure below), and third, the salicylate calibration assay was run. The TDx and TDxFLx software generated a best-fit curve equation and used this equation to prepare a calibration curve. The analyzer then applied calibration curve acceptance criteria including the criterion that all the controls were within the specified range. The accepted curve was stored in memory and salicylate concentrations in unknown samples were calculated from this curve using polarisastion values generated from each sample in the assay.

The curve was checked against quality control samples from a different source (Randox Laboratories Ltd, Crumlin Co. Antrim, UK), and controls were included in every run of patient samples. Recalibration was performed whenever any of the quality control samples was outside the specified range or whenever a new lot number of reagent or buffer was used.

Assay procedure

The Abbott TDx analyzer has a sample carousel with twenty positions for cuvettes and corresponding sample cartridges labeled 1 to 20. This allows analysis of up to 20

samples in a single run. Sample identification was recorded against the corresponding carousel position in the laboratory Daybook. Sample cartridges and cuvettes were loaded on the carousel before a minimum of 50µl of sample was dispensed into the corresponding sample cartridge on the carouse. After all samples were dispensed, the cuvettes were secured by turning the carousel to a lock position. Samples in cuvettes were inspected for any bubbles, which were removed using an applicator stick (different for each sample). The salicylate reagent pack was identified, and its three wells: antibody (S), tracer-antigen (T), and pretreatment solution (P) were opened and inspected for any bubbles. The carousel and the reagent pack were then loaded to their corresponding positions in the analyzer. The analyzer access door was closed, and the file for salicylate assay was selected and opened. Sample identification numbers were keyed in against the corresponding carousel position, before the salicylate assay method was run.

The analyzer went through initiation checks, and upon clearance of all checks, automatically dispensed appropriate amount of reagent to the cuvettes. Next, the probe diluted and dispensed appropriate amount of sample into the corresponding cuvettes, washing in buffer after each sample dispensing. After dispensing reagent and sample, the analyzer did background and blank readings, before incubating the sample-reagent mixture for the competitive binding reaction to reach a steady state. After incubation, the analyser performed final intensity reading (correcting for background intensities) and calculated the level of polarization. At this stage, using the stored calibration curve, the

analyser automatically converted the polarization result to concentration in preset units (mg/l) and assay results were printed.

2.2.2.8 Calculation of parameters for characterization of metabolic acidosis

For the purpose of this study, metabolic acidosis was defined *a priori* as an admission base excess of ≤ -10mmol/l. This definition is indicative of a more marked acidosis than in other studies (English, Sauerwein et al. 1997; Day, Phu et al. 2000; Moviat, van Haren et al. 2003). It was selected because it is close to a readily clinically identifiable threshold (English, Waruiru et al. 1996a), and unequivocally identifies children with a significant metabolic acidosis.

The formulae used for calculating the classic anion gap (AG), albumin corrected anion gap (AG_c), the apparent strong ion difference (SID_{app}) and the effective strong ion difference (SID_{eff}) are provided in appendix I(Fencl and Leith 1993). Since calcium and magnesium concentrations were not measured, the lower limit of standard reference ranges in our laboratory (1.75mmol/l and 0.63mmol/l respectively), were assigned to every patient for the calculation of the apparent strong ion difference. Imputing these low values in each case was a conservative approach likely to underestimate the mean strong ion difference in each group (Maitland, Pamba et al. 2005b).

SIG (strong ion gap) was calculated by subtracting effective SID (SID_{eff}) from apparent SID (SID_{app}). Including lactate and 3-hydroxybutyrate concentrations in the calculation of the apparent strong ion difference (see appendix I) allowed estimation of unidentified strong anions.

2.2.3 Statistical analysis

Data were analyzed using STATA software version 6.0 and 8.0 (Stata corp., College Station, TX, USA). Non-normally distributed data were transformed prior to analysis. Proportions were compared using Chi square test, and forward stepwise regression models were used to identify biochemical parameters associated with acidosis.

2.3 RESULTS

A total of 1902 children were admitted during the study period. Two hundred and fifty-nine did not meet the inclusion criteria. Complete data were available for 1375/1644 (84 %) of the total eligible children. The most common reason for failure to recruit children was the temporary unavailability of blood gas analysis in the laboratory. Despite the limited study period and minor problems with recruitment, the population studied was representative of admissions at KDH. Clinical characteristics on admission, and electrolytes and acid-base data for children with the four commonest conditions are shown on table 2.1 and 2.2 respectively.

Table 2.1: Baseline clinical characteristics of patients admitted with malaria, GE, LRTI and severe malnutrition

	Malaria (n = 624)	GE (n = 127)	LRTI (n = 184)	Severe malnutrition (n = 95)
Median age in months (IQR)	25 (13, 42)	12 (8, 18)	13 (5, 23)	24 (15, 36)
Male [n (%)]	317 (51)	74 (58)	92 (50)	48 (51)
Median weight in kg (IQR)	9.9 (7.7, 12.3)	7.5 (6.2, 8.8)	7.9 (6.1, 9.4)	6.6 (5.7, 8.7)
Mean pulse rate (95 % CI) [a]	156 (154, 159)	146 (140, 151)	160 (155, 165)	141 (135, 147)
Reduced skin turgor [n (%)]	6 (1)	37 (29)	4 (2)	35 (37)
Prolonged capillary refill [n (%)]	94 (18)[b]	32 (29)[c]	26 (17)[d]	25 (28)[e]
Mean weight for age Z score (95 % CI)	-1.7 (-1.8, -1.6)	-1.9 (-2.2, -1.8)	-1.6 (-1.8, 1.4)	-4.0 (-4.3, -3.8)
Mean haemoglobin in g/dl (95 % CI)	7.7 (7.5, 7.9)	9.6 (9.3, 9.9)	9.0 (8.7, 9.3)	7.6(7.2, 8.1)
Severe disease [n (%)]	89 (14)	7 (6)	7 (4)	6 (6)
History of seizures (current illness) [n (%)]	223 (36)	5 (4)	12 (7)	1 (1)
Mortality [n (%)]	9 (1)	2 (2)	4 (2)	18 (19)

abcde

IQR: interquartile range.
[a] Measured using a pulse oxymeter
[b] Malaria (n = 531)
[c] GE (n = 115)
[d] LRTI (n = 156)
[e] Severe malnutrition (n = 90)

Table 2.2: Mean (95 % CI) baseline biochemical characteristics by acid-base status

	Sodium[b]	Potassium[b]	Creatinine[a] (μmol/l)	Lactate[a, b]	pH	pCO2 (kPa)	Bicarbonate[b]
Malaria							
Acidotic (n = 117)	133 (132, 134)	4.6 (4.4, 4.8)	74 (68, 80)	[g]2.9 (2.5, 3.4)	7.24 (7.22, 7.27)	3.2 (3.0, 3.5)	10.6 (10.0, 11.3)
Non-aciotic (n = 507)	134 (133, 134)	4.1 (4.0, 4.2)	47 (45, 48)	[g]2.1 (1.9, 2.2)	7.37 (7.37, 7.38)	4.2 (4.1, 4.2)	18.2 (18.0, 18.5)
Gastroenteritis							
Acidotic (n = 77)	136 (135, 138)	3.5 (3.2, 3.7)	65 (60, 71)	[g]1.6 (1.4, 1.8)	7.24 (7.22, 7.26)	3.1 (2.9, 3.2)	10.2 (9.7, 10.8)
Non-acidotic (n = 50)	135 (133, 136)	3.8 (3.6, 4.1)	54 (48, 59)	[g]1.9(1.6, 2.3)	7.36 (7.35, 7.38)	4.0 (3.8, 4.2)	17.6 (16.9, 18.3)
LRTI							
Acidotic (n = 26)	136 (134, 137)	4.3 (3.9, 4.7)	52 (41, 65)	[g]1.9 (1.5, 2.5)	7.23 (7.18, 7.28)	3.4 (2.9, 3.9)	10.8 (9.4, 12.2)
Non-acidotic (n = 158)	136 (135, 137)	4.4 (4.3, 4.5)	38 (35, 40)	[g]1.8 (1.7, 2.0)	7.36 (7.36, 7.37)	4.4 (4.2, 4.5)	19.0 (18.6, 19.5)
Severe malnutrition							
Acidotic (n = 23)	133 (130, 135)	3.4 (2.9, 3.8)	58 (46, 73)	[g]1.9 (1.4, 2.6)	7.27 (7.22, 7.31)	2.8 (2.5, 3.2)	9.9 (8.7, 11.2)
Non-acidotic (n = 72)	133 (132, 134)	3.6 (3.4, 3.9)	31 (26, 36)	[g]2.0 (1.8, 2.3)	7.39 (7.38, 7.40)	4.0 (3.9, 4.2)	18.6 (17.9, 19.3)

LRTI: lower respiratory tract infection.
[a] Geometric mean
[b] mmol/l

2.3.1 Prevalence of metabolic acidosis in paediatric admissions

Two hundred and eighty-five (21 %) of the 1375 patients with complete data had acidosis as defined in this study. Gastroenteritis had the highest percentage of children with acidosis (figure 2.1). In malaria cases, acidosis was observed to be more common in children meeting the definition of severe malaria than in those not meeting this definition - moderate malaria (57 % versus 12 % P <0.001). In the other diagnostic groups, the prevalence of severe disease was low to permit meaningful within group comparisons.

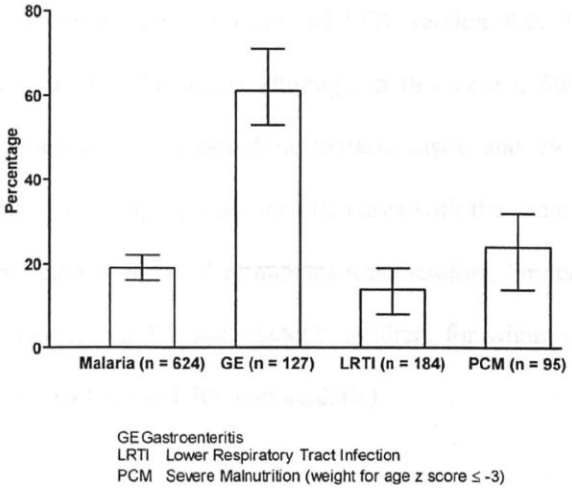


Figure 2.1: Percentage of patients with acidosis (Whiskers: 95 % CI)

2.3.2 Characterization of metabolic acidosis

At the end of the study, frozen samples from all cases of malaria (n =117), LRTI (n= 26), gastroenteritis (n= 77) and severe malnutrition (n = 23) that met our pre-defined criterion of metabolic acidosis were included for further assay to characterise acidosis. As the total numbers of non-acidotic gastroenteritis (n= 50) and severe malnutrition (n= 72) cases were relatively small, all of these were selected for further assay. However, to reduce the total number of assays to be performed while still permitting within group comparisons of reasonable precision, sub-samples of frozen specimens from non-

acidotic malaria and LRTI cases were selected for further assay. To do this, the unique case record numbers for non-acidotic malaria patients in the study were listed and a 25% sub-sample was randomly selected using STATA version 8.0. The procedure was repeated for non-acidotic LRTI cases although in this case a 50% sub-sample was selected. This resulted in 127 non-acidotic malaria cases, and 79 non-acidotic LRTI cases being identified to compare with acidotic cases with the same diagnosis. In total, characterization of acidosis and its determinants was therefore, limited to a population of 571 cases: 243 acidotic, and 328 non-acidotic children, for whom complete data were present in 93 % (223 acidotic and 306 non-acidotic).

Albumin-corrected anion gap, AG_c, and strong ion gap, SIG, were higher than normal (> 13mEq/l and > 0 mmol/l (i. e. positive), respectively) in most children with acidosis in malaria, gastroenteritis, LRTI and malnutrition. Mean (95 % CI) AG_c was 22 (20 – 23), 18 (17 – 20), 21(18 – 23), and 21(17 – 24), and mean SIG was15 (14 –17), 10 (9 – 12), 14 (11 – 16) and 13 (10 – 17) mmol/l, respectively. When all deaths were pooled, mean SIG (95 % CI) was significantly higher in those with fatal outcome compared with survivors, 14.8 (11.2 – 18.4) and 9.7 (9.1 – 10.4) respectively. In malaria patients with acidosis, the mean SID was 38mmol/l (95 % CI = 37 – 39; normal = 48)..

Complete data for analysis of unidentified anions were present in 175 of the 223 acidotic children. Clinically significant unidentified anions (> 5mmol/L)(Durward, Skellett et al. 2001) were present in 51/81 (63 %), 27/55 (49 %), 17/21(81 %), and 11/18(61 %) of

acidotic children in malaria, gastroenteritis, LRTI, and severe malnutrition respectively ($p = 0.076$). Mean (95 % CI) concentration of unidentified anions was 8 (7 – 10), 5 (3 – 7), 10 (7 – 13), and 10 (6 – 23) mmol/l for malaria, gastroenteritis, LRTI and malnutrition respectively.

2.3.3 Determinants of metabolic acidosis

Overall, clinically significant lactate accumulation (blood lactate ≥ 5mmol/L) was more common in children with acidosis than in those without (18 %) versus 7 %; $p < 0.001$). In acidotic children, hyperlacatataemia was significantly more common in children with malaria than in those with the other three conditions 28 % compared with 6 % in gastroenteritis, 13 % in LRTI, and 13 % in severe malnutrition; $p = 0.001$. Similarly, overall, ketosis (plasma 3-hydroxybutyrate (≥ 4 mmol/l) was more common in children with acidosis than those without (39 % versus 18 %; $p < 0.001$). In this case, however, there was no significant difference in the percentage of acidotic children with elevated 3-hydroxybutyrate in the four diagnosis categories (46 %, 35 %, 26 %, 26 % for malaria, gastroenteritis, LRTI and severe malnutrition respectively; $p = 0.112$). Likewise, hyperchloraemia (plasma chloride > 107 mmol/l) was more common in acidotic than in nonacidotic children (25 % versus 17 %; $p = 0.029$). In acidotic children, hyperchloraemia was significantly more common in gastroenteritis than in the other three diseases (36 % versus 18 %, 22 % and 26 % in malaria, LRTI and malnutrition respectively; $p = 0.045$).

Plasma salicylate data were available for 475 (276 non-acidotic and 199 acidotic children), 83 % of the target 571 children. Fifty (11 %) had detectable salicylate in plasma (≥ 5mg/l, detection limit for FPIA method). There was no difference overall in prevalence of salicylate detection in acidotic and non-acidotic children (22/199 (11 %) versus 28/276 (10 %); p = 0.75). In malaria (the largest clinical subgroup) there was similarly no difference in salicylate detection between children with acidosis and those without (14 %) versus (17 %; p = 0.46). Overall, however, after adjusting for the presence of malaria, an association between salicylate detection and the presence of signs indicative of severe illness on admission was observed (odds ratio (OR) = 2.11, 95% CI 1.1 – 4.2).

Following univariable regression analyses for malaria cases only, $\log_{10}$ plasma creatinine (coefficient = –14.66; p <0.001), $\log_{10}$ blood lactate (coefficient = –6.55; p <0.001) and $\log_{10}$ plasma 3-hydroxybutyrate (coefficient = -4.0; p <0.001) were significant predictors of base excess (acidosis). Other significant predictors were age (coefficient = 0.05; p = 0.004) and plasma inorganic phosphorus (coefficient = -2.877; p <0.001). Sodium, albumin, and chloride were not significant predictors. In a forward stepwise regression model, $\log_{10}$ plasma creatinine, $\log_{10}$ blood lactate and plasma inorganic phosphorus were the best independent predictors of acidosis, together accounting for approximately 40 % of variation in base excess (adjusted R^2 = 0.397). The inclusion of plasma 3-

hydroxybutyrate in the model did not improve the fit because of the strong correlation between plasma 3-hydroxybutyrate and creatinine (figure. 2.2).

When the same approach was applied in gastroenteritis cases only, $\log_{10}$ plasma 3-hydroxybutyrate (coefficient = –4.8; $p < 0.001$), chloride (coefficient = –0.2; $p<0.001$) and albumin (coefficient = –0.26; $p = 0.006$) were significant predictors of acidosis. There was also a strong positive correlation between plasma creatinine and 3-hydroxybutyrate in children with gastroenteritis (figure 2.3). In forward stepwise regression model, $\log_{10}$ 3-hydroxybutyrate, chloride and albumin levels accounted for 38 % of variation in base excess (adjusted $R^2 = 0.3834$).

In LRTI and severe malnutrition, $\log_{10}$ plasma creatinine and $\log_{10}$ plasma 3-hydroxybutyrate were the only significant predictors of acidosis, accounting for 20 % and 28 % of variability in base excess (adjusted $R^2 = 0.20$ and 0.276, respectively).

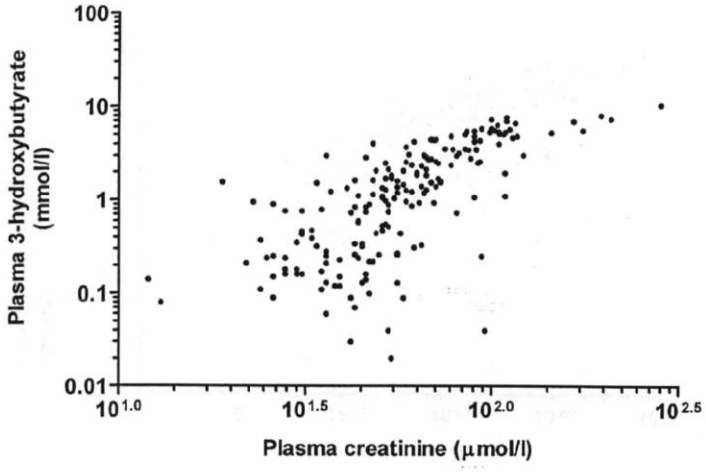


Figure 2.2: Correlation between (log) 3-hydroxybutyrate and creatinine in malaria patients
($r = 0.68$, $P = 0.001$)

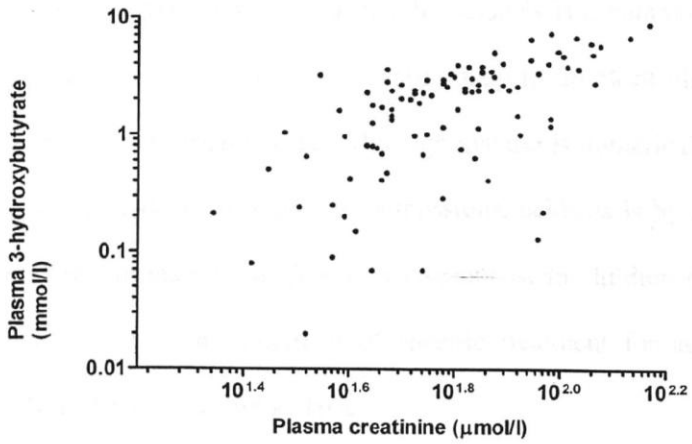


Figure 2.3: Correlation between (log) 3-hydroxybutyrate and creatinine in patients with gastroenteritis
($r = 0.6$, $P = 0.001$)

2.4 DISCUSSION

The findings in this study show that metabolic acidosis is a common complication of severe childhood illnesses in Africa, occurring in up to 20 % of all admissions even when a conservative definition is used. Although malaria is numerically the commonest cause of metabolic acidosis in paedriatric admissions, acidosis is by no mean a unique presentation of severe malaria; in fact, it is commonest in children admitted for acute gastroenteritis. Therefore, development of specific treatment for acidosis as adjunct therapy may be of benefit beyond malaria.

In addition, results presented in this chapter show that metabolic acidosis in malaria, gastroenteritis, LRTI and malnutrition share a common underlying mechanism – the presence of anions in plasma including lactate and ketones that are otherwise absent in healthy subjects. 3-hydroxybutyrate was associated with acidosis in all the four diagnostic groups. This suggests that the underlying mechanism for elevated ketones is not malaria specific. In the background of poor nutritional status in most African children, food deprivation associated with loss of appetite and vomiting during acute illness is a possible explanation for excess ketone in plasma. Excess lactate, however, appears to be malaria specific and this may be explained by more pronounced impairment of tissue oxygenation associated with the key pathogenetic mechanisms of falciparum malaria, sequestration and severe anaemia.

2.4.1 Acidosis in malaria

In malaria, lactate, ketones and salicylates did not account for the entire measured SIG indicating significant amounts of unidentified anions. The presence and prognostic significance of unidentified anions has recently been reported in Thai adults with severe malaria (Dondorp, Chau et al. 2004). In this study, the number of deaths was small, but when all deaths were pooled the mean SIG was higher in those with a fatal outcome than in survivors. Therefore, establishing the identity of these strong anions may have major therapeutic implications.

Experience from critical care units suggest that the choice of specific treatment for metabolic acidosis depends on the nature of acids involved (Levraut and Grimaud 2003). There are suggestions that optimum treatment of the underlying disease in combination with supportive care with the aim of normalising blood volume, cardiac output and tissue oxygenation may be the best treatment for organic metabolic acidosis. Organic acids, (e. g. lactate and ketones) are metabolisable and the acidosis will resolve with time provided the underlying disease is adequately treated and life is supported. On the other hand, alkali therapy or renal replacement therapy may be of benefit in acidosis associated with the presence of non-metabolisable acids or mineral acidosis (Rehm and Finsterer 2003). Conversely, the benefit of alkali therapy is doubtful (Cooper, Walley et al. 1990) and may be harmful in organic acidosis (Hale, Crase et al. 1984).

Results presented in this chapter also demonstrate the important association between plasma creatinine and acidosis in malaria. This association may in large part result from the strong correlation between creatinine and ketones (represented by plasma 3-hydroxybutyrate). Two explanations for this association are possible. First, a longer period of severe illness prior to admission may result in ketoacidosis and an elevated creatinine as a result of catabolism and mild to moderate dehydration, the latter potentially reducing excretion of ketoacids. Alternatively, or in addition, there may be a direct effect of malaria in impairing renal function, resulting in an elevation in creatinine and a reduced capacity to excrete ketoacids and other unidentified acids. The latter suggests that the unidentified anions might be mineral acids associated with renal impairment (e.g. sulphate, urate, hippurate) (Niwa 1996). Although frank acute renal failure is rare in African children with severe malaria(Waller, Krishna et al. 1995), significantly reduced glomerular filtration rates have been documented (English, Sauerwein et al. 1997).

2.4.2 The role of salicylates

Salicylate exposure did not commonly underlie acidosis among the patients included in this study, allaying previous concerns that chronic salicylate poisoning or exposure may be a common important co-factor in the development of acidosis in severe malaria. However, occasional inadvertent salicylate toxicity does occur (English, Sauerwein et al. 1997); personal observation at KDH) and can still cause diagnostic confusion with

severe malaria. However, the prevalence of salicylate intake confirmed by detectable plasma concentration was lower in this study than previously observed in a small study at the KDH (English, Marsh et al. 1996). Change in childhood fever treatment practices in the community following a shopkeeper training programme may explain the reduction in the prevalence of prior salicylate intake in paediatric admissions (Marsh, Mutemi et al. 1999). Therefore, it is possible that salicylate intake may be an important factor in areas where the use of salicylates for treating childhood fever episodes in the household is still common. Interestingly, post hoc analysis in this study did suggest an association between salicylate exposure and the presence of severe illness. However, the numbers of cases included in these analyses were small and this question is specifically addressed in chapter 4 of the thesis.

2.4.3 Acidosis in gastroenteritis

Traditionally, metabolic acidosis in gastroenteritis is of normal anion gap and is characterised by a raised plasma chloride (hyperchloraemic acidosis) (Hill, Morris et al. 1971). Surprisingly, the results of this study suggest that the presence of strong anions other than chloride underlie acidosis in African children with gastroenteritis. According to the traditional approach, loss of bicarbonate in stools is associated with a shift of chloride ions from the intracellular to the extracellular compartment (to maintain electroneutrality), leading to increased plasma chloride concentration. According to the Stewart's approach, loss of cations in stools, rather than bicarbonate, is responsible for

acidosis (decrease in SID) and chloride shift (Kellum 2000). Diarrhoea is associated with dehydration, which causes haemoconcentration leading to an increase in plasma albumin concentration and thus an increase in total plasma weak acid. According to the Stewart's theory, this has an acidifying effect. Therefore, the present finding that plasma chloride and albumin are significant determinants of acidosis in gastroenteritis but not in the other three diseases was expected. Additionally, however, ketones (3-hydroxybutyrate), but not lactate were associated with acidosis in this group of children and unidentified anions were common. This implies that in African children with gastroenteritis the accumulation of acids in plasma is a more pronounced mechanism than bicarbonate or cation loss in the development of acidosis.

2.4.4 Conclusions

The results presented in this chapter indicate that metabolic acidosis in African children with malaria is characterised by the presence of strong anions in plasma, which are otherwise absent in healthy subjects. The strong anions include lactate, 3-hydroxybutyrate and currently unidentified anions. Elevated blood lactate concentration appears to be a more pronounced feature of metabolic acidosis in children with malaria. However, currently unidentified anions are also present and because the nature of these anions is still unknown, it was not possible to determine whether metabolic acidosis in children with malaria is predominantly organic or mineral.

The finding of the presence of unidentified anions in metabolic acidosis associated with malaria is a first step towards establishing the identity and the role of different anions in malarial acidosis. This will have major implications for the development of specific treatment. These implications and the underlying mechanism for metabolic acidosis in children with malaria is discussed in chapter 6.

The presence of detectable plasma salicylates as a marker of salicylate intake was not associated with metabolic acidosis. Therefore, salicylate intake before hospitalization is not a major factor for the presence of metabolic acidosis on admission, although, there may be a possible association between intake of salicylate and signs of severe disease. This possible association is further investigated and discussed in chapter 5.

CHAPTER THREE

3 EFFECT OF ACIDOSIS ON THE EFFICACY OF ARTEMETHER-LUMEFANTRINE

3.1 INTRODUCTION

As discussed in section 1.3 of chapter 1, it is important to understand how acidosis might cause death in children admitted with malaria because such understanding may lead to the design of interventions targeting specific mechanisms or factors.

In chapter 2, it was indicated that approximately12 % of children admitted for malaria but do not show any of the features of severe disease had moderate to severe acidosis, and this suggested that acidosis is not unique to patients with features of severe disease. In this chapter, the effect of acidosis on the efficacy of artemether-lumefantrine was investigated.

Twenty-one African countries have now adopted artemther-lumefantrine as the first antimalarial treatment for uncomplicated malaria. However, paediatric formulation of artemether-lumefantrine is still under development and will not be available until 2008.

Available pharmacokinetic data for artemether-lumefantrine in-patients with malaria suggest that the lumefantrine area under the plasma concentration curve (AUC), which is a measure of bioavailability, determines cure (Ezzet, Mull et al. 1998). However, lumefantrine is highly lipophilic and its oral absorption is highly variable between individuals (White, van Vugt et al. 1999; Ezzet, van Vugt et al. 2000). As described under section 1.2.2.7 (chapter 1), to achieved radical cure for a *P. falciparum* malaria infection, drug concentration above the *in vivo* MIC should be maintained for at least six days from the start of treatment (White and Krishna 1989), and in artemether-lumefantrine-treated patients, lumefantrine plasma concentration 7 days after start of treatment has been found to be a good predictor of cure. In addition, day 7 lumefantrine concentration is a simple surrogate of the lumefantrine total AUC (White, van Vugt et al. 1999). Concentrations less than 280 ng/ml on day 7 were associated with increased risk of treatment failure in Thailand (Ezzet, Mull et al. 1998). Subsequently, this concentration has been judged to be the *in vivo* MIC for multi-drug resistant *P. falciparum* parasites (White and Olliaro 1996).

Therefore, this study had the following aims:

1. *To assess the efficacy of artemether-lumefantrine in children admitted for malaria of moderate severity*
2. *To assess the bioavailability of lumefantrine using day 7 concentration as a simple surrogate of oral bioavailability in paediatric patients with moderate malaria.*

These aims were achieved within the context of a large comparative study of the efficacy of Artemether-lumefantrine, and sulphadoxine-pyrimethamine (SP), which until early 2004 was the standard treatment in patients admitted for moderate malaria at the KDH. The results of this study will be useful in view of the fact that artemether lumefantrine has now been adopted as first line treatment for uncomplicated malaria in several countries in sub-Saharan Africa and its use may be extended to the treatment of moderate malaria in paediatric patients.

3.2 METHODS

3.2.1 Design

This was an open-label randomized controlled trial of the efficacy of the six-dose regimen of artemether-lumefantrine (Coartem®) and pyrimethamine-sulphadoxine (Fansidar®) in children with moderate malaria admitted to the general paediatric ward at the KDH.

3.2.2 Study patients and procedures

3.2.2.1 Inclusion criteria

Patients were eligible if they satisfied the following inclusion criteria:

- Resident in the study area
- Age between six months and 13 years
- History of fever within the previous 24 hours or presence of axillary temperature ≥ 37.5 °C
- Microscopically confirmed *P. falciparum* mono-infection (parasitaemia between 2,000 and 900, 000 per µl)
- Weight ≥ 5 kg
- Signed informed consent by the accompanying parent or guardian (appendix IV)

3.2.2.2 Exclusion criteria

Patients with any of the following features were excluded

- Presence of any of the signs of severe malaria(prostration, coma, deep breathing or inability to feed or drink for children less than 9 months of age (Marsh, Forster et al. 1995)

- Presence of intercurrent illness that may interfere with the assessment of efficacy (otitis, throat infection, skin infection or chest infection – chest signs)
- Severe malnutrition (WAZ score $\leq$ -3 SD or overt Kwashiorkor)
- Presence of chronic disease (e.g. Sickle Cell Disease or suspected symptomatic HIV infection)
- Prescription of i. v. antibiotics by the attending clinician
- Use of SP or any other antimarials within the previous 14 days

3.2.2.3 Study procedures

At the time of the design of this study, an online system for the paediatric admissions had been implemented at the KDH. As the result, screening for eligibility and randomization for this study were done using a computer (FileMaker Pro version 5).

Eligible children were randomized to receive either a single dose of Fansidar® (F. Hoffmann-La Roche Ltd, Basel, Switzerland); each tablet containing 25 mg pyrimethamine and 500 mg sulphadoxine or the six-dose regimen of Artemether-lumefantrine (Coartem®, Norvatis Pharma, Basel Switzerland); each tablet containing 20 mg artemether and 120 mg lumefantrine, given at enrolment and at 8, 24, 36, 48, and 60 h after the first dose.

In this pragmatic trial, the nurse on duty on the general paediatric ward administered the study medications according to body weight as indicated on Table 3.1. Where it was judged that the child was too young or unable to take tablets, the tablet(s) was first crushed into powder, and then administered after mixing with a small quantity of water in a teaspoon. If the patient vomited within 2 h after a dose of artemether-lumefantrine (within 30 minutes for SP), re-dosing was done. A maximum of two replacement doses were permitted medications and vomiting of the second replacement dose was an indication for withdrawal from the study and the patient was given parenteral treatment. Mothers were encouraged to breast feed their child or to give any available food before artemether-lumefantrine administration.

Study patients were hospitalized for a minimum of three days and active follow-up at the KEMRI OPD clinic was done on day 7, 14 and 28 from the start of antimalarial treatment. During hospitalization, study patients were closely monitored for development of severe disease, and upon observation of any such sign the patient was transferred to the high dependence unit for appropriate management for severe malaria. In addition, during hospitalization, patients were evaluated daily for treatment response and occurrence of adverse events. Patients who failed treatment with study medications were given a full course of amodiaquine (Malaratab®, COSMOS Pharmaceuticals Ltd, Nairobi, 10 mg/kg per day for 3 days) as rescue treatment and reviewed after one week.

Response to treatment was assessed using the WHO protocol for in vivo test of antimalarial drugs efficacy (WHO/HTM/RBM/2003.50; http://www.who.int/malaria/includes_en/whomalariapublications19982004.htm) and the day 14 adequate clinical and parasitological response (per protocol analysis, PP) was the principal outcome measure. Secondary outcome measures included the day 28 clinical and parasitological response and the haematological recovery by day 28.

The decision to admit patients was taken by clinical officers who were independent of the study and working at the KDH out patient department. Before enrolment into the study, patients went through the routine admission procedure as described in section 1.5.2 in chapter1. During this process screening for potentially eligible children was done while data was being entered on the computer. KEMRI field workers conducted informed consent discussions and obtained written consent from parents or guardians of eligible children whereas admitting KEMRI clinicians randomised eligible patients after checking that all parameters on the inclusion criteria were present and all parameters on the exclusion were absent.

Baseline blood tests included a blood smear for asexual *P. falciparum* parasite density; 0.5ml blood sample for haematology (hemoglobin and haematocrit; AcT .10 haematology analyzer, coulter corporation florida, U.S.A.); 0.5ml venous whole blood for acid-base status (IL 1620 Blood Gas System, Instrumentation Laboratories, Milan Italy).; and 50 µl of whole blood was spotted on filter paper (Whatman 17 CR,

Maidstone, England), dried at room temperature, and stored in plastic bags with silica gel desiccants at ambient temperature for PCR.

A blood smear for parasite density and a blood sample for haematology was repeated on day 3 and on all active follow-up visits or on any other day if the child presented with fever in which case, a filter paper spot for PCR was also done. For those patients randomised to artemether-lumefantrine, 1ml of blood for plasma lumefantrine concentration was taken on day 7. These samples were collected in lithium-heparin tubes, centrifuged immediately and plasma stored at -80 °C until analysed for plasma lumefantrine.

Table 3.1: Dosing per body weight group

Pyrimethamine-sulfadoxine (Fansidar®) (Each tablet: 25mg pyrimethamine; 500mg sulfadoxine)	
Weight group	**Dose**
5 to < 10 kg	½ tab
10 to<20 kg	1 tab
20 – 30 kg	1½ tab
30+ kg	2 tab
Artemether-lumefantrine (Coartem®) (Each tablet: 10mg artemether; 60mg lumefantrine)	
5 to < 10kg	1 tab
10 to < 15kg	1 tab
15 to < 25kg	2 tab
25 to < 35kg	3 tab

3.2.2.4 Laboratory procedures

3.2.2.4.1 Plasmodium falciparum genotyping

Parasite genomic DNA preparation

Parasite genomic material was prepared according to the methanol fixation method (Nzila, Mberu et al. 2000). Briefly, a small piece (approximately 0.02cm^2) of the blood-impregnated filter paper was snipped and put into a 0.5-ml micro centrifuge tube containing 150 µl of absolute methanol and left in place for 15 min at ambient temperature. Thereafter, the methanol was poured off, and the paper was allowed to dry so that the entire methanol evaporated. Sterilised water (50 µl) was then added to the tube, which was heated at 95°C for 15 min in a polymerase chain reaction (PCR) machine [Model PTC-100, MJ Research, Inc, Massachusetts, U.S.A], and the tube was vortexed every 3 min during this incubation. Following centrifugation (15,000 × *g*, 2 min), 2.5 to 5 µl of the solution was used as the template for the PCR analysis.

PCR analysis for MSP-1 and MSP-2

a) Primary PCR

A nested Polymerase chain reaction technique was applied for detection of the length polymorphism of the *P.falciparum* merozoite surface protein-1and 2 (MSP-1 and MSP-2) genes. A 5µl aliquot of the genomic DNA extract was used as the template for the primary PCR analysis targeting the MSP-1 block2, and MSP-2 block 3 domains. The following PCR conditions were used: 0.2µM outer primers, M103a (5'-GTGTAAACACATGAAAGTTATCAAGAACTTGTC-3) and M103b (5'-CGTAGGATCTTGTGATTTAGGTGTTACATCAAC-3') for *MSP-1*, and m2b3a (5'-GATCATGAAGGTAATTAAAACATTGTCTATTATA-3') and m2b3b (5'-AGCTTTATATGAATATGGCAAAAGATAAAACAAG-3') for *MSP-2*, Mg^{++} free Buffer (1X), $MgCl_2$ (2.5mM), dNTPs (0.2mM), and 1.5U of *Taq* DNA polymerase [Promega, Madison, USA] in a final volume of 50µl. The PCR reaction was performed using the PTC-100™ Programmable Thermal Controller (Model PTC-100, MJ Research, Inc, Massachusetts, U.S.A.) under the following profiles: primary denaturation phase for 45 sec at 92°C, followed by 25 cycles of secondary denaturation at 92°C for 30s, annealing at 55°C for 60sec, extension at 70°C for 2 min and then a final extension at 70°C for 5min.

b) Secondary PCR

In the second round of PCR aimed at amplifying the blocks of varying size polymorphisms, a 2.5µl aliquot of the primary PCR DNA template was used under the following PCR conditions: 0.5µM of inner primers m1b2a (5'-CTAGAAGCTTTAGAAGATGCAGTATTG-3') and m1b2b (5'-GTACGTCTAATTCATTTGCACGAAT-3') for *MSP-1 block 2* while for *MSP-2 block 3*, the inner primers were m2 5a(5'-GAGTATAAGGAGAAGTATGGCAGAAAG-3') and m2 5b (5'-ATGAGGGATGTTGCTGCTCCACAGAGTTTTG-3'), Mg^{++} free Buffer (1.0X), $MgCl_2$ (2.5mM), dNTPs (0.2mM) and 1.5U of Taq DNA polymerase [Promega, Madison, USA] in a final volume of 50µl. The PCR reaction was performed using the PTC-100™ Programmable Thermal Controller (Model PTC-100, MJ Research, Inc, Massachusetts, U.S.A.) under the following profiles: primary denaturation phase for 45 sec at 92^{0}C, secondary denaturation at 92°C for 30s, annealing at 55°C for 60sec, extension at 70°C for 2 min for 35 cycles and then a final extension at 70°C for 2min.

c) Gel electrophoresis

To verify the amplification, the PCR products of MSP-1 block 2, and MSP-2 block 3, were run side by side (before and after treatment) on a 1.5% w/v (1.5g of agarose powder, Promega in a 100ml of 1X TAE buffer) agarose gel electrophoresis at 80V for

two hours. The gel was then stained with a 0.5µg/ml Ethidium-bromide, and viewed under UV transillumination. The sizes of the bands were established using the 100bp and 50bp molecular markers.

A recrudescence was defined as bands of same sizes before and after treatment in both MSP-1 and MSP-2, and new infection was defined as bands of different sizes in both MSP-1 and MSP-2 or of same size in one MSP locus but different in the other.

3.2.2.4.2 Determination of plasma lumefantrine by HPLC

Plasma lumefantrine (beflumentol) concentrations were determined by high-performance liquid chromatography using a modification of the method by Zeng and colleagues (Zeng, Lu et al. 1996) as described below.

a) Chemicals and reagents

Lumefantrine (beflumentol, BFM; MW 528.95; no 3001872, batch # 801195) and its metabolite, desbutyl-lumefantrine (dBFM); and Halofantrine hydrochloride (Lot # 4619-5687; internal standard) were kindly provided by Dr. Geofrey Edwards, University of Liverpool. Acetonitrile, ethyl acetate, methanol and glacial acetic acid (all HPLC-grade), and citric acid (AnalaR® grade) were purchased from BDH Laboratory Supplies (Poole, UK). Boric acid (H_3BO_3, ACS reagent) was obtained from Sigma Chemical Company

(St. Louis, MO, USA). Tri-sodium orthophosphate was obtained from Alpha Chemicals Ltd (Nairobi, Kenya). Deionized water was obtained in-house using an Elgacan C-114 Ultrapure water system (Elga Ltd, Buckinghamshire, UK).

b) Preparation of standard solutions

Stock solution of BFM and desbutyl-BFM (1mg/ml) was prepared by dissolving the compounds separately in glacial acetic acid (2%, v/v) in methanol (acidified methanol). BFM working solutions (100, 10 and 1 μg/ml) were prepared by appropriate serial dilution of the stock solution in acidified methanol. Internal standard (HFN) stock solution (1 mg/ml) was prepared by dissolving HFN hydrochloride in 1% (v/v) glacial acetic acid in methanol. Working solutions (100, 10 and 1 μg/ml) of the internal standard (IS) were prepared by appropriate serial dilution of the stock solution in acidified methanol. The stock solutions were stored in amber glass bottles at about −20 °C in the dark, and the working solutions were prepared freshly before use. Buffer solution (pH 4.0) was prepared by mixing 40 ml of solution A (containing 1.24 g of boric acid and 1.05 g of citric acid dissolved in 100 ml with distilled water; pH = 1.5) and 9 ml of solution B (containing 3.8 g of tri-sodium orthophosphate dissolved in 100 ml of deionised water; pH = 12.2).

c) Extraction procedure

Aliquots of drug-free plasma (250 µl) were spiked with various amounts (10–1800 ng) each of BFM and desbutyl-BFM and a constant amount (3.13 µg) of the IS. Acetonitrile (0.5 ml) was added and contents vortex mixed for 30 sec to precipitate plasma proteins. Following centrifugation (1500 x g; 10 min), the clear supernatant was transferred into a clean silanized glass tube. Buffer solution (pH 4; 0.5 ml) was added, followed by vortex-mixing for 10 sec. Extracting solvent (ethyl acetate-glacial acetic acid; 100:1, v/v; 5 ml) was then added, and extraction effected by end-over-end mechanical tumbling (30 min, speed 5) on a Stuart STR4 blood tube rotator (Stuart Scientific, UK) followed by centrifugation (1500 x g; 10 min; 4 ^{0}C). The upper organic phase was transferred to a clean 15 ml glass centrifuge tube and evaporated to dryness in a water bath (37 ^{0}C) under a gentle stream of white spot nitrogen gas (BOC Ltd, Nairobi, Kenya). The residue was reconstituted in mobile phase (100 µl) and a 50-µl aliquot was injected onto the chromatographic system as described below.

d) Chromatography

Chromatography was performed under isocratic conditions at ambient temperatures. An isochrom delivery system (Isochrom LC; Spectra Physics, San Jose, CA, USA) fitted with a Model 7125 Rheodyne (Cotati, CA, USA) valve injector (50 µl loop) was used.

Chromatographic separation was performed on a reversed-phase column (Hypersil BDS C18 5µ; 250 x 4.6 mm; ThermoQuest Hypersil Division, Rucorn, Cheshire, England) preceeded by a guard column (LiChrospher 100 RP–18e, 5 µm, 10 ×4.6 mm i.d.; Merck, Darmstadt, Germany), using a mobile phase comprising methanol/deionised water/glacial acetic acid (85:14:1 %; v/v) containing 0.03% (v/v) diethyl amine. The flow rate was 1.0 ml/min. The column effluent was monitored with a variable wavelength UV-vis detector (model Spectra Series UV100; Spectra Physics, San Jose, CA, USA) set at 335 nm. Chromatographic peaks were recorded on an integrator (*ChromJet* CH-1 data integrator, Thermo Separation Products, San Jose, California). Peak area ratios (drug/IS were plotted as a function of the known concentrations of the BFM. The results were analyzed by least squares linear regression.

f) Analysis of patient samples

Plasma lumefantrine concentrations were analysed after a successful validation study of the assay method. A constant amount (3.13µg) of IS, was added to 250µl of patient's plasma and the mixture was treated and drugs extracted and the reconstituted solution analysed as described above.

g) Method validation

Precision was determined by assessing the degree of agreement of individual test results for quality control (QC) samples. A constant amount (3.13 μg) of IS was added to 250 μl of quality control samples at three levels: low, mid and high (n =7) at each level and the mixture was treated and drugs extracted as described above. The experiments were repeated to assess intra-assay (within day) and inter-assay (between days) variability.

QC samples were prepared from the stock solutions at concentrations corresponding to the low (LQC), medium (MQC) and high (HQC) levels of the calibration range. Recommended amounts for this assay are 30, 900 and 1800 ng of BFM and desbutyl-BFM (Karnes, Shiu et al. 1991). Both QC and patient samples were stored at –80°C until analyzed. QC samples were assayed in duplicate at the three concentrations. It is recommended that for an acceptable assay performance, at least 4 out of 6 results should fall within ±15% of their nominal concentrations, with at least one of each concentration (Shah, Midha et al. 1991).

Recovery of BFM and desbutyl-BFM was assessed by analyzing duplicate 250–μl aliquots of plasma spiked with various amounts (30, 900 and 1800 ng) of BFM and desbutyl-BFM and a constant amount (3.13 μg) of the IS (n = 7 for each level). The samples were processed as described above. In another set of tubes, equivalent amounts

(3.13 μg) of BFM and desbutyl-BFM and IS were added directly into extracting solvent followed by evaporation of the solvent under a gentle stream of nitrogen. The residue was reconstituted and injected onto the chromatograph as described above. Recovery was assessed by comparing peak area ratios of BFM and desbutyl-BFM to IS for extracted plasma blood samples, with those from equivalent amounts of BFM and desbutyl-BFM spiked directly into the organic phase.

Reproducibility of the assay was assessed by determining the intra- and inter-day coefficients of variation (%CV) for amounts of BFM and desbutyl-BFM in spiked plasma. Intra-assay (within-day) variability was assessed by analyzing seven replicates of plasma spiked with 30, 900 and 1800 ng/ml of BFM and desbutyl-BFM. Inter-assay (day-to-day) variability was assessed by analyzing duplicates of each amount over a period of 5 days.

Selectivity was evaluated by analysis of blank plasma from different sources and evaluating various anti-malarial drugs (chloroquine and its metabolite desethylchloroquine, quinine, sulfadoxine, pyrimethamine, proguanil, cycloguanil, artesunate), and analgesics (acetaminophen, salicylate) used in management of severe malaria for interference with the assay for BFM and desbutyl-BFM. Drug-free plasma was spiked with therapeutic concentrations of these drugs followed by extraction and analysis as described.

Accuracy was evaluated by assessing the degree of agreement of the results that were obtained from spiking known amounts to blank plasma and results that were obtained from pure standards (not subjected to sample pretreatment). To prepare pure standards, equivalent amounts analytes (ng) to those spiked on blank plasma including a constant amount (3.13 μg) of internal standard were added to appropriately labeled tubes and 5 ml of extracting solvent was added and mixed by several gentle inversions. The extracting solvent was then evaporated in a water bath (37 °C) under a slow stream of nitrogen. The residue was then reconstituted in 100μl of mobile phase and 50μl of the solution reconstituted injected into the chromatographic system.

Limits of detection and quantification were determined by spiking known amounts of BFM and desbutyl-BFM near the lower end of the calibration curve (5 – 40 ng) and a constant amount (3.13μg) of internal standard (HFN) to 250–μl of blank plasma. Samples were extracted and analysed as described above. And the experiment was repeated to determine the amount associated with reproducible peak area ratios equivalent to known amounts (lower limit of quantification) and the lowest amount producing non-intergratable peak (lower limit of detection).

3.2.3 Statistical analysis

Based on limited published data on artemether-lumefantrine and none on the six-dose regimen in African children at the time of designing this study, day 14 adequate clinical and parasitological response for the six-dose regimen of artemether-lumefantrine was assumed to be between 90 and 95 %. Given the 80 % day14 parasitological cure rate for SP(Mutabigwa 2002) a sample size of 100 children on each arm was selected to give precision for the estimates of between 4 and 6 % (95 % CI). The intention-to-treat analysis (ITT) population was defined as all patients who received at least one dose of study medication irrespective of study end point. ITT analysis was by worst-case scenario where all patients who violated the protocol or who were withdrawn or discontinued from the study (e. g. loss to follow-up) were considered as treatment failures. Per protocol population (PP) was defined as children who completed full course of study medication according to protocol and completed the 28-day follow up period as described in the protocol. Protocol violations and loss to follow-up were excluded from the PP analysis and patients with missing or indeterminate PCR result were excluded from the calculation of PCR-adjusted ACPR. Mean and standard deviation were used to compare normally distributed base line data whereas median and intequatile range were used for skewed data

3.3 RESULTS

Kenya adopted artemether-lumefantrine as the first-line treatment for uncomplicated malaria half-way through the planned sample size of the trial. At this point, it was considered unethical to continue randomising patients to SP and randomisation was stopped. However, the study continued as a single arm study to assess Coartem© efficacy.

A total of 1420 patients had been screened for eligibility at the time of change from a two arm randomized control trial to a single arm study. The trial profile, including the number of patients included in the interim intention-to-treat analysis for the primary outcome measure (adequate clinical and parasitological response on day 14) are summarised on figure 3.1. Protocol violations during the trial included two children who were randomized to receive Artemether-lumefantrine, but did not get it, because the drug was not available on the ward at the time of enrolment. One child was randomized to receive SP, but was erroneously given Artemether-lumefantrine. Three children on the SP arm were given alternative treatment (amodiaquine) without fulfilling the criteria for alternative treatment as per study protocol. One patient was randomized to the SP arm, but was not given the study drug.

Two patients vomited their second replacement dose of Artemether-lumefantrine, and one child was randomized to receive SP but was uncooperative for oral medication

(repeatedly sput out medication). Three children on the SP arm developed signs of severe malaria within 24 h of starting treatment and were given appropriate treatment for severe malaria including intravenous quinine. One patient on the SP arm developed signs of lower respiratory tract infection within 48h of admission, and was started on intravenous benzyl penicillin. One patient on the SP arm absconded before evaluation on the second day of treatment.

Baseline clinical and demographic characteristics were not significantly different between the SP-threatened and artemether-lumefantrine treated children during the two arm trial and overall (table 3.2). Fifty-five patients had been randomised to receive artemether-lumefantrine and 65 to receive SP at the time of change from two arm to single arm study. The treatment responses for the two arms of the study are presented on table 3.3. Fever had resolved by day seven after start of treatment in all patients. However, on the SP arm, 5/65 (7.7 %) patients had asymptomatic parasitaemia on day 7 compared to none on the artemether-lumefantrine arm.

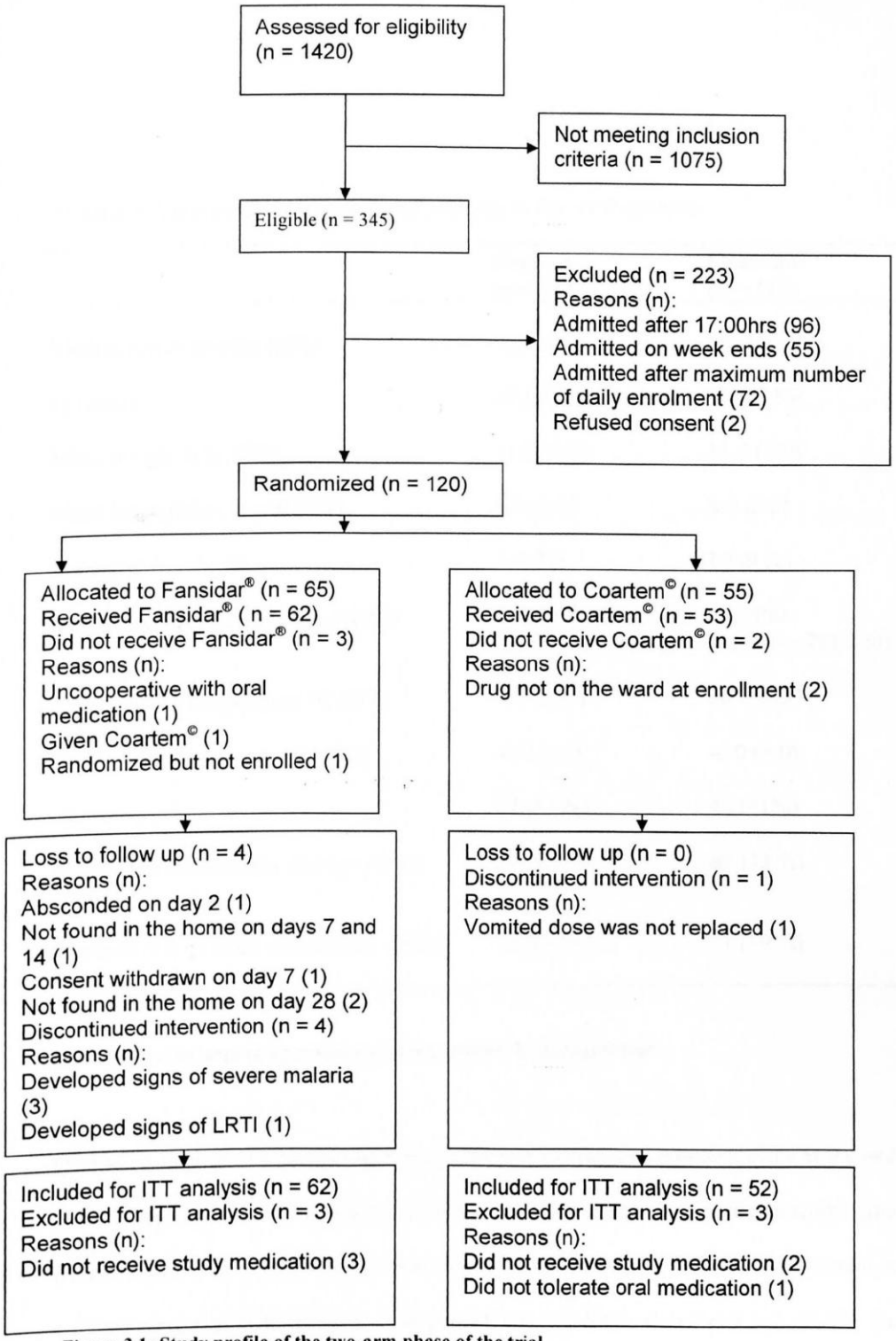


Figure 3.1: Study profile of the two-arm phase of the trial

Table 3.2: Baseline characteristics of patients in the ITT analysis

	Fansidar® **(n = 62)**	**Coartem®** **(n = 113)**
Median age in months (IQR)	28 (14 – 45)	28 (13 – 42)
% female	32 (52 %)	46 (41 %)
Mean weight in kg (SD)	11.8 (4.7)	11.3 (4.0)
Mean hemoglobin in g/dl (SD)	8.8 (2.1)	8.3 (2.2)
Hemoglobin ≤ 5 g/dl	4 (6 %)	10 (9 %)
Median parasite density per µl (IQR)	147 540 (66 800 – 368 920)	152 900 (51 800 – 281 520)
Mean axillary temperature °C (SD)	39.0 (1.0)	38.7 (1.2)
Mean base excess in mmol/l (SD)	-6.8 (3.5)	-6.0 (4.0)
Clinically pale	17 (27 %)	46 (41%)
Presence of convulsions during current illness	33 (53 %)	48 (43 %)
Presence of 2 or more convulsions within previous 24 h	18 (29 %)	21 (19 %)

3.3.1 Treatment response to artemether-lumefantrine

Over all, a total of 118 patients had been allocated to artemether-lumefantrine at the end of the study. There were nine (8 %) protocol violations during the single arm study: One patient vomited two replacement doses of artemether-lumefantrine and in one patient, a vomited dose was not replaced. One patient was randomised but did not receive the

study medication because artemether-lumefantrine could not be available on the ward at the time of enrolment. In two patients it was discovered after recruitment, that the exclusion criteria had been violated. One patient had a clinically significant drop in hemoglobin and developed a prolonged convulsion two days after start of treatment. The patient was treated according to the standard medical care for severe malaria at the KDH including a blood transfusion and recovered fully. Consent was withdrawn in two (2 %) children whereas three (3 %) children were lost to follow-up on day 7. Six (5 %) patients were lost to follow-up on day 14 and five (4 %) on day 28.

Except for one child who remained with fever and parasitaemia on day 3, fever had resolved and parasites cleared by day 3 from start of treatment in all the 110 patients in the PP population, and parasites had not reappeared by day 7. However, two (2 %) patients had reappearance of parasitaemia associated with fever on day 14, and one had reappearance of parasitaemia and fever on day 26. In addition, 4(4 %) patients had reappearance of parasitaemia associated with fever on day twenty-eight. Adequate clinical and parasitological response is presented on table 3.3. In addition, a steady haematological recovery during the twenty-eight days of follow up period was observed in these paediatric patients treated with artemether-lumefantrine (figure 3.2).

Table 3.3: Treatment response to SP and artemether-lumefantrine

	Fansidar®	[g]Coartem®	Coartem® total[f]
ETF			
ITT	15/62 (24 %)	3/52 (6 %)	4/113 (4 %)
PP	10/57 (18 %)	1/49 (2 %)	1/110 (1 %)
Day 14 ACPR			
ITT	34/62 (55 %)	44/52 (85 %)	96/113 (85 %)
PP	34/45 (76 %)	44/46 (96 %)	96/99 (97 %)
Day 28 ACPR			
ITT	21/62 (34 %)	36/52 (69 %)	76/113 (67 %)
PP	21/36 (58 %)	36/44 (82 %)	76/98 (78 %)
PCR-adjusted day 28 ACPR			
ITT	ND*	ND	84/104 (81 %)
PP	ND	ND	83/88 (94 %)

Twelve children (11 %) of the patients included in the ITT analysis had acidosis (base excess ≤ -10mmol/l). Only one of these artemether-lumefantrine-treated patients with acidosis had parasite reappearance on day 28 and none deteriorated or had early treatment failure.

[g] Patients treated with Coartem© during the two arm phase of the study
[f] Includes patients during the two arm phase of the study.
* Not done

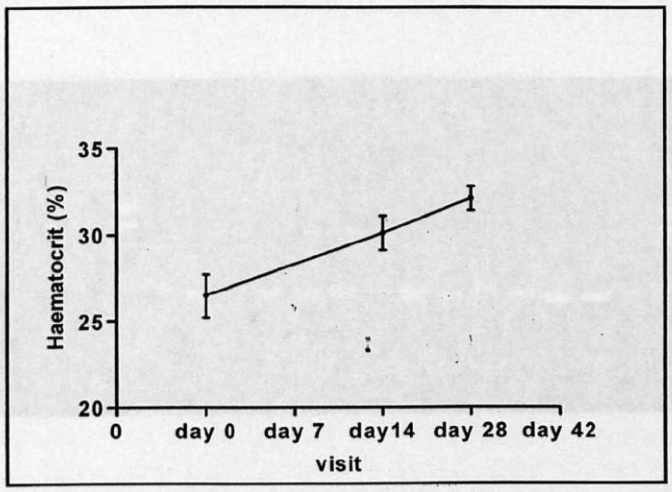


Figure 3.2: Mean haematocrit (95 % CI) over 28 days of follow-up

3.3.2 Parasite genotyping

Parasites reappeared in 20 (18 %) out of 113 artemether-lumefantrine treated patients in this study. Filter paper strips for PCR before and after treatment was available in 14 (70%) patients. Eight of these patients had new infection at parasite reappearance; two children had recrudescence; and PCR result was indeterminate in 4 patients and missing in six patients. The median age of children with parasite reappearance was lower than the median age in children who did not have parasite reappearance during the 28 days of follow up (median age (IQR): 22 (11 – 38) versus 30.5 (14 – 43) months).

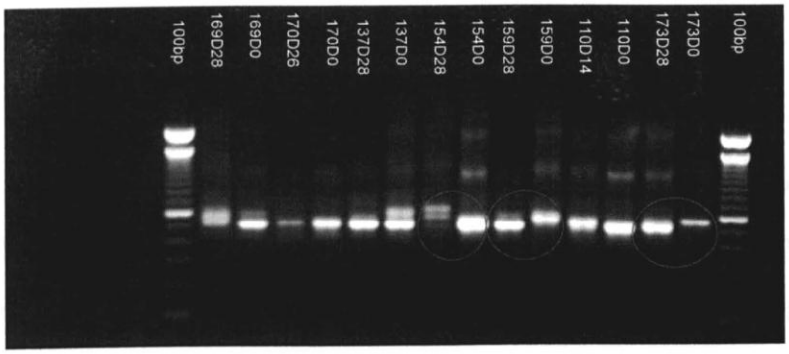


Figure 3.3: An illustration of agarose gel electrophoresis results showing recrudescence and new infections for MSP-1

The parasite genotype was different before treatment (Day 0) and after treatment (Day of parasite reappearance) for patient numbers: 173, 159, and 154; and are examples of new infections (circled in red). The remaining patients had same parasite genotype before and after treatment and are examples of recrudescence. Patient number 137 had two parasite clones before treatment (Day 0); one clone had been cleared at the time of reappearance but one clone persisted.

3.3.3 HPLC assay of plasma lumefantrine on Day 7 samples

3.3.3.1 Chromatography

HFN (internal standard), desbutyl-BFM and BFM were resolved to baseline with retention times of 6.5, 7.5 and 9.0 min, respectively. This is in contrast to one previous method that depended on pH changes in the mobile phase to achieve good peak resolution between BFM and desbutyl-BFM. In the method described in this thesis, good resolution between the peaks was achieved without the need to change the pH of the mobile phase. The limit of detection was 5 ng for BFM and 10 ng for desbbutyl-BFM, respectively at a detector attenuation of 0.005 a.u.f.s.

3.3.3.2 Recovery, calibration curves and reproducibility

The mean (% ±S.D) recovery of BFM (n = 7) was 83.0 ± 3.7, 84.4 ± 4.2 and 86.5 ± 2.5.) at 200, 2000 and 15000 ng/mL, respectively. The recovery of IS was 85.8 ± 2.6 (% ± S.D.) and independent upon BFM concentration. The lower limits of quantification (LLOQ) was determined as 30 ng/mL with R.S.D. and accuracy <20. The intra-assay coefficients of variation at 30, 900 and 1800 ng/ml of BFM and desbutyl-BFM were 7.7, 8.9 and 6.2% (BFM) and 10.1, 5.4 and 3.9% (desbutyl-BFM), respectively. The inter-assay coefficients of variation at the same concentrations were 5.2, 8.1 and 6.9% (BFM)

and 3.3, 2.3 and 4.6% (desbutyl-BFM), respectively. The calibration curves in spiked human plasma were linear within the range 30–2500 ng/ml (figure 3.4).

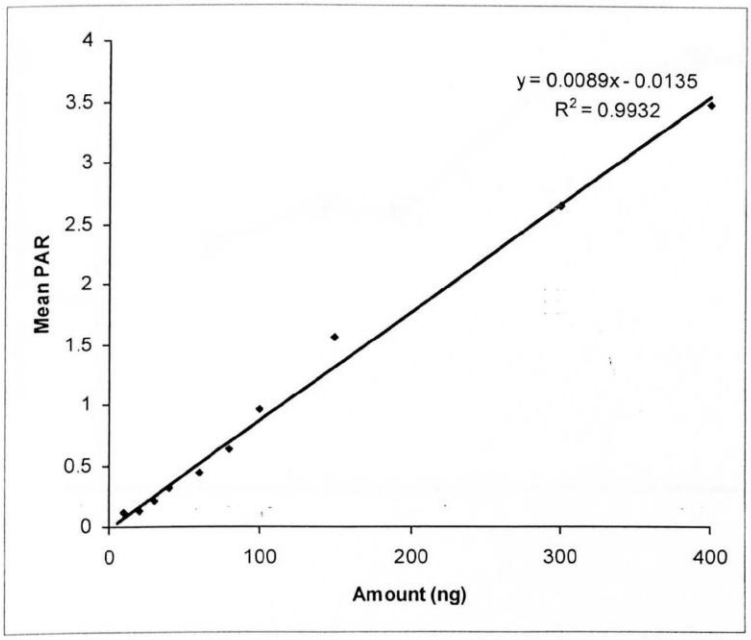


Figure 3.4: A typical calibration curve for the lumefantrine assay method

3.3.3.3 Validation study

The assay method was used to measure the concentrations of lumefantrine and desbutyl-lumefantrine in plasma samples obtained from a child following administration of two doses of BFM 200 mg each at 0 and 8 hours. A plasma concentration versus time profile for BFM is shown in Figure 3.6. BFM maximum plasma concentration of 1861 ng/ml was achieved at 16 h, whereas desbutyl-BFM was not detected in all sampling points.

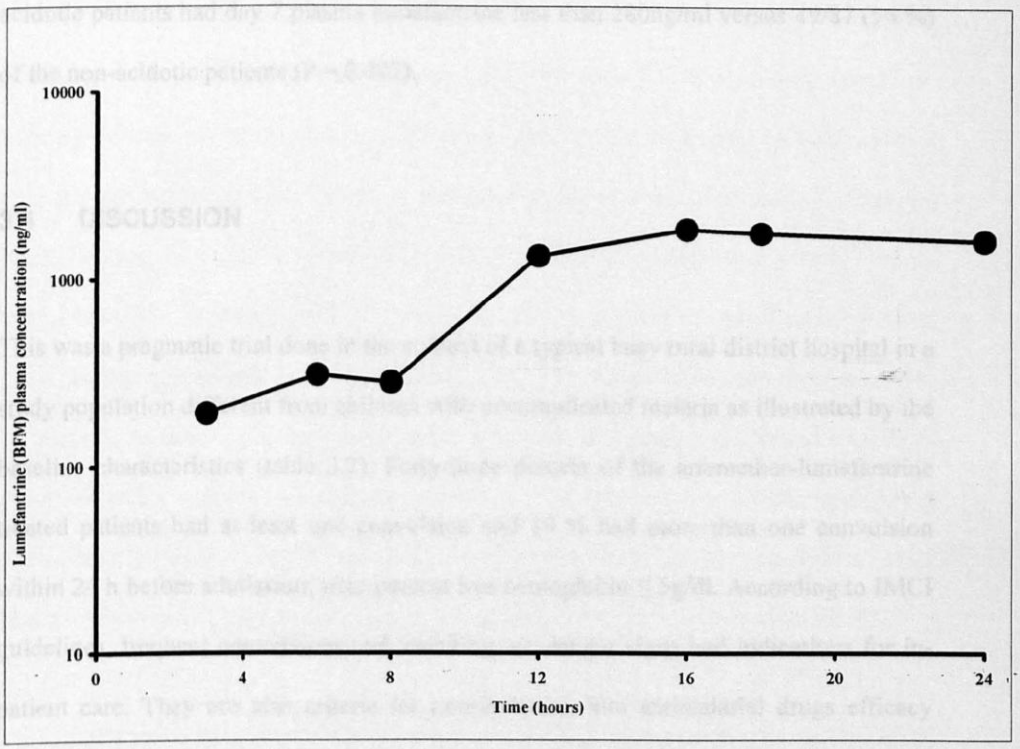


Figure 3.5: Plasma concentration versus time profile of lumefantrine following administration of two doses 200 mg lumefantrine each at 0 and 8 h

3.3.3.4 Day 7 plasma lumefantrine concentration

Ninety-nine (88 %) of patients that were included in the ITT analysis had a plasma sample for lumefantrine concentration on day 7. The median (interquatile range) lumefantrine concentration was 236.9 (129.8 – 344.8) ng/ml. The concentrations were not significantly different between nonacidotic and acidotic patients, mean (95 % CI) 279.4 (243.0 – 316.0) and 266.4 (107.2 – 425.7) respectively. However, 8/12 (67 %) of

acidotic patients had day 7 plasma lumefantrine less than 280ng/ml versus 49/87 (56 %) of the non-acidotic patients (P = 0.497).

3.4 DISCUSSION

This was a pragmatic trial done in the context of a typical busy rural district hospital in a study population different from children with uncomplicated malaria as illustrated by the baseline characteristics (table 3.2). Forty-three percent of the artemether-lumefantrine treated patients had at least one convulsion and 19 % had more than one convulsion within 24 h before admission; nine percent had hemoglobin $\leq$ 5g/dl. According to IMCI guidelines, frequent convulsions and vomiting are danger signs and indications for in-patient care. They are also criteria for non-inclusion into antimalarial drugs efficacy studies in patients with uncomplicated malaria according to the current WHO protocol (WHO/HTM/RBM/2003.50; http://www.who.int/malaria/includes_en/whomalariapublications19982004.htm). As such the WHO definition of severe malaria includes these features, which are not features of severe malaria in the more strict definition based on prognostic significance (table 1.8).

The efficacy of artemether-lumefantrine by day 14 in the PP population was excellent and comparable to data reported in patients with uncomplicated malaria (Falade,

Makanga et al. 2005; Piola, Fogg et al. 2005). However, day 14 efficacy data is now considered not to reflect the true picture as it is too soon for all recrudescence or re-infection to have taken place. As the result, it is now recommended that children enroled into antimalarial drug trials should be followed-up to 42 days after start of treatment (Stepniewska, Taylor et al. 2004). Therefore, day 28 efficacy data in this study is more useful than day 14 data. Even by day twenty 28 the efficacy of six-dose artemether-lumefantrine regimen in these moderately severely ill children was excellent and comparable to data in uncomplicated malaria (Falade, Makanga et al. 2005; Piola, Fogg et al. 2005). In addition, the PCR-unadjusted day 28 efficacy data for the randomised controlled clinical trial part of this study is similar to the data for the overall artemether-lumefantrine-treated population (table 3.3) suggesting that these data are reproducible.

Like in children with uncomplicated malaria, artemether-lumefantrine was well tolerated by the vast majority of children with moderate severe disease in this trial. Only 2/113 (2 %) did not tolerate the drug and required parenteral medication. Artemether-lumefantrine has been shown to be safe both in children and adults (Bakshi, Hermeling-Fritz et al. 2000), and all serious adverse events observed during this trial were related to the disease (malaria) rather than the drug suggesting that artemether-lumefantrine is also safe and well tolerated in children with moderate malaria.

In this study, lumefantrine bioavailability (measured by day 7 plasma lumefantrine concentration) was inadequate in more than 50 % of children despite excellent efficacy.

Fifty-seven percent of the artemether-lumefantrine-treated children did not achieve greater than 280ng/ml cut-off reported to be associated with radical cure. These findings are similar to those that have recently been reported in Ugandan children with uncomplicated malaria who received the six-dose regimen unsupervised (Piola, Fogg et al. 2005)

Lumefantrine absorption is enhanced by co administration with food especially fatty foods. In this study, food intake before or shortly after artemether-lumefantrine administration was not rigorous, and mothers were encouraged but left to decide wheather or not to feed or breastfeed their child, and the type of food given did not matter. This can explain in part, the observed low Day 7 lumfantrine concentrations. In the Uganda study of paediatric patients with uncomplicated malaria, fatty food was co administered in the supervised group and these children achieved higher lumefantrine levels than the unsupervised group (Piola, Fogg et al. 2005). Although the authors attribute the difference in lumefantrine levels in supervised and unsupervised treatment groups to the supervision of treatment, fatty meal co administration may also explain this difference. However, the presence rather than the quantity of fat may be more important (Mayxay, Khanthavong et al. 2004). Nevertheless, as this study demonstrates, Day 7 lumefantrine concentration does not seem to be related to treatment outcome of the six-dose regimen of artemether-lumefantrine in African paediatric patients.

Theoretically, the presence of disease severity factors, in particular acidosis, may affect oral bioavailability due to reduced gut perfusion. However, in this study, the presence of acidosis did not seem to affect efficacy. Even so, a higher percentage of acidotic children did not achieve the 280ng/ml cut-off compared to non-aciodtic patients. However, the number of children with moderate to severe acidosis (base excess < or = -10mmol/l) in this study was small and these findings call for a cautious interpretation. Nevertheless, if artemether-lumefantrine is used in paediatric patients with moderate malaria, it is important to identify those with severe acidosis (clinically evident deep breathing) as this is a feature of severe disease, which requires treatment with parenteral quinine.

The presence of relatively strong background acquired immunity to malaria in African paediatric patients is considered to explain the observation of excellent efficacy despite low Day 7 lumefantrine concentration (Piola, Fogg et al. 2005). Host immunity complements the clearance of remaining parasites by lumefantrine after the rapid effect of artemether. Age is a proxy measure of acquired immunity to malaria and children with parasite reappearance in this study were younger than those with successful artemether-lumefantrine treatment. However, this study was not specifically designed to evaluate the effect of age. Most patients treated with artemether-lumefantrine were below five years of age 102 (89 %) precluding meaningful comparison by age. *P.falciparum* isolates from Africa have been reported to be extremely sensitive to lumefantrine (Wernsdorfer 2004) and this may further explain excellent efficacy in the

background of inadequate lumefantrine bioavailability. Therefore, day 7 plasma lumefantrine concentration with 280ng/ml cut-off as good predictor of radical cure may not be appropriate in Africa where background immunity is high and parasites extremely sensitive. The effect of acidosis on lumefantrine bioavailability is further examined in chapter 4.

In conclusion, artemether-lumefantrine is as highly efficacious in children with moderate malaria as it is in those with uncomplicated malaria. It can be used in children admitted for malaria of moderate severity with excellent results. Even in these children with moderately severe disease, parasites are completely cleared and symptoms are resolved by day three after start of treatment. Practically, children can be discharged 24 hours after start of treatment and given the remaining three doses (every 12 hours) by mothers at home. There is now evidence that artemether-lumefantrine efficacy is not reduced when drug administration is unsupervised (Mutabingwa, Anthony et al. 2005; Piola, Fogg et al. 2005).

This study has also demonstrated a steady haematological recovery associated with artemether-lumefantrine treatment and this would be an important advantage of using artemether-lumefantrine in paediatric patients with moderate malaria who would, in general, have higher parasite load than patients with uncomplicated malaria. In addition, a relatively low recrudescence rate which has been previously reported with artemether-lumefantrine (Mutabingwa, Anthony et al. 2005) was also observed in this study and

adds to the advantages of using artemether-lumefantrine in childhood moderate malaria. Cost is still an impediment to the implementation of artemether-lumefantrine as first-line treatment for uncomplicated malaria in many African countries. However, for national malaria control programmes that are able to implement this policy or when this policy is implemented, it would be worth extending the use of artemether-lumefantrine to in-patients with moderate malaria.

CHAPTER FOUR

4 EFFECT OF ACIDOSIS ON LUMEFANTRINE BIOAVAILABILITY IN PAEDIATRIC PATIENTS WITH MODERATE MALARIA

4.1 INTRODUCTION

Pharmacokinetic information is important in the evaluation of new drugs in the intended patient population. Conventionally, pharmacokinetic parameters are established by rich data from adult volunteers involving multiple blood samples per volunteer over time following administration of a single dose of the drug under investigation. It may be difficult to obtain rich data for some patients especially in paediatric patients. Population pharmacokinetics is a relatively new concept and increasingly used to solve the problem of obtaining rich data especially in children. Each patient contributes a few samples and the data is modeled to produce pharmacokinetic parameters for that patient population.

In this thesis the possible use of artemether-lumefantrine for the treatment of moderate malaria in paediatric patients was investigated. The pharmacokinetics of artemether-lumefantrine in African paediatric patients has not been studied, and it was important to examine the pharmacokinetics of artemether-lumefantrine in moderate childhood malaria. In particular, the bioavailability of lumefantrine in relation to acidosis (a disease

severity factor in malaria) during the first 24 h of treatment when acidosis is still present was investigated.

The area under the plasma concentration-time curve (AUC) represents the amount of drug available systemically and for antimalarial drugs, the amount to which parasites in blood may be poententially exposed. In malaria patients treated with artemether-lumefantrine, the total lumefantrine AUC ($AUC_{0-\infty}$) has been shown to be a key pharmacokinetic parameter influencing Day 28 cure rate (Ezzet, Mull et al. 1998). In chapter 3, Day 7 plasma lumefantrine, a surrogate of the total AUC was used to evaluate the relationship between lumefantrine bioavailability and therapeutic response in paediatric patients with moderate malaria.

Day 7 plasma lumefantrine as a surrogate for AUC was derived in population pharmacokinetic studies of Thai patients most of them adults (Ezzet, Mull et al. 1998; Ezzet, van Vugt et al. 2000), and there has been no similar studies reported in African paediatric malaria patients particulary those with moderate malaria. *Therefore, in this chapter, the effect of acidosis on lumefantrine AUC and any subsequent effect on treatment response were studied in more detail using plasma lumefantrine concentration-time profiles*. Pharmacokinetic sampling was done during the first 24 h after start of treatment, the time when acidosis would not have resolved in most children under treatment (Maitland, Pamba et al. 2003).

Lumefantrine is lipophilic and hydrophobic and almost insoluble in water and aqueous acids (White, van Vugt et al. 1999) leading to erratic absorption. Since the presence of food in the stomach stimulates splachnic and hepatic blood flow and induces bile acids release from the gall bladder, artemether-lumefantrine co administration with food improves lumefantrine bioavailability (White, van Vugt et al. 1999). The fraction of lumefantrine oral dose absorbed has been shown to increase with the resolution of malaria symptoms and the AUCs for the first two doses are significantly less than for the third and fourth dose (Ezzet, Mull et al. 1998). With the six-dose regimen, the highest lumefantrine concentration is expected six hours after the last dose (Ezzet, van Vugt et al. 2000), and in this study, an additional sample was taken at this point to evaluate maximum lumefantrine concentration achieved after the six doses in paediatric patients with moderate malaria.

4.2 METHODS

During the single-arm phase of the study reported in chapter 3, 12 consecutive eligible children (6 acidotic and 6 non-acidotic) were also recruited into this bioavailability study after their parents had consented for both the clinical trial and pharmacokinetic studies (Appendix IV). The additional procedure for the pharmacokinetic study was an intensive blood-sampling scheme to establish the lumefantrine plasma concentration-time profile during the first 24 h after starting treatment. Teflon sampling cannula was inserted into one of the major veins on the antecubital fossa and was connected to a 5ml syringe

(containing heparinised normal saline). From this sampling line, blood samples (approximately 0.7 ml each) were taken at times 0 (just before the first dose), and at 1, 3, 6, 8, 12, 16, 18, 24 and 66 h after the first dose. To avoid infection, the sampling cannula was removed after 24 h, and the additional sample, six hours after the last dose (66 h) was taken through a fresh venipuncture.

These samples were collected in lithium-heparin tubes, and were immediately centrifuged (1,500 x g; 10 min), and plasma was separated and stored at –70 ^{0}C until assayed for lumefantrine by HPLC as described in section 3.2.3.2 chapter 3.

4.2.1 Pharmacokinetic and statistical analysis

Pharmacokinetic parameters were calculated using TopFit software version 2.0 (Heinzel, Woloszczak et al. 1993). Mean dose-corrected AUCs were calculated as follows: first, weight corrected lumefantrine dose (mg/kg) for each patient was calculated by dividing the total dose the patient received (mg) by the patient's weight. Second, the weight-corrected doses for all patients in a weight range (e. g. 5 – 14.9 kg) were added up and the sum divided by the number of children in that weight rage to get the mean weight-corrected dose (mg/kg) for the patients in that weight range. The mean weight-corrected dose was then used to calculate mean dose-corrected AUC according to the following formula:

Mean dose-corrected AUC = {[Mean weight-corrected dose (mg/kg)/Actual dose (mg/kg)] X Actual AUC}

The mean dose-corrected AUCs and other pharmacokinetic parameters between non acidotic and acidotic patients were compared using Confidence Interval Analysis Software (Altman, Machin et al. 2000)

4.3 RESULTS:

Parents of sixteen eligible children were asked to volunteer for their children to participate in the pharmacokinetic study. Consent for the clinical trial and pharmacokinetic study was obtained from thirteen parents or guardians and there were three refusals. Therefore, thirteen children were included in the pharmacokinetic study.

Blood sampling problems were encountered in three children: in one of the patients, it was discovered at the time of taking the 16 h sample that the sampling line was no longer patent and it was not possible to re-establish patency. In one of the patients, a clinical trial Day 3 blood sample was not taken simultaneously with a 66h pharmacokinetic sample and the child could not be bled again for the pharmacokinetic sample. In another patient, the plasma volume for an 18 h sample was below the minimum volume needed for the assay (100µl). Samples from one child were used as part of method validation and there was not enough volume during analysis of patient

samples for sampling points 0h, 1h, 3h, 6h, 8h, and the 12h. This profile was incomplete and was excluded from analysis.

The baseline characteristics for individual children included in this study are presented on table 4.1. There was marked individual variability in plasma lumefantrine concentration at different sampling time points in this study (figure 4.1). On average lumefantrine appeared in plasma 3 hours after the first dose, and during the first 24 hours of treatment a maximum concentration [mean (SD) 1404.6 (829.6)ng/ml] was achieved 16 hours after the first dose or 8 hours after the second dose (figure 4.2).

Table 4.1: Baseline characteristics of patients in the pharmacokinetics study

Patient number	Age (months)	Gender	Weight (kg)	Temperature (°C)	Number of parasites per (µl)	Base excess (mmol/l)	Haemoglobin (g/dl)	Clinically pale	Number of convulsions past 24 hours
65857	43	f	12.9	40.2	157 920	-7.5	9.6	no	1
65880	14	m	8.3	39.9	151 920	-9.8	8.2	yes	2
65890	49	m	13.86	37.6	84 600	-3.8	7.4	yes	0
65843	26	m	10.42	39.6	9 555	-4.4	10.2	no	1
65917	60	f	13.7	39.5	460 800	-6.4	6.7	yes	0
62769	11	f	6.7	37.3	12 672	-13.9	5.8	yes	0
65935	32	f	12.36	39.6	12 118	-4.8	6.3	yes	0
65955	13	f	10.4	40.1	303 800	-6.5	9.4	no	0
65959	34	m	10.2	39.5	970 200	-18.7	7.1	no	0
65960	16	m	9.6	36.7	6 120	-12.0	8.6	yes	1
65988	16	m	8.2	39.6	34 000	-7.4	8.4	no	0
66032	52	f	13.9	39.2	183 700	-1.6	4.9	yes	0
66036	28	m	11.1	37.2	160 400	-3.3	9.2	no	3

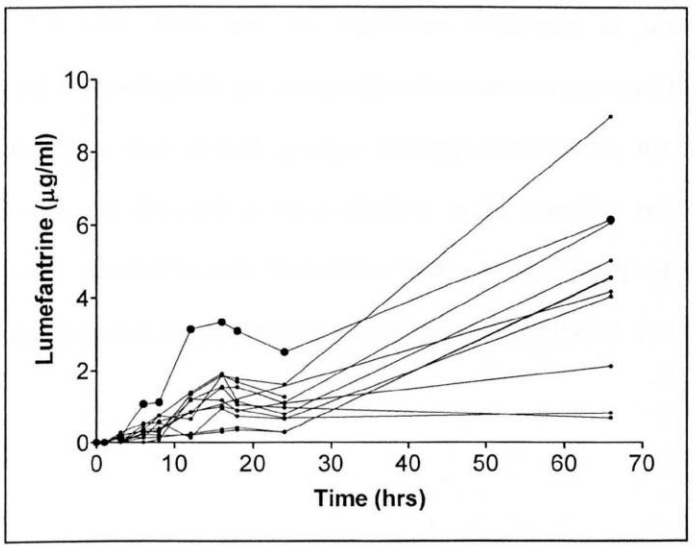


Figure 4.1: Plasma lumefantrine concentration-time profiles over 66 h from start of artemether-lumefantrine treatment

With the artemether-lumefantrine 6-dose regimen, the highest plasma lumefantrine concentration is expected six hours after the last dose, as bioavailability has improved considerably during this time after complete resolution of malaria symptoms and the patient is eating normally. As expected, plasma lumefantrine was highest at this point in all patients in this study. However, there was considerable individual variability (figure 4.1). The mean plasma lumefantrine concentration six hours after the last dose was not significantly different in the two groups (table 4.2).

Lumefantrine was bioequivalent in the two groups during the first 24 h of treatment (table 4.2). Likewise, there was no significant difference in mean maximum concentration (Cmax) achieved, and median time to maximum concentration in the two groups (median T_{max} 16 h in both groups). Desbutyl-lumefantrine, the metabolite of lumefantrine was not detected in most children at all sampling points. Desbutyl-lumefantrine was detected in only five of the twelve patients in the pharmacokinetic study and where detected, the concentration was low ranging from 51- 125 ng/ml.

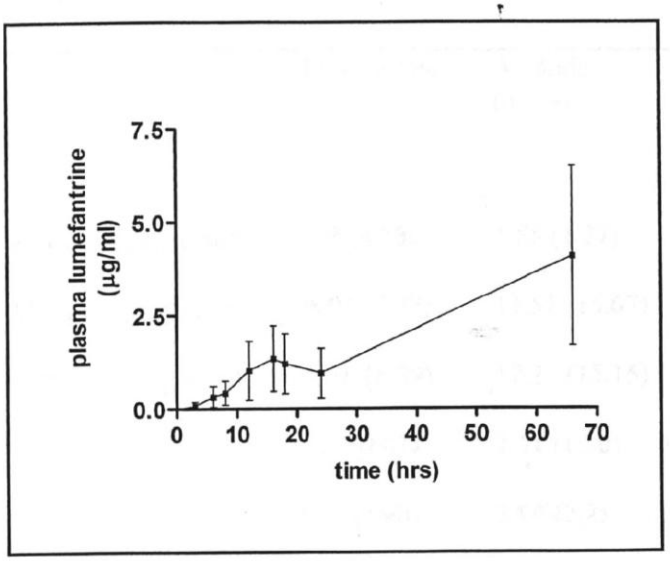


Figure 4.2: Mean (SD) plasma lumefantrine concentration-time profile over 66 h from start of artemether-lumefantrine treatment

Table 4.2: Mean (SD) lumefantrine pharmacokinetic parameters by acid-base status

	Non-acidotic (n = 8)	Acidotic (n = 4)	95 % CI for difference between means
Dose-corrected $AUC_{0-8\,h}$ (μg hr/ml)	1.39 (0.78)	1.85 (1.27)	-1.83 – 0.90
Dose-corrected $AUC_{8-24\,h}$ (μg hr/ml)	16.97 (7.77)	13.51 (15.67)	-11.23 – 18.14
Dose-corrected $AUC_{0-24\,h}$ (μg hr/ml)	18.16 (8.39)	17.21 (15.15)	-13.87 – 15.79
Cmax (μg/ml)	1.35 (0.61)	1.51 (1.30)	-1.3 – 1.0
C_{66h} (μg/ml)	4.19 (2.90)	3.90 (2.2)	-3.5 – 4.1

4.4 DISCUSSION AND CONCLUSIONS

The pharmacokinetic sampling schedule in this study was designed to collect information on the bioavailability of lumefantrine in the first 24 h of treatment. The results indicate that moderate acidosis may not have an effect on lumefantrine bioavailability during this period. There was a tendency for achieving a higher plasma lumefantrine concentration six hours after the last dose in children with acidosis compared to non-acidotic children. However, the number of patients in each group was too small to draw any firm conclusion. The required minimum sample size in each group for a comparative bioavailability study is 12 (Ueda 1979). This was not achieved because of time constraints and therefore, the results need to be interpreted cautiously.

However, lumefantrine is more soluble in acid medium (Zeng, Lu et al. 1996) and acidosis might improve its intestinal absorption. However, the major significance of the findings of this bioavailability study, is the assurance that acidosis may not be an impediment for the use of Artemether-lumefantrine in children with moderate malaria.

The observed plasma lumefantrine concentration on Day 2 (66 h after start of treatment) in this study are comparable to those reported on Day 3 after start of treatment in Ugandan children (Fogg, Bajunirwe et al. 2004). However, the time of blood sampling in this study was less variable between children than in the Uganda study where sampling took place at any time on Day 3. In addition, drug administration for all the six doses in this study was supervised whereas in the Ugandan study only the first dose was supervised.

This study confirms the slow absorption of lumefantrine previously reported in adult patients (Ezzet, Mull et al. 1998; Ezzet, van Vugt et al. 2000), with the maximum concentration during the first 24 h being achieved 16 hours after the first dose or 8 h after the second dose. On average however, lumefantrine plasma concentrations were above the *in vivo* MIC (280ng/ml) starting from 6 h after the first dose to 66 h providing adequate lumefantrine exposure to parasites. Since this study was not designed to estimate the total AUC after the six doses (reported data for adults), a comparison of lumefantrine bioavailability between paediatric and adult malaria patients cannot be

drawn. However, this study confirms reports that little lumefantrine metabolite is formed *in vivo* (White, van Vugt et al. 1999).

In conclusion, the results of this study suggest that moderate acidosis may not affect the oral bioavailability of lumefantrine in African paediatric patients with malaria.

CHAPTER FIVE

5 SALICYLATE EXPOSURE AND THE RISK FOR MALARIA SEVERITY AND MORTALITY

5.1 INTRODUCTION

The determinants of a *Plasmodium falciparum* infection progressing to severe malaria in children living in malaria endemic countries was discussed in section 1.2.5 of chapter 1. Unfortunately, there are still no known risk factors that can be easily identified and used to preditic who is likely to progress to severe malaria, and thus apply preventive measures. Theoretically, targeting socioeconomic factors such those associated with access to effective treatment is more practical and may have immediate public health impact. However, findings from some studies have indicated that socioeconomic factors may not be major determinants of progression to severe disease (Koram, Bennett et al. 1995; Luckner, Lell et al. 1998). Despite such reports, socioeconomic factors such as treatment seeking behaviour and practices may be important. For example, delayed administration of effective treatment provides the opportunity for the infecting parasite

to multiply, mature, and sequester leading to severe disease. This is probably why early and effective treatment has been reported to reduce severe malaria morbidity (Sirima, Konate et al. 2003).

More recently, OTC drugs used to treat childhood fever episodes before the child is seen at a health facility, such as salicylates, have been implicated in the progression of *P. falciparum* infection to severe malaria (Clark, Whitten et al. 2001). The results of the study in chapter 2 of this thesis suggest for an association between the intake of salicylates before hospitalization and the presences of features of severe disease on admission. If this association truly exists, changing drug policies such as the introduction and enforcement of mandatory safety packaging and labeling for OTCs may lead to reduced severe malaria morbidity.

Therefore, the aim of the studies reported in this chapter was to test two hypotheses: a) that intake of salicylate is associated with severe malaria, and b) that intake of salicylate might determine outcome of a severe malaria episode as well as other common infections in children.

5.2 METHODS

The two case-control studies reported in this chapter were done at the KEMRI Center for Geographic Medicine Research-Coast and the KDH. The protocol was reviewed and

approved by the KEMRI Scientific Steering and National Ethical Review Committees (Appendix V). The studies involved archived plasma samples from well-documented clinical cases and controls.

5.2.1 Study I: Salicylate exposure and malaria severity

This study used archived admission-plasma samples from children enrolled in a previous (1994) malaria case-control study (Snow, Schellenberg et al. 1993). The same definitions for cases and controls in the previous study were used in this study. Cases were children who lived in the study area and were admitted to the KDH with severe malaria as defined by the presence of any of the features on table 5.1. Controls were age-matched patients with uncomplicated malaria who lived in the study area and who were seen at the Maternal and Child Health Clinic within 14 days of the presentation of the case.

Table 5.1: Definitions for cases in the original malaria case-control study

(Snow, Schellenberg et al. 1993)

- Inability to localise a painful stimulus
- Anaemia (Hb < 5.1g/dl) with a peripheral parasitaemia ≥ 10 000 per µl
- Two or more generalised convulsions within the 24 h period before admission
- Hyperparasitaemia (≥ 20 % infected red cells)
- Prostrate (unable to sit or stand unaided)
- Death with a confirmed diagnosis of malaria

The current study had 80 % power to detect an Odds ratio of 2.0 to be significant at 5 % level of significant. To achieve this, a minimum of 307 samples from each group (cases and controls were required. However, the original study enrolled 300 cases and 300 controls. Therefore, all the cases and controls from the original study were eligible for inclusion. Plasma chloroquine concentration on these samples was determined (competitive ELISA method using PAQ 13 antibody) in the previous study. The result was used in the current study to examine confounding attributable to a greater tendency in the household to treat severe disease with any available drug. Chloroquine was considered suitable for this purpose because it was a common self-administered antimalarial drug at the time of the original study.

A list of the sample identification record together with the chloroquine concentration result for all children enrolled in the original study was printed out. The samples were retrieved and physically checked. Samples with inadequate volume (< 100µl) and those without a chloroquine result were excluded.

5.2.1.1 Salicylate assay

All included samples were analysed for salicylate by fluorescence polarization immunoassay (FPIA) using the Abbot TDx analyser (Abbot Laboratories, Diagnostic Division, Abbot Park, IL, USA) as described in chapter 2. Salicylate exposure was defined as plasma concentration of ≥ 5µg/ml, the limit of detection for the salicylate

assay method. Exposure to chloroquine was defined as a plasma concentration above 7.9nmol/l, the limit of detection for the competitive ELISA method.

5.2.2 Study II: Salicylate exposure and the outcome of hospital admission

In this study, archived admission-samples from children enrolled to bacteraemia surveillance amongst paediatric admissions at the KDH were used (Berkley, Lowe et al. 2005). These were an extra 0.5 to 1 ml of blood taken from all children whose parents or guardians consented for their participation in the bacteraemia study. Blood was immediately centrifuged, plasma separated and stored at –20 ^{0}C before transferred to –80 ^{0}C within 24 h of collection. For the study reported here, all samples from children admitted in the period between September 1999 and December 2001 were eligible if the final diagnosis was malaria, acute gastroenteritis, pneumonia or acute bacterial meningitis. Samples from children below one month of age (neonates) were excluded. This was a case-control study where deaths were cases, and the controls were selected from children admitted during the same period with same conditions as the cases but survived.

The study had 80 % power to detect an Odds ratio of 2.5 as significant at 5 % level of significance. To achieve this, taking the proportion of children with salicylate exposure on admission at the KDH as 11 % (chapter 2), a minimum of 105 samples from deaths and 315 samples from survivors was required.

The inclusion criteria were applied to the clinical database at the KDH, and 157 potential cases (deaths), and 338 (i. e. a 6% random sample of all eligible controls) controls (survivors) were identified within the pre-defined time period. A list of the sample identification record of these 495 children was printed out, and the samples were retrieved and physically checked. Children with missing sample or inadequate volume (< 100μl) were excluded. Included samples were analysed for salicylate and acetaminophen. Plasma acetaminophen concentration was used to examine confounding attributable to a greater tendency in the household to treat severe disease with any available drug. Salicylate assay was done as described in chapter 2 and exposure to salicylate was defined as in study I above. Acetaminophen exposure was defined as plasma concentration of ≥ 1.0 μg/ml i.e., the limit of detection for the assay method used.

5.2.2.1 Acetaminophen assay

Like salicylate, acetaminophen in plasma was determined by fluorescence polarization immunoassay (FPIA) using the Abbot TDx analyser (Abbot Laboratories, Diagnostic Division, Abbot Park, IL USA). Acetaminophen assay also uses fluorescence polarisation and competitive binding immunoassay technologies and the principle of operation for this test system is described in chapter2, section 2.2.8. For acetaminophen assay, the following ready-to-use items were provided:

- Reagent pack containing the following preserved in Sodium Azide

Antibody (S), < 25 % Acetaminophen Antiserum (Sheep) in buffer with protein stabilizer

- Tracer (T), < 0.01 % Acetaminophen Fluorescein Tracer in buffer with surfactant and protein stabilizer (3ml)
- Pretreatment Solution (P), buffer with surfactant and protein stabiliser (3 ml)

- Six calibrators A to F (2.5ml) in human serum preserved in Sodium Azide (with known concentrations: 0.0, 10, 20, 50, 100, and 200).

- Three controls in human serum preserved in Sodium Azide (2.5ml) at three levels spanning the calibration curve (target value in μg/ml (range): Low 15 (12.70 – 17.30), Mid 35 (31.50), High 150 (135.00 – 165).

- Dilution buffer

- Cuvettes and sample cartridges

The acetaminophen assay parameters on the analyser are pre-set, and before the initial calibration and running of samples these parameters were verified according to the manufacturer's instruction. After verification of assay parameters, the calibrators were

loaded and the calibration assay was run. The analyser software calculated a best-fit curve equation, which it used to generate a calibration curve. This curve was stored in memory and acetaminophen concentrations in unknown samples were calculated from this curve using polarisation values generated for each sample.

Analysing all the controls after acceptance of the calibration curve checked the reagent system, and patient samples were only analysed when all the controls were within the specified range. Controls were included in each run of patient samples and recalibration was done whenever any of the control was out of the specified range, and whenever a new lot number of reagent or buffer was used. The instrument procedure for the analyser was the same as for salicylate assay (Chapter 2).

5.2.3 Statistical analysis

Laboratory and basic demographic and clinical data were linked and analysed using STATA v8.1 (Statacorp, USA). MacNemar's test was used to estimate the Odds ratio for matched data whereas Mantel-Haensel Odds ratios were used to explore potential confounding effects of age, sex, chloroquine exposure and nutritional status in stratified analyses. Conditional and ordinary logistic regressions were used for estimating adjusted Odds ratios for matched and unmatched data respectively. Although the samples collected for the original malaria case control study were age-and-period matched, for this study they were analyzed both preserving the matching and after breaking the

matching. This was primarily because of a failure to achieve the desired sample size, but was considered reasonable as the age matching was poor (data not shown), and because period matching would be less appropriate for an acute exposure (salicylate ingestion) not obviously confounded by calendar time.

5.3 RESULTS

5.3.1 Study I

From the 600 archived samples, 490 samples were selected as suitable for the current study. Complete data were available for 133 matched pairs (about 38 % of the number in the original study). The main reason for sample exclusion was inadequate sample volume. Although age matching was poor in matched pairs, there was no significant difference in age when controls and cases were regarded as independent groups (table 5.2). When matching was preserved, salicylate exposure was associated with severe malaria, unadjusted odds ratio (95 % CI) 3.8 (2.36 – 6.27), with an odds ratio of 2.8 (1.4, 5.4) after adjustment for chloroquine exposure, age, sex, and nutritional status (table 5.3). Complete data were also available for a further 178 unmatched samples (26 Cases, 152 controls). The risk of exposure to salicylate was higher in boys than girls, and increased with increasing age and also with the presence of chloroquine exposure (table 5.3). Unmatched analyses including these data also demonstrated association between

Salicylate or chloroquine exposure and severe malaria (table 5.3). There was no interaction between salicylate and chloroquine exposure in either matched or unmatched analyses.

5.3.2 Study II

In the second study, complete data were available for 117 cases and 285 controls. On average, cases had poorer nutritional status (table 5.2). In this analysis, salicylate exposure did not appear to be associated with death even after adjustment for sex, age, acetaminophen exposure and nutritional status (table 5.3). Analyses restricted to malaria (cases = 62, controls = 182) also showed no association between salicylate exposure (OR 1.1, 0.47 – 2.57) and death although the power to detect an effect was small.

Table 5.2: Demographic characteristics and exposure status of patients in the case-control studies

	Severe malaria[i]		Mortality[j]	
	Matched data Cases (n=133)	Controls (n=133)	Cases (n= 117)	Controls (n = 285)
Female	58 (44 %)	64 (48 %)	59 (50 %)	136 (48 %)
Age > 23 months	82 (62 %)	75 (56 %)	49 (42 %)	114 (40 %)
Salicylate exposed	46 (35 %)	23 (17 %)	21 (18 %)	47 (16 %)
Chloroquine exposed	105 (79 %)	95 (71 %)		
Paracetamol exposed			102 (87 %)	229 (80 %)
Mean WA Z score	-1.9(-2.1, -1.6)	-1.7(-1.9, -1.5)	-2.2(-2.5, -1.96)	-1.6(-1.7, -1.4)
	Unmatched data Cases (n =151)	Controls (n = 283)		
Female	72 (48%)	130(46 %)		
Age > 23 months	94 (62 %)	148 (52 %)		
Salicylate exposed	51 (34 %)	53 (19 %)		
Chloroquine exposed	123 (81 %)	187 (66 %)		
Mean WAZ score	-1.6(-1.9, -1.2)	-1.5(1.7, -1.3)		

[i] Study I
[j] Study II

Table 5.3: Odds ratios of salicylate exposure among patients with severe malaria and deaths

	Univariate analysis			Multivariable analysis		
	OR	P value	95 % CI	OR	P value	95 % CI
Severe malaria:						
Matched data						
Salicylate	3.8	< 0.001	2.3 – 6.2	2.8	0.002	1.4 – 5.4
Chloroquine	0.3	< 0.001	0.2 – 0.4	1.6	0.103	0.5 – 1.4
Nutritional status	8.0	< 0.001	4.6 – 15.2	1.3	0.465	0.6 – 2.8
Severe malaria:						
Non matched data						
Salicylate	2.2	< 0.001	1.4 – 3.5	2.2	0.001	1.4 – 3.5
Chloroquine	2.2	< 0.001	1.4 – 3.8	2.3	0.001	1.4 – 3.8
Nutritional status	1.7	0.0501	0.9 – 3.2	1.7	0.083	0.9 – 3.0
Mortality data:						
Salicylate	1.1	0.7020	0.6 – 2.0	1.2	0.512	0.7 – 2.2
Paracetamol	1.6	0.9910	0.8 – 3.3	1.9	0.059	0.9 – 3.6
Nutritional status	4.6	< 0.001	2.5 – 8.5	5.2	< 0.001	2.9 – 9.4

5.4 DISCUSSION AND CONCLUSIONS

Despite the presence of a scientifically plausible hypothesis linking salicylate intake and severe malaria in children, the association has not been previously examined in clinical studies. Thus, the true magnitude of the danger of developing severe malaria that might be associated with the use of aspirin or salicylate-containing antipyretics to treat malaria fever episodes in children remained unknown. The studies reported here are probably the first clinical studies to try and estimate the risk. The results in the first study support the hypothesis for an association between salicylate intake and severe malaria in children. In this study, children admitted with severe malaria were about 2.8 times more likely to have taken salicylate in the home. However, since children admitted with severe malaria were also more likely (2.2 times) to have taken chloroquine (unmatched data), the greater chance that more severely ill children are administered any shop bought medication remains a plausible explanation for these results. However, the association with the intake of chloroquine was only observed with unmatched data, and the estimate of the association was significantly greater with matched than unmatched data suggesting that matching factors had an effect on the association. As a result, the estimate of association using unmatched data may be in part or totally attributed to the influence of potential confounding factors on this association.

Nevertheless, these results need to be interpreted with caution for three reasons. First, the original malaria case-control study was not specifically designed to investigate the association between salicylate exposure and severe malaria. Second, in the current study,

it was only possible to investigate a modest proportion of the matched pairs recruited into the original study. Thirdly, only samples with enough volume were included and this might have introduced some selection bias. Unmeasured confounding influences such as the degree of fever, and the duration of illness, might account for these findings. In addition, the inherent limitation of case-control studies, notably the temporal relationship between exposure and disease is worth noting when interpreting these results. In some cases, salicylate may have been taken after development of signs of severe malaria. However, these findings remain compatible with the hypothesis that the intake of salicylates increases the risk of severe malaria in children although not necessarily their risk of death if reasonable treatment is available.

As discussed in chapter1, the intake of salicylate in febrile children is associated with a risk for Reye's syndrome as well as therapeutic or chronic poisoning. In conclusion, results from this study suggest an added risk of exacerbating *P. falciparum* infection. Since there is no proven benefit of using salicylates to treat fever in children, there seems no good reason to continue using salicylates in febrile children, including those with malaria.

CHAPTER SIX

6 GENERAL DISCUSSION AND CONCLUSIONS

6.1 INTRODUCTION

For many years, cerebral malaria has been considered the major cause of severe malaria case fatality, and severe malarial anaemia considered responsible for the remaining childhood malaria deaths. However, in recent years, metabolic acidosis has emerged as a common complication of severe malaria in children, and as the best independent predictor of poor outcome. Most of these deaths occur within 12 to 24 hours after admission, the time at which antimalarial action has not reached maximum. Therefore, there are arguments that improved supportive care such as interventions targeting amelioration of acidosis at and or immediately after admission may reduce mortality.

For this reason, studies leading to improved understanding of metabolic acidosis in children with malaria are a high priority research. Such studies are critical for developing specific interventions for acidosis as adjunct supportive care in the management of severe childhood malaria. The overall aim of the studies presented in this thesis was to contribute towards a better understanding of metabolic acidosis in African children with malaria. This chapter summarises the major findings and conclusions that can be drawn from the work described in this thesis.

6.2 The nature and underlying pathophysiologic mechanism for metabolic acidosis in malaria

The underlying pathophysiological mechanisms for metabolic acidosis were discussed in chapter 1 and the advantages and limitations of using both the traditional and Stewart's approach to characterise metabolic acidosis were described. Using the Stewart's approach, results in chapter 2 suggest that in African paediatric patients with malaria, metabolic acidosis is commonly due to a decrease in plasma SID. There are three mechanisms that may lead to a decrease in plasma SID: an increase in plasma water content; an increase in chloride concentration; and the accumulation of other acidic anions such as lactate, keto acids, sulphate, hippurate or exogenous acids.

6.3 Increase in plasma water content and accumulation of organic strong anions

On average, plasma sodium was normal in malaria patients described in chapter 2. This suggests that there is no increase in the water content of plasma, which would be invariably associated with hyponatremia. In general, children with severe malaria are water depleted (dehydrated) (English, Waruiru et al. 1997). However, there is disagreement on the degree of dehydration in these children, and whether volume depletion is a major factor in the pathogenesis of metabolic acidosis in children with

severe malaria (Planche, Onanga et al. 2004). According to Stewart's theory any change in plasma volume that does not primarily involve change in the water content does not affect SID and will not cause a change in acid-base status (Fencl and Leith 1993).

The argument for hypovolemia as a major factor in the aetiology of metabolic acidosis in severe childhood malaria is based on the presence of high blood lactate level in most children with malarial acidosis together with the demonstration of clinical features of shock or hypovolemia in these children (Maitland, Pamba et al. 2005b). In addition, there is improvement in clinical parameters upon fluid administration (Maitland, Pamba et al. 2003). However, some authors have argued for micro vascular obstruction resulting from sequestration of parasitised red cells as the cause of elevated blood lactate level having demonstrated absence of severe depletion of total body water (Planche, Onanga et al. 2004).

In this thesis, acidosis in paediatric patients with malaria was compared with that of those with acute gastroenteritis, where hypovolemia is expected to be more prevalent and pronounced. Interestingly, elevated blood lactate was more common in children with malaria than those with gastroenteritis. This supports the microvascular obstruction theory, i. e; microvascular obstruction by sequestered parasites is responsible for reduced organ blood flow, hypoxia and lactate generation. This pathophysiological mechanism is present in malaria but absent in gastroenteritis. However, results presented in chapter 2 indicate that lactate may not be the only significant organic acidic anion in malarial acidosis; ketones and currently unidentified anions are also significant. Ketones have also

been previously reported and implicated in the pathogenesis of acidosis in malaria patients (White, Miller et al. 1987; Allen, O'Donnell et al. 1996). Interestingly, in this thesis however, ketones were also associated with acidosis in patients with gastroenteritis, lower respiratory tract infection and severe malnutrition. This suggests for absence of malaria-specific factors for ketosis and this might be associated with the effect of symptoms of clinical malaria on the availability of glucose in the background poor nutritional status in African children.

6.4 Increase in chloride concentration

Like for sodium, in results presented in chapter 2, chloride concentrations were on average within normal limits for most malaria patients. This observation indicates that hyperchloraemia is not a cause of metabolic acidosis in malaria patients.

6.5 Accumulation of inorganic (mineral) anions

The strong anions that when in excess (in plasma) cause a decrease in SID and metabolic acidosis can be classified into organic or inorganic (mineral anions). Organic anions include lactate ($_{L}$-lactic acid and $_{D}$-lactic acid) and ketoacids. These are cleared through metabolic pathways. Mineral anions include chloride and acidic anions resulting from protein (nitrogen) metabolism such as phosphoric acids, sulfuric acids and hippuric acid, and are cleared through renal elimination.

Frank renal failure is rare in African children with severe malaria (Waller, Krishna et al. 1995) and mineral acids have previously not been implicated in metabolic acidosis in children with severe malaria. However, renal impairment measured by plasma creatinine is a consistent finding in studies of severe malaria and is consistently associated with acidosis (Waller, Krishna et al. 1995; English, Sauerwein et al. 1997; Day, Phu et al. 2000). It is likely that some degree of renal impairment is a significant factor in the pathogenesis of acidosis in children with malaria. Renal impairment in these children could be a result of mild to moderate dehydration or possibly a direct effect of malaria infection on renal function. None of the mineral anions associated with renal failure was measured in this thesis, but results presented in chapter 2 suggest a role for currently unidentified anions. These anions might be mineral anions associated with reduced renal capacity to eliminate acids. In addition, a strong positive correlation between plasma creatinine and ketones was observed in the work described in this thesis. Therefore, it can be speculated that severe malaria causes increased catabolism and moderate dehydration. The former causing an increase in the mineral acid load and the later causing renal impairment, and reduced food intake associated with malaria illness in the background of poor nutritional status may be associated with elevated ketones.

6.6 Exogenous acids

Salicylates are weak acids and in toxic concentrations marginally increase the non-volatile weak acid load. More importantly, at toxic levels they impair oxidative metabolism of energy substrate with subsequent increase in lactate production. As described in chapter 1, in tropical Africa, salicylates are still widely available as OTC and

are used for treatment of fever episodes in homes (van der Geest 1987; Slutsker, Chitsulo et al. 1994; Marsh, V. M., Mutemi et al. 1999). Salicylate elimination pathways become saturable at relatively low amount in the body and toxicity can quickly result from repeated administration (Levy 1978). Thus, there is always a risk for toxicity when salicylates are used in children where mothers are usually not sure of the paediatric dose, and thus, salicylates are the most likely exogenous acids that may cause metabolic acidosis in children with malaria.

However, results presented in chapter 2 indicated that salicylates did not commonly underlie acidosis; allaying previous concerns that salicylate consumption before hospital admission (chronic salicylate poisoning) may be a common and important co-factor in the development of acidosis in children with severe malaria. However, occasional inadvertent salicylate toxicity occurs (English, Marsh et al. 1996; Musumba, Pamba et al. 2004). The clinical presentation of chronic salicylate toxicity in children is similar to that of severe malaria and still causes diagnostic confusion with severe malaria.

From the findings presented in chapter 2 of this thesis, it can be concluded that the principal underlying pathophysiologic mechanism for acidosis in severe malaria is the presence of strong anions other than chloride, which include organic acids: lactate and ketones; and currently unidentified anions. Apart from increased lactate production, which could result from moderate volume depletion or perhaps principally from micro vascular obstruction secondary to sequestration, impairment of renal excretion of acids may be an additional factor. This could explain the presence of currently unidentified

anions particularly if the identity of these anions proves to be those associated with renal failure. However, the findings in thesis have limitations: the malaria population was mainly of moderate cases and relatively few cases of severe disease. In addition, calcium and magnesium, important cations for the calculation of SID and estimation of unidentified anions were not directly measured in children studied in this thesis. Thus, these data need to be confirmed in a relatively large series of children with severe malaria.

6.7 Implications for future treatment of metabolic acidosis in malaria patients

A conceptual classification of acute non-toxic metabolic acidosis (which seems to be the nature of acidosis in children with malaria) into organic and mineral is essential when considering treatment options (Levraut and Grimaud 2003). Accumulation of organic acids gives evidence that a metabolic distress is in process whereas accumulation of mineral acids simply shows that protons, normally eliminated by the kidneys are in excess. The principles of treatment for metabolic acidosis as adjunct management for the underlying disease include correction of acidosis by administration of alkali, and measures to accelerate the clearance of accumulating acidic anions. Experience from intensive care units suggests that alkali therapy may be of benefit only in patients with severe mineral metabolic acidosis (base excess < -10mmol/l) in whom anuria is absent (Levraut and Grimaud 2003). No benefit for alkali therapy has been demonstrated in patients with organic metabolic acidosis. Since organic acids are cleared through

metabolic pathways and bicarbonate is generated through this process, there is a risk of overshoot alkalosis once the acidosis is over if alkali is administered. In patients with diabetic ketoacidosis alkali therapy has been associated with increased time to pH normalization and a decrease in the rate of ketone bodies metabolism (Hale, Crase et al. 1984; Morris, Murphy et al. 1986). In addition, alkali therapy has not been proved to be superior to isotonic saline in patients with lactic acidosis (Mathieu, Neviere et al. 1991).

Therefore, there is an argument that specific acidosis treatment with alkali may have no value in organic metabolic acidosis (Levraut and Grimaud 2003). Accumulated organic acids will be cleared through metabolic pathways and the management of an organic metabolic acidosis should be primarily based on the treatment of the underlying disease. By contrast, accumulated mineral acids have to be cleared through the kidneys and correction of acidosis by alkali therapy or measures to accelerate their renal elimination such as renal replacement therapy are probably beneficial.

Work presented in this thesis, has demonstrated that metabolic acidosis in children with malaria is associated with accumulation of organic acids and currently unidentified anions. Unidentified anions have also recently been reported in adults with severe malaria (Dondorp, Chau et al. 2004). Therefore, the identity of the currently unidentified anions in metabolic acidosis associated with severe malaria will have major implications for the choice of future potential treatment targets. If these currently unidentified anions are proven to be mineral acid anions in future studies, metabolic acidosis in malaria would be a mixed organic and mineral acidosis. In this case, development of alkali products

without the side effect of CO_2 release such as Tribonat (a mixture of tromethamine, acetate, bicarbonate and phosphate, Pharmacia and Upjohn AB, Stockholm, Sweden) for the treatment of acidosis in severe malaria might be justified. In addition, the value of measures to accelerate renal elimination such as renal replacement therapy could be explored. Therefore, future studies to establish the identity of currently unidentified anions and the effect of *P. falciparum* malaria infection on renal acid excretion are a high priority.

On the basis of known association between metabolic acidosis in severe malaria and elevated blood lactate, dichloacetate, a molecule that promotes utilization of pyruvate through the enzyme complex pyruvate-dehydrogenase has recently been tested in clinical trials in African children with severe malaria (Krishna, Agbenyega et al. 1995; Agbenyega, Planche et al. 2003). These trials and other clinical studies have shown improvement in lactic acidosis following dichloacetate administration. However, a large clinical trial showed no mortality reduction in critically ill adults with severe lactic acidosis (Stacpoole, Wright et al. 1992). Work presented in this thesis has demonstrated that metabolic acidosis in paediatric malaria patients is not simply lactic acidosis and it would not be surprising if dichloacetate administration was not associated with mortality reduction in future multi-center trials.

One major scientific achievement described in this thesis is the demonstration using the Stewart's approach that metabolic acidosis in paediatric malaria patients is associated with reduced SID. According to this approach, the essence of all therapeutic interventions

is the adjustment of the independent variables for pH, in this case, the SID. According to Stewart's theory increasing the sodium concentration or decreasing chloride concentration will have an alkalinizing effect by increasing the SID. Administration of normal saline is currently used on the account that paediatric severe malaria patients with acidosis are volume depleted. However, despite clinical improvement in most cases, acidosis does not resolve quickly until up to 48 hr after admission in some children (Maitland, Pamba et al. 2003). In addition, normal saline being a crystalloid is considered potentially dangerous for its tendency to leave the intravascular compartment and move into the interstitial compartment. This would, for example, exacerbate cerebral oedema. Thus, there have recently been efforts to investigate what is the optimum or ideal intravenous fluid in these patients, and preliminary work is suggesting benefit from the use of colloids such as human albumin (Maitland, Pamba et al. 2005a). However, according to Stewart's approach, albumin would increase the total concentration of weak acids (an independent variable) and will have an acidifying effect and might worsen the acidosis. Normal saline would improve the SID and acidosis, but chloride concentration will increase simultaneously and increased chloride has an acidifying effect. This explains in part why acidosis does not resolve quickly in response to normal saline or albumin treatment (Maitland, Pamba et al. 2003; Maitland, Pamba et al. 2005a).

Saline contains equal amounts of sodium and chloride, whereas plasma does not. For this reason, when large amounts of saline are administered, the chloride concentration increases much more than sodium does. This has a greater effect on total body chloride than total body sodium, and the SID is decreased resulting in metabolic acidosis. Indeed,

saline has been shown to cause metabolic acidosis in animal model of sepsis (Kellum, Bellomo et al. 1998) and in patients undergoing surgery (Scheingraber, Rehm et al. 1999; Waters, Miller et al. 1999). However, it seems that saline might be beneficial in severe malaria if only the amount required to normalize SID is administered. Therefore, saline dose-finding studies might be useful in determining the right amount of saline for the treatment of metabolic acidosis in children with severe malaria. The other approach might be the development of new saline solutions with sodium and chloride ratio similar to that in plasma.

The role of ketoacids has also been demonstrated in this thesis. Like lactate, these are organic acids and are cleared through metabolic pathways and the correction of their levels may not be justified. However, the administration of dextrose even in the absence of hypoglycaemia might reduce ketone production. In fact, dextrose with added sodium would be an interesting fluid to evaluate for the treatment of metabolic acidosis. Sodium will adjust the SID and dextrose will reduce the production of ketoacids.

6.8 Acidosis and the use of artemether-lumefantrine in paediatric patients with moderate malaria

Artemether-lumefantrine (Coartem®) is currently the only co-formulated atreminsinin-based combination therapy for uncomplicated malaria on the market. Several African countries have now adopted artemether-lumefantrine as the first line treatment for uncomplicated malaria. Extending its use to hospitalized paediatric patients with moderate malaria is potentially advantageous for the following reasons: first, its rapid

parasite killing rate would reduce the risk of unrestricted parasite multiplication and prevent progression to severe disease. Second, all drug administration will be supervised solving the problem of a complicated dosing schedule and improving compliance.

However, moderate acidosis that may be associated with moderate malaria as the results presented in chapter 2 of this thesis indicate may be a potential disadvantage.

Both hypovolemia and micro vascular obstruction as possible underlying pathophysiologic mechanisms for acidosis in malaria patients would be associated with impaired blood flow to the slpanchnic bed (Dondorp, Pongponratn et al. 2004). In theory, this would lead to poor lumefantrine oral absorption. Tolerability to oral medication of these hospitalized paediatric patients is another potential disadvantage of using artemether-lumefantrine for the treatment of paediatric moderate malaria.

Work presented in chapter 3 and 4 in this thesis however, indicates that in practice, poor lumefantrine absorption may not be a major problem. Furthermore, orally administered artemether-lumefantrine seems to be well tolerated by most paediatric moderate malaria patients. For example, only 2 % of the 113 patients presented in chapter 3 vomited replacement oral dose and required parenteral medication.

The results presented in chapter 3 suggest an additional advantage of using artemether-lumefantrine in paediatric patients with moderate malaria. There was steady haematological recovery in most patients after artemether lumefantrine. Children in resource poor countries where malaria is endemic are surrounded by several factors associated with anaemia including repeated *P.falciparum* malaria attacks, helminthic

infestation and poor nutrition. Artemether-lumefantrine prevents recrudescence of the infecting malaria parasites but does not prevent re-infection. Thus, patients treated with artemether-lumefantrine still have the risk for re-infection; the use of artemether-lumefantrine in paediatric patients with moderate malaria (a patient population more likely to have low haemoglobin); will ensure satisfactory haemoglobin level at re-infection reducing the risk for malaria severity and mortality.

Lack of defined criteria for paediatric moderate malaria is a limitation for the results on the use of artemether-lumefantrine presented in chapter 3 of this thesis. Clinical malaria is commonly classified as uncomplicated or severe and there are currently two defining criteria for severe malaria. The WHO criteria is much broader and includes features that have not necessary been proven to reliably predict poor outcome such as severe anaemia without respiratory distress and multiple convulsions without neurological impairment. Some studies in African paediatric patients with moderate malaria have included prostrated children (Barnes, Mwenechanya et al. 2004), a feature of severe disease according to the more strict criteria derived from studies of African paediatric patients with severe malaria (table 1.8). Severe anaemia (Hb≤ 5 g/dl) without respiratory distress, multiple convulsion and frequent vomiting are danger signs according to the IMCI guidelines. In the work presented in this thesis it is argued that malaria disease is a clinical spectrum with uncomplicated malaria and severe malaria in the extreme ends and moderate malaria in between. Paediatric malaria patients with danger signs have an increased risk for clinical deterioration (White 2003) and require in-patient care.

The criterion used to define moderate malaria in the results presented in chapter 3 was the decision by the attending clinician (independent from the study) that the patient required hospiatalization and in some of the patients, the criteria for moderate malaria being proposed may not have been satisfied. However, from the results presented in this thesis, it can be concluded that the six-dose regimen of artemether-lumefantrine can be safely used in paediatric patients admitted with moderate malaria. However, further studies involving patients fulfilling the criteria being proposed are needed to confirm the findings described in this work.

6.9 Day 7 plasma lumefantrine and artemether-lumefantrine treatment response

In agreement with other studies in African paediatric malaria patients (Piola, Fogg et al. 2005), work described in this thesis has shown that Day 7 lumefantrine concentration cut-off for radical cure (280ng/ml) may not be valid in African paediatric malaria patients. The different findings between studies in South-East Asia and Africa may be due to the presence in African paediatric patients of higher background immunity and more sensitive parasites to lumefantrine. However, further studies are needed to specifically investigate the role of the Day 7 lumefantrine concentration in predicting cure and parasite reappearance in African paediatric patients.

6.10 Plasma lumefantrine assay

There are currently few published liquid-liquid extraction HPLC with UV detection methods for the assay of lumefantrine in plasma (Mansor, Navaratnam et al. 1996; Zeng, Lu et al. 1996). However, with implementation of artemether-lumefantrine as first line treatment for uncomplicated malaria in many countries, measurement of plasma lumefantrine will be increasingly needed in various studies evaluating efficacy, effectiveness or compliance. One limitation of these methods in paediatric patients is the requirement for large sample volumes. Both of the published methods required 1ml of plasma to achieve limit of quantification at 0.025 μg/ml and 0.012μg/ml respectively (Mansor, Navaratnam et al. 1996; Zeng, Lu et al. 1996). In addition, the linearity limit achieved by calibration curves in both of these methods was insufficient, with the highest calibration concentration of 0.8μg/ml and 4.0μg/ml respectively. This is insufficient because therapeutic concentrations as high as 12.0μg/ml have been reported in clinical trials (Ezzet, Mull et al. 1998).

One innovation of the work presented in this thesis was a validation and modification of the published method by Zeng and others (Zeng, Lu et al. 1996) to enable assays using only 250μl of plasma. In addition, measurement of des-butyl-lumefantrine (lumefantrine metabolite) using this method was validated. The modification of the method worked well in the local environment in the Pharmacology laboratory at the KEMRI/Wellcome Trust unit in Nairobi. The modified method reported in this thesis, can be adapted by other laboratories in Africa including the Clinical Pharmacokinetics laboratory at the

Muhimbili University College acquired through the project for the PhD work leading to this thesis. The laboratory is an important research tool for the college that will be very useful for future studies in clinical pharmacology.

6.11 Salicytates intake and malaria severity

Available data from in vivo and ex vivo studies has lead to the hypothesis that salicylates may exacerbate *P.falciparum* malaria through their effect on the expression of interferon gamma-induced inducible nitric oxide synthase (iNOS) (Clark, Whitten et al. 2001). Clinical data presented in chapter two of this thesis suggested support for this hypothesis. One of the aims of this study was to establish whether there was a relationship between the presence of detectable salicylate in plasma (on admission), a marker of salicylate ingestion, and the presence of acidosis. Post-hoc analysis of data in this study showed a relationship between salicylate ingestion and the presence of signs of severe disease on admission. This relationship was not limited to malaria; it was observed in the other three common diseases studied. This relationship was further explored in chapter six of this thesis. However, of utmost importance is whether salicylate ingestion would influence the outcome of severe malaria or other common (in our setting) severe childhood infections such as severe pneumonia and acute bacterial meningitis. An attempt to answer this question was made in chapter 5.

To find the best study design to address these questions is an enormous challenge. However, in this thesis, a case-control study design using archived plasma samples from

a previous malaria case-control study; and from well-documented in-patient cases of malaria, pneumonia and meningitis was used. Given the reality in rural tropical Africa of uncontrolled salicylate access; unknown and variation in time and size of dose; and co-medication, it was always going to be hard to extract clean data on the association between home salicylate consumption and severity of malaria disease or outcome. Results presented in chapter 5 suggesting that there is an association between the intake of salicylates and severity of malaria and that the intake of salicylate did not have any influence on the outcome of hospitalization for malaria, pneumonia or acute bacterial meningitis may have limitations. However, these results have practical value when seen in the light of *in vitro* data with salicylates that predicts *in vivo* harm (Clark, Whitten et al. 2001).

The lack of influence of intake of salicylates on outcome might have been due to several factors operating after hospital admission. Of particular interest is the prompt and quality management offered at Kilifi District Hospital. In addition, for malaria, acidosis seem to be the best determinant of outcome, and in chapter 1 of this thesis lack of association between salicylate intake and acidosis was demonstrated, which might explain in part, the lack of influence on outcome by the intake of salicylates. This means, the intake salicylate might operate at the level of progression of clinical malaria to severe disease but not necessarily at the level between severe disease and outcome.

The limitations of the work described in chapter 5 on the relationship between salicylate intake and malaria severity are several and were highlighted in chapter 5 section 5.4.

Future studies might be justified to investigate this relationship in a prospective design in areas where salicylates are still widely available and used for treating childhood fever episodes in homes. However, the available scientifically plausible hypothesis on this relationship; the results presented in this thesis; and the known risk for Reye's syndrome and unwitting therapeutically induced salicylate toxicity associated with the use of salicylates in children when taken together may be enough evidence to embark on preventive measures. Specifically for East Africa, there is need for mandatory safety packaging, labeling and advertising. For example, a statement: "not to be used in children" on all OTCs containing aspirin or salicylates.

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