Treatment of *Plasmodium falciparum* malaria in the Democratic Republic of the Congo

VOLUME I

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Abstract

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Despite international efforts, the malaria burden remains high worldwide with half of all malaria-attributable deaths occurring in the Democratic Republic of Congo and Nigeria. Children under five and pregnant women bear the heaviest burden. New treatment options for *falciparum* malaria are urgently needed due to potential wide spreading plasmodium resistance to artemisinin derivatives.

In the first study, amodiaquine-artesunate, artemether-lumefantrine (AL) and dihydroartemisinin-piperaquine (DHA-PQ) were assessed in 684 Congolese children under 5. The efficacy was good and comparable for all combinations. The short parasitaemia clearance half-life suggested that the local parasite populations are still susceptible to the artemisinin derivatives. DHA-PQ provided the greatest prophylactic effect making it a good candidate for Intermittent Preventive Treatment in pregnancy. Plasma level of piperaquine and lumefantrine in small children at day 7 was however suboptimal indicating the necessity of adjusting the current dosage.

Artemether-lumefantrine is the most recommended ACT for malaria treatment in pregnancy although its pharmacokinetics properties are altered in this group. In the second study, the PK, efficacy, safety and tolerability of a 5 days regimen of AL were

tested in a group of 48 pregnant women and a control group of 48 non-pregnant women with uncomplicated *falciparum* malaria *versus* the standard 3 day regimen. The day 42 PCR corrected efficacy was 100% in both treatment arms. Pregnancy was associated with reduced exposure to both lumefantrine and dihydroartemisinin. The extended regimen improved the exposure to lumefantrine, artemether and DHA in pregnancy. The QTc duration remained normal, but transient hematological or biochemical changes were observed in both groups. Babies born from the women treated in the study displayed a normal physical and neurological development in the first 12 months of life. At delivery 38% of women had placental malaria. The extended AL regimen is a promising option for those areas with emerging artemisinin resistance.

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Finally, I dedicate this thesis to my father, Professor Damase Onyamboko who has always wanted me to follow in his footsteps as an academic.

Attributions

This submission is entirely my own work except for the specific contributions that are acknowledged in this section.

My research was conducted in Kinshasa, the Democratic Republic of Congo from 2011 to 2014. I was supervised by Professor Nicholas Day (Nuffield Department of Clinical Medicine, University of Oxford and Mahidol-Oxford Research Unit) and Dr. Caterina I. Fanello (Nuffield Department of Clinical Medicine, University of Oxford and Mahidol-Oxford Research Unit).

I designed the two clinical trials, wrote the study protocol, the Case Report Form, the Standard Operating Procedures and coordinated the data collection under the supervision of Professor Day and Dr. Fanello. I analyzed the data and generated the tables and figures reported (unless stated otherwise) with the support of Dr. Fanello and of a statistician, Dr Sue Lee. I wrote the thesis which was revised by my supervisors.

Several people contributed to the clinical activities and laboratory analysis.

In the field, the team of KIMORU (Kinshasa Mahidol Oxford Research Unit) contributed to my research i/ in the laboratory: by preparing and reading malaria slides, collecting Dried Blood Spots, collecting and processing blood samples for the pharmacokinetic analysis and assisting me in the collection and preparation of placental biopsies and ii/ for the clinical part by monitoring the patients and collecting clinical data. I thank the doctors C. Kabedi, E. Kato-kale, D. Kayembe and P Ndjowo, the nurses, M. Sakina, O. Muniaka, ADD Fela, J. Omari, E. Biyela and A. Boyanga and laboratory technicians B. Badjanga, Y. Kambashi, M. Inwen and G. Simi. Dr Makwani, a registered obstetrician, conducted all the ultrasound exams in pregnant women.

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The data entry of the ACT trial was done at KIMORU and the one in pregnancy at MORU.

The population PK modeling analysis was carried out by Richard Hogland and Joel Tarning of the Pharmacology Department Mahidol-Oxford Research Unit in Bangkok.

This dissertation is a printed single-sided on A4 paper in double –spaced type, font 12 point Time New Roman, as required by the Graduate Studies, University of Oxford and doesn't exceed 50,000 words after excluding figures, pictures, tables, appendices and bibliography.

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LIST OF ABBREVIATIONS AND ACRONYMS

AC	Abdominal Circumference
ACPR	Adequate Clinical and Parasitological Response
ACT	Artemisinin-based Combination Therapy
AE	Adverse Event
AL	Artemether - lumefantrine
ALT	Alanine transaminase
ANC	Antenatal Care
ANOVA	Analysis of variance
AQ + AS	Amodiaquine + artesunate (loose formulation)
AQAS	Amodiaquine-artesunate (fixed dose combination)
ARI	Acute Respiratory Infection
ASSP	Artesunate-sulfadoxine-pyrimethamine
AST	Aspartate transaminase
AUC	Area Under the concentration-time Curve
BCZS	Bureau Central de la Zone de Sante
BMI	Body Mass Index
BPD	Biparietal Diameter
CHK	Centre Hospitalier de Kinshasa
CI	Confidence Interval
CL/F	Oral Clearance
Cmax	Maximum Concentration
CQ	Chloroquine
CQ + SPIRA	Chloroquine-spiramycin
CSA	Chondroitin Sulfate A
CYP3A4	Cytochrome P450 3A4
DBS	Dried Blood Spot
DHA	Dihydroartemisinin
DHA -PQ	Dihydroartemisinin-piperaquine
DHS II	Demographic Health Survey
DNA	Deoxyribonucleic acid
DRC	The Democratic Republic of the Congo
DRC-NMCP	Congolese National Malaria Program
ECG	Electrocardiograph
EDPT	Early Detection and Prompt Treatment
EGA	Estimated Gestational Age
EIR	Entomological Inoculation Rate
ETF	Early Therapeutic Failure
FDA	Food and Drug Administration
FL	Femur Length
GA	Gestational Age
GCP	Good Clinical Practice

GLURP	Glutamate rich protein
H&E	Hematoxylin and eosin stain
HDI	Human Development Index
HIV	Human Immunodeficiency Virus
HZs	Health Zones
ICH	International Conference on Harmonisation
IPTp	Intermittent Presumptive Treatment in pregnancy
IQR	Interquartile Range
***i*RBC**	Infected Red Blood Cells
ISRCTN	International Standard Randomised Controlled Trial Number
ITNs	Insecticid Treated bedNets
ITT	Intent to treat
IUGR	Intra Uterine Growth Retardation
IV	Intravenous
IVS	Intervillous space
KSPH	Kinshasa School of Public Health
LBW	Low Birth Weight
LCF	Late Clinical Failure
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LF	Lumefantrine
LLOQ	Lower Level of Quantification
LOD	Lower level of Detection
LPF	Late Parasitological Failure
MIC	Minimal Inhibitory Concentration
MICS2	Multiple Indicator Cluster Survey 2
MIS	Malaria Indicator Survey
MORU	Mahidol Oxford Tropical Medicine Research Unit
MSP1	Merozoite surface protein 1
MSP2	Merozoite surface protein 2
NCA	Non Compartmental Analysis
NPM	Non Placental Malaria
NPW	Non pregnant woman
OR	Odd Ratio
OXTREC	Oxford Tropical Research Ethics Committee
P+AS	Pyronaridine-artesunate
PC50	Estimated time in hours for parasitemia to reduce by 50% of initial value
PCR	Polymerase Chain Reaction
PCt1/2	Parasite Clearance half-life
PCV	Packed Cell Volume
PfEMP1	*Plasmodium falciparum* erythrocyte membrane protein 1
PfHRP2	Plasmodium falciparum Histidine Rich Protein
PK	Pharmacokinetic
PLDH	*Plasmodium* Lactate Dehydrogenase

PM	Placental Malaria
PNDS	Plan National de Développement Sanitaire
PPA	Per Protocol Analysis
PW	Pregnant Woman
QTc	Corrected QT Interval
RBC	Red Blood Cell
RDT	Rapid Diagnosis Test
SD	Standard Deviation
SMRU	Shoklo Malaria Research Unit
SOPs	Standard Operating Procedures
SP	Sulfadoxine-pyrimethamine
SSA	Sub-Saharan Africa
T1/2	Terminal Elimination Half-life
Tmax	Time to Maximum Concentration
TNF	Tumor Necrosis Factor
TRAC	Tracking Resistance to Artemisinin Collaboration
U/L	Units/Litre
UNDP	United Nations Development Program
UNICEF	United Nations International Children's Emergency Fund
UV	Ultraviolet
V/F	Apparent oral Volume of Distribution
WBC	White Blood Cells
WHO	World Health Organization
WWARN	World Wide Antimalarial Resistance Network

OVERVIEW OF THE THESIS

The first chapter of this thesis describes the epidemiology of malaria in the Democratic Republic of Congo (DRC) and the past and current history of malaria treatment in the country with particular attention to the treatment of malaria in pregnancy.

The second chapter describes the first clinical trial I carried out on the efficacy, safety and tolerability of three artemisinin–based combination therapies in a paediatric population. The main aim of this trial was to collect baseline data and to assess the efficacy of amodiaquine-artesunate (AQAS), five years after its introduction as a first line treatment of uncomplicated *Plasmodium falciparum* malaria in the DRC, and to compare this with the efficacies of potential alternatives, dihydroartemisinin-piperaquine (DHA-PQ) and artemether-lumefantrine (AL).

The third chapter describes the second clinical trial I carried out. With this trial I studied the pharmacokinetics of a 5-day regimen of artemether–lumefantrine compared to the current standard 3-day regimen in a group of pregnant and a group of non-pregnant women with *falciparum* malaria in the DRC. The main aim was to collect data that could help optimize the dosage of antimalarial therapy with this combination in pregnancy.

The histopathology of the placenta biopsies collected during this trial is described in chapter 4. The aim of this chapter was to describe the histopathological features of placental samples in the study population and correlate them with the clinical outcome.

In the conclusions I discuss my research findings and how the questions generated by these finding could be addressed by further future research.

1 Introduction

1.1 Malaria

Malaria is a preventable and treatable infectious disease caused by a protozoa of the genus *Plasmodium* (Garnham 1966). Only five species are infectious to humans, namely *Plasmodium falciparum, malariae, vivax, ovale* and *knowlesi*. The latter is normally found in long-tailed and pig-tailed macaques living in forested areas of South East Asia, but more recently it has been recognized to be a causative agent of malaria in humans (Singh *et al.* 2004; Cox Singh *et al.* 2008; White 2008). *Plasmodium falciparum* is the most pathogenic (White *et al.* 1983; Warrell *et al*. 1990; Miller *et al*. 1994) and the most prevalent in Sub-Saharan Africa (SSA) (Wernsdorfer 1980; Guerin *et al.* 2002). Only the female mosquitoes of the genus *Anopheles* are able to transmit malaria to humans. About 70 species are implicated in the transmission, but only 40 are considered important vectors. In Africa, the *Anopheles gambiae complex, An. funestus*, *An. nilii* and *An. moucheti* are the most common (White 1974).

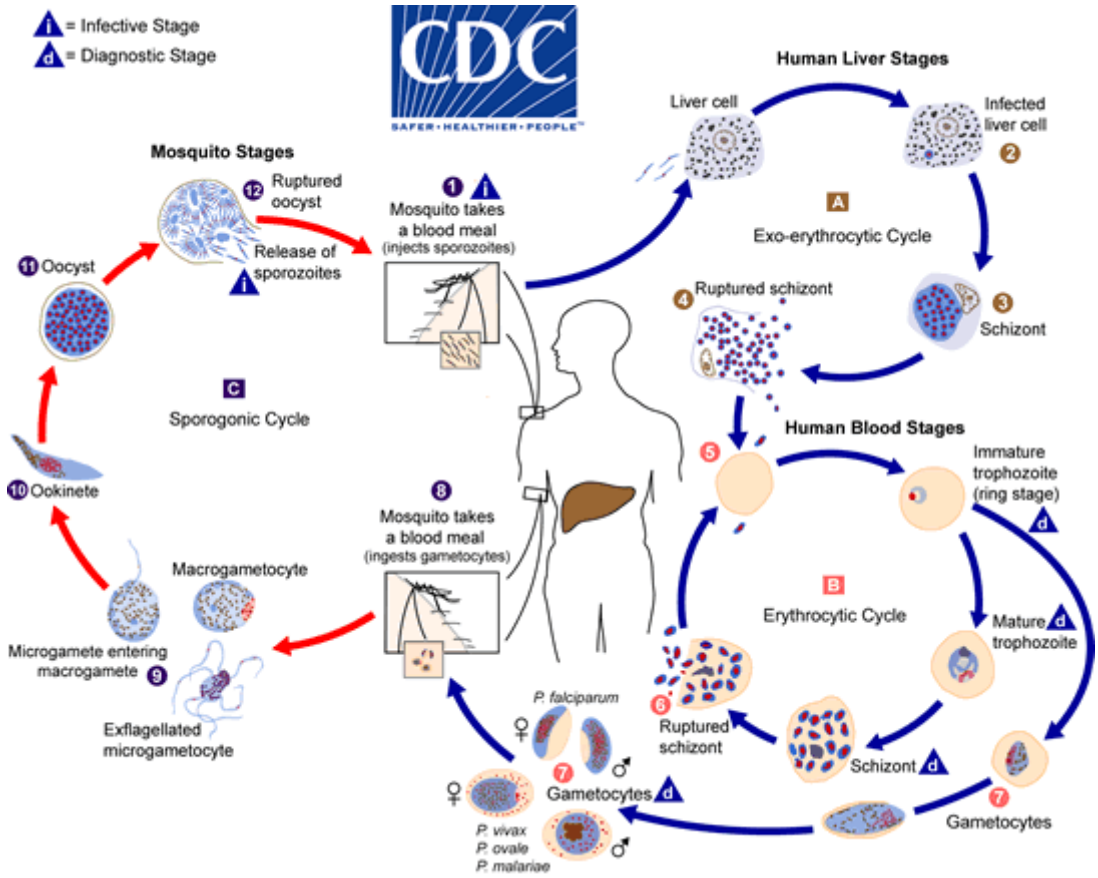
1.1.1 Malaria life cycle

The malaria parasite life cycle involves two different hosts, a mosquito and a human. In the mosquito, the parasite goes through the sexual phase of its cycle. The female *Anopheles* becomes infected by ingesting the sexual stages of the parasite while feeding on an infected person. The male microgametes fuse with the female macrogametes to form diploid zygotes which develop into mobile ookinetes. The ookinetes penetrate the midgut wall of the mosquito where develop into oocysts. The oocysts mature and rupture releasing sporozoites which migrate to the mosquito salivary glands. The

duration of the sporogonic phase varies according to the *Plasmodium* species and environmental conditions and at 28°C can last between 8 and 16 days.

The sporozoites are inoculated into the human bloodstream during the blood meal and migrate to liver cells where they mature into hepatic schizonts. These schizonts rupture and release merozoites into the circulation. One schizont can produce up to 30,000 merozoites (Amino *et al.* 2006; Tuteja 2007). In *P. vivax* some parasites remain dormant in the liver (hypnozoites) causing occasional relapses (Shute 1946; Shortt *et al.* 1948; Garnham *et al.* 1975; Krotoski *et al.* 1982). *Plasmodium ovale* was previously regarded as closely related to *vivax* and assumed to have hypnozoites. Molecular studies have however demonstrated that the two species are not closely related (Carter and Mendis 2002) and do not support the presence of a dormant stage in this species (Richter *et al.* 2010).

The exo-erythrocytic period varies between 6 and 16 days depending on the *Plasmodium* species. The merozoites released from the liver cells infect the red blood cells and start the erythrocytic cycle. The ring stages mature into schizonts, which contain multiple merozoites. These are released into the circulation when the erythrocyte ruptures and invade new erythrocytes. Some of these will differentiate into gametocytes, the sexual erythrocytic stage, and if ingested by a mosquito will complete the cycle.



*Picture from http://www.cdc.gov/malaria/about/biology/ , accessed 10/12/2015

Figure 1.1. The Plasmodium life cycle*

1.1.2 Malaria transmission

The severity of malaria and its clinical presentation in specific geographic areas and age-groups is determined by the intensity of transmission. Different categories are used to describe the different levels of malaria transmission and thus acquired immunity.

Ross-McDonald (MacDonald 1957) proposed the classification of stable and unstable malaria. In areas with stable malaria, transmission is high and occurs without marked fluctuations during the year, although minor seasonal fluctuations might occur. People living in these areas develop high levels of immunity with age, and the severe form of the disease affects primarily young children and pregnant women. In areas of unstable

malaria, transmission is variable in space and time. This category includes areas prone to epidemics. People have low levels of immunity and all age groups are similarly affected by the disease.

Other classifications used are based on the spleen rate (i.e. number of individuals with a palpable enlarged spleen per 100 individuals of similar age) and the parasite rate (i.e. number of persons with parasites on the peripheral blood film per 100 individuals of similar ages). These classifications were revised by Metselaar *et al.* (1959) to define four categories of malaria transmission intensity: hypoendemic, mesoendemic, and hyperendemic and holoendemic malaria (Table 1.1).

Another method for describing malaria endemicity is the use of the Entomological Inoculation Rate (EIR) which measures the number of infective mosquito bites per person per unit of time (MacDonald 1957). The annual EIR is the most used for measuring endemicity. Broadly if the EIR is between 10 to 100 infective bites per person per yearthe transmission of malaria is considered stable, whereas in areas where the EIR is< 10 infective bites per year malaria transmission is unstable or seasonal (Hay *et al.* 2000; WHO 2015a).

Immunity to malaria, defined as the ability to tolerate parasitaemia without clinical symptoms and to minimize the likelihood of developing the severe form of the disease (Marsh and Snow 1997) is acquired more rapidly in holoendemic regions compared to mesoendemic region (Doolan *et al.* 2009).

Table 1.1 Classification of endemicity and malaria transmission

Type	Spleen rates	Parasite rates	Transmission	Description
Hypoendemicity	Not exceeding 10% in children aged 2-9 years	Not exceeding 10% in children aged 2-9 years but may be higher for part of the year	Unstable	Areas where there is little transmission and the effects, during an average year, upon the general population are unimportant
Mesoendemicity	Between 11% and 50% in children aged 2-9 years	Between 11% and 50% in children aged 2-9 years	Unstable	Typically found among rural communities in subtropical zones where wide geographic variations in transmission risk exist
Hyperendemicity	Constantly over 50% in children aged 2-9 years; also high in adults (over 25%)	Constantly over 50% in children aged 2-9 years	Stable	Areas where transmission is intense but seasonal where the immunity is insufficient in all age group
Holoendemicity	Constantly over 75 % in children aged 2-9 years but low in adults	Constantly over 75 % among infants aged 0-11 months	Stable	Perennial, intense transmission resulting in a considerable degree of immunity outside early childhood.

1.1.3 Malaria burden world wide

In 2013, 124 to 283 million confirmed cases of malaria were reported worldwide and 584,000 deaths were attributed to the disease (ranging from 367,000 to 755,000) (WHO 2014a). These figures are still very high, despite a 30% reduction of malaria incidence and a 47% reduction of mortality worldwide observed since 2000 associated mainly to the scaling up of malaria interventions (Snow *et al.* 2008; O'Meara *et al.* 2010; Steketee and Campbell 2010). In 2014 WHO estimated that 3.3 billion people in 97 countries were at risk of contracting the disease (WHO 2014a). Sub-Saharan Africa carries the heaviest burden with the majority of deaths (90%) occurring in this region, where children under-five and pregnant women represent the most important group at risk (Murray *et al.* 2014; WHO 2014a). DRC along with India and Nigeria accounted for approximately 50% of all malaria deaths in 2013 (Murray *et al.* 2014).

1.1.4 Malaria in pregnancy

Malaria in pregnancy is a major public health problem due to its deleterious consequences for the mother and the fetus, such as maternal anaemia, intrauterine growth retardation (IUGR), prematurity, Low Birth Weight (LBW) and death (Steketee *et al.* 1996, 2001; Shulman and Dorman 2003; Sevene, Gonzalez and Menendez 2010).

In SSA, particularly in areas of stable endemicity, adults acquire a specific clinical immunity to malaria as a result of the repeated exposure to the parasite from birth. Adults consequently do not suffer severe malaria when infected. In pregnancy, this immunity is altered (Shulman and Dorman 2003) and particularly in primigravidae there is increased susceptibility to malaria (Desai *et al.* 2007; Uneke 2007a; Boel *et al.* 2012). However in high transmission areas, malaria infection is often asymptomatic

due to this partial immunity (Desai *et al.* 2007) and thus the infection often remains untreated. On the other hand, in areas of low endemicity pregnant women are symptomatic irrespective of parity (Nosten, McGready, *et al.* 1999; Nosten *et al.* 2007). Independently from the level of endemicity, malaria has the potential to impair the development of the fetus and all pregnancies are at risk; it is therefore important to prevent and treat all cases.

There is a limited number of antimalarials that have been authorized for use in pregnancy (Nosten *et al.* 2006). This is due to the absence of adequate safety data for many antimalarials in this vulnerable period, and especially in the first trimester when drugs are potentially more harmful to the fetus. Because of these concerns pregnant women are in fact normally excluded from clinical trials (Blehar *et al.* 2014). This combined with the lack of an adverse events (AEs) reporting system in developing countries results in a scarcity of data on drug safety of antimalarials in pregnancy.

1.1.5 Review of the antimalarials used in pregnancy

In this next section a review of the antimalarial used in pregnancy either for prevention or treatment of *falciparum* malaria is presented.

Chloroquine is one of the safest drugs used for treating malaria in pregnancy, and can be given to all gestational ages and during breastfeeding (Steketee, Wirima, Hightower, *et al.* 1996; Motta *et al.* 2002). However, due to the high level of resistance in *P. falciparum* its use is no longer recommended.

Sulfadoxine-pyrimethamine (SP) was considered until recently a valuable alternative to chloroquine for treatment, although the pharmacokinetics of sulfadoxine in pregnancy is suboptimal (Nyunt *et al.* 2010; Parikh and Rosenthal 2010). This combination, except for the hypersensitivity to the sulpha component associated with Steven-Johnson

syndrome (Hernborg 1985), has a good safety profile in pregnancy. As with chloroquine the efficacy of SP is now affected by the widespread presence of mutations associated with antifolate resistance in Africa (Sridaran *et al.* 2010).

Quinine is safe for use in all trimesters when taken at normal therapeutic doses, but its long treatment regimen (seven days) and the side effects such as hypoglycaemia, reduce compliance (Looareesuwan *et al.* 1985; Phillips-Howard 1999; McGready *et al.* 2000).

In the first trimester in particular, oral quinine remains the recommended drug (WHO 2015a). In some areas quinine is given with clindamycin. The latter has a good safety profile, unaltered pharmacokinetic properties in pregnancy, and has been suggested to reduce the rate of failure compared to quinine alone, although results are inconsistent and more studies should be carried out (Mcgready *et al.* 2001; Lell and Kremsner 2002; Obonyo and Juma 2012). Moreover this combination is not widely used because of the poor availability and high cost of clindamycin (Obonyo and Juma 2012).

Artemisinin derivatives are highly potent and fast acting antimalarial drugs with schizontocidal and gametocytocidal action (Luo and Shen 1987; Hien and White 1993; Woerdenbag *et al.* 1994; Kamchonwongpaisan and Meshnick 1996; Meshnick *et al.* 1996). They are available in a variety of oral (artemisinin, artesunate, artemether and dihydroartemisinin) and parenteral (artemether, arteether and artesunate) formulations (Klayman 1985; Brossi *et al.* 1988; Hien and White 1993; Woerdenbag *et al.* 1994). While safe in non-pregnant adults, mutagenic and teratogenic effects were observed in rodents in the first period of gestation corresponding to the first trimester in humans (Xu and Zhang 1996; Clark *et al.* 2004, 2008; Longo *et al.* 2006; White and Clark 2008; Clark 2009). Thus, in 2006, WHO recommended the use artemisinin-based combination therapies (ACTs) exclusively in the 2nd and 3rdtrimester.

Among the ACTs, artemether-lumefantrine has been the most widely recommended (WHO 2010a). This is because until 2007, the AL combination was the only fixed-dose ACT approved by WHO. It has proven to be highly effective against falciparum malaria and safe for use at the current standard dosage in the general population and in pregnancy (McGready *et al.* 2011). AL will be discussed in detail in another section as it is the ACT we tested in pregnancy in our second clinical trial.

Amodiaquine in combination with artesunate is indicated for the treatment of uncomplicated malaria and is considered to be effective against all the human plasmodial species. Many pregnant women have been treated with the combination, either co-administered or co-formulated (Orton and Omari 2008; WHO 2015a). The combination is safe for use in pregnancy at therapeutic dosage in the 2nd and 3rd trimester of pregnancy but prolonged use (such as in prophylaxis) increases the risks of hepatotoxicity and agranulocytosis (Hatton *et al.* 1986; Gasasira *et al.* 2008; Guevart and Aguemon 2009).

Dihydroartemisinin–piperaquine (DHA-PQ) has been successfully used to treat malaria in children and adults with good efficacy and safety profiles (Denis *et al.* 2002; Gargano *et al.* 2011). Piperaquine is a 4-aminoquinoline structurally similar to chloroquine. It showed no teratogenicity in animals studies (Chen *et al.* 1993). Because of the long elimination half life of piperaquine the combination is a potential candidate for intermittent preventive treatment in pregnant women with malaria (White 2005; Briand *et al.* 2007).

Artesunate–mefloquine is considered safe for use in the last two trimesters of pregnancy (Bounyasong 2001) despite reports of possible increased risk of stillbirth with the use of mefloquine in pregnancy. While it has been associated with an increased risk of stillbirth in large observational studies in Thailand (Nosten, Vincenti,

et al. 1999; McGready *et al.* 2000), this effect was not seen in Malawi (Steketee, Wirima, Slutsker, *et al.* 1996) nor in other studies in pregnancy in Africa (Okeyeh *et al.* 1996; Adam *et al.* 2004). The drug is now being considered a potential candidate for treatment and for IPTp (Vallely *et al.* 2007). The combination has been more used in Asia than in Africa.

1.1.6 Artemether-lumefantrine

AL is a fixed-dose combination of artemether (the artemisinin derivative) and lumefantrine (the partner drug). The two components, artemether and lumefantrine have a complementary antimalarial action. Arthemether has high antimalarial activity and reduces the asexual stage parasite biomass by 10,000–fold per reproductive parasite cycle (Ezzet and Karbwang 1998; White *et al.* 1999). Artemether absorption is fast and it is rapidly hydrolysed to its main active metabolite dihydroartemisinin (DHA) which consequently is responsible for the main antimalarial activity. Lumefantrine is on the contrary slowly absorbed and slowly eliminated. By staying longer in the body, it clears the residual parasites thus preventing a recrudescence of the infection (White *et al.* 1999; Price *et al.* 2006).

Artemether is lipid-soluble and it is available either as an oil-based formulation for intramuscular injection or combined with lumefantrine for oral administration. The drug is metabolized through cytochrome P450 CYP3A4 and is 95% bound to plasma proteins (Van Agtmael *et al.* 1999).

After oral administration it reaches a peak of concentration in median (range) 1.0 h (0.5 to 3.0) in the acute phase of malaria and 1.5 h (0.5 to 4.0) in the convalescent phase (Silamut *et al.* 2003).

Its metabolite DHA, which is active against the erythrocytic stage of *falciparum*, reaches a peak in the blood after 2-3 hours (mean) in healthy volunterers and its mean (range) terminal elimination half-life is 1.9 h (1 to 3)(White *et al.* 1999).

After intramuscular administration in severe malaria, the absorption of artemether is erratic and slow with a median peak of plasma concentration of 8 h (4 to 24)(Silamut *et al.* 2003).

The median elimination half-life after intramuscular administration is reported to be longer than after oral administration: 7 hours (3.5 to 10), due to continuous absorption (WHO 2006).

Lumefantrine is a racemic fluorine derivative similar to halofantrine which with halofantrine, quinine and mefloquine belongs to the aryl amino-alcohol group of antimalarials. The component is highly lipophilic and insoluble in water. Halofantrine is reported to be cardiotoxic by prolonging the QTc interval when administered in high doses (Nosten *et al.* 1993; White 1998). Lumefantrine is structurally similar to halofantrine, but is reported to be safe when administered at standard dose (Ward *et al.* 2007; White 2007).

Compared to artemether, the absorption of lumefantrine is slow and erratic (Ezzet and Karbwang 1998; White *et al.* 1999). There is an absorption lag phase of approximately 2 hours and the peak concentration in plasma is reached 6 to 8 hours after administration of the first dose (White *et al.* 1999; Ezzet *et al.* 2000). The drug is slowly eliminated from the body with a terminal elimination half-life of 3 to 6 days (Ezzet and Karbwang 1998; White *et al.* 1999). Lumefantrine absorption is described as dose-dependent (Ezzet *et al.* 2000; Ashley, Stepniewska, Lindegårdh, McGready, *et al.* 2007) and at the recommended dose of 4 tablets of AL twice daily for 3 days,

lumefantrine has reached its maximal absorption (Ashley, Stepniewska, Lindegårdh, McGready, *et al.* 2007). However, due to its relatively long elimination half–life of 4.5 days (Ezzet and Karbwang 1998), lumefantrine after successive doses accumulates in the blood resulting in progressively higher concentration throughout the treatment (Djimdé and Lefèvre 2009). As a result, parasites are constantly exposed to high levels of lumefantrine during the course of the therapy long after the rapid elimination of artemether and DHA (Djimdé and Lefèvre 2009).

Lumefantrine eliminates residual parasites left after the rapid biomass clearance by artemether/DHA, and consequently reduces the risk of recrudescence (White *et al.* 1999).

The plasma concentration of lumefantrine at day 7 is considered a good proxy of drug exposure, as represented by the area under the curve of plasma drug concentrations *versus* time. A lumefantrine level at day 7 below the value of 280 ng/ml has been associated with an increased risk of therapeutic failure (Ezzet and Karbwang 1998; Ezzet *et al.* 2000)

Treatment with AL has some limitations. First, as with many antimalarial drugs, thedistribution and metabolism of both components are altered in pregnancy resulting in reduced drug exposure (McGready, Stepniewska, Lindegardh, *et al.* 2006; McGready *et al.* 2011; Wilby and Ensom 2011; Tarning, Kloprogge, *et al.* 2012). Moreover lumefantrine absorption is fat-dependent (White *et al.* 1999). Secondly, the compliance can be sub-optimal due to the complex regimen of twice daily doses for three days (Bloland 2003). It is therefore particularly important to describe further the pharmacokinetics of this ACT in pregnant women and to identify the optimum safe dosage.

1.2 Malaria in DRC

1.2.1 The parasite and the vector

In DRC only three species are found: *Plasmodium malariae*, *P. ovale* and *P. falciparum*, the latter accounting for 97% of all malaria infections. As in the rest of SSA outside of the horn of Africa, *P. vivax* is not found in the country (DRC- NMCP 2011) This is due to the high frequency of the Duffy negative blood group antigen in the population, which is considered necessary to mediate the invasion of erythrocytes by this parasite species (Langhi and Bordin 2006; Rosenberg 2007). Nevertheless, cases of vivax infection in individuals with negative Duffy trait have been reported (Ménard *et al.* 2010; Mendes *et al.* 2011; Wurtz *et al.* 2011; Woldearegai *et al.* 2013) and a case of vivax infection in a migrant from DRC to Europe has been reported in literature (Comellini *et al.* 1998). However an error in the diagnosis cannot be ruled out as ovale and vivax are morphologically very similar.

In DRC, the most common vector for malaria are *Anopheles gambiae sensu stricto (ss)* and *An. arabiensis* and to a less extent *An. funestus, An. nilii, An. moucheti*, *An. brunnipes* and *An. Paludis* (Karch *et al.* 1992; DRC- NMCP 2011).

1.2.2 Ecological patterns of malaria in DRC

In DRC three specific epidemiological patterns can be identified: the equatorial, the tropical and the highland pattern (Mouchet *et al.* 1993).

The equatorial zone covers the largest part of the country in the so-called Central Basin ranging from forested to savannah areas. Malaria transmission in this zone is intense, stable and perennial with an EIR of 1,000 infectious bites per person per year. The

main malaria vectors are considered to be *An. gambiae ss, An. funestus, An. nilii* and *An. moucheti* (DRC- NMCP 2007).

The tropical zone consists essentially of savannahs areas. Malaria transmission is intense and seasonal, peaking in the rainy season that lasts five to eight months. The transmission is considered stable and the EIR ranges from 100 to 400 infectious bites per person per year. The vector population is similar to the one of the equatorial zone, but for the presence of *An*. *arabiensis* instead of *An*. *moucheti* (DRC- NMCP 2007).

The highland zone, in the eastern part of the country, is a relatively small area where malaria transmission is unstable occurring only in short periods of the year and thus prone to outbreaks. The EIR is less than 2 infectious bites per person per year. The main vectors are considered *An. arabiensis* and *An. funestus* (DRC- NMCP 2007).

Ninety-seven percent of the population lives in the areas with stable transmission (equatorial and tropical) and only 3% in the highlands.

Kinshasa, the capital city of the DRC, is located in the western part of the country and the estimated population is about 10 million residents. The city is divided into 35 health zones. Of those 30 are considered urban, 4 suburban (Maluku I, Nsele, Mont-Ngafula and Kisenso) and 1 rural (Maluku II). In Kinshasa there are two main seasons, the dry season from mid-May to mid-September and the rainy season from mid-September to mid-May. There is also a short dry season from January to February and a short rainy season from October to December (Crabbé 1980). There is considerable hydrographic coverage surrounding the city, sustained by abundant rainfall which favours the development of suitable breeding sites for anophelines. The transmission of malaria is intense and perennial in most of the city with an estimated EIR ranging from 2.8 to 620.5 infectious bites per person per year (DRC- NMCP 2007). The sporozoitic index,

a measure of the percentage of female anophelines of a particular species infected with sporozoites is about 7.2% (DRC- NMCP 2007).

1.2.3 Socio-economic and health indicators

The Democratic Republic of the Congo is the third largest country in Africa (after Algeria and Sudan) covering an area of two million square kilometres and with an estimated population of 68 million people. The country shares boundaries with Rwanda, Burundi, Uganda, Tanzania, Zambia, Angola, Congo, Central African Republic and Sudan.

The socioeconomic indicators classify DRC as one of the poorest African countries (UNDP 2013) with an estimated 88% of the population living below the international poverty line of US 1.25 per day (World Bank 2013). In 2013, the Human Development Index (HDI), a composite indicator measuring average achievement in three basic dimensions of human development i.e. a long and healthy life, knowledge, and a decent standard of living was 0.304, the lowest in the world, equally placed with Niger (UNDP 2013).

Regarding the health system, the territory is divided into more than 500 Health Zones (HZs). Each zone includes approximately 100,000 to 250,000 inhabitants and represents one operational unit for planning, implementation and integration of health activities. However less than 35% of the population has access to a health structure within a distance of 5 kilometres (PNDS RDC 2010). Malaria is the major cause of morbidity and mortality in the country and pregnant women and children under-five bear the heaviest burden.

The DRC is considered, along with India, Nigeria and Mozambique, the country with the heaviest malaria burden in the world (WHO 2013a; Murray *et al.* 2014). The

National Malaria Control Programme (NMCP) reported in 2012, 9 million clinical cases of malaria confirmed either by Rapid Diagnostic Test (RDT) or microscopy (data from passive surveillance at the sentinel sites). Of these, 49% were children under-five and 6% pregnant women. Thirty-seven percent of all hospital consultations were suspected cases of malaria and 34% of all hospital admissions were confirmed cases of malaria. Eighty-five percent of all blood transfusions were administered for the treatment of malaria related anaemia and most of them (75%) in children under-five. Twenty-two thousands deaths were attributed to malaria (about a third of total deaths reported) and 75% of them in children under-five (DRC-NMCP 2012a).

Key indicators for malaria prevention and control are consistent with the poor socio-economic conditions. It is important to point out that the surveys performed to measure these indicators were conducted in small areas of the country selected because they were accessible and / or where international funding agencies supported their projects. Therefore the results do not represent the entire country and especially do not represent remote areas. Only two national surveys, the Demographic and Health Survey II (DHS II) conducted in 2013-2014 and the Multiple Indicator Cluster Survey (MICS 2) in 2010 covered the entire territory. National surveys are not repeated frequently and often use a different methodology compared to small surveys, making data comparison difficult and results inconsistent. A summary of the Key Malaria Indicators from two national surveys, DHS II and MICS 2 and the Malaria Indicator Survey (MIS) conducted in 2010 by the Kinshasa School of Public Health in 119 Health Zones and funded by the Global Fund, is presented in Table 1.2.

Table 1.2. Summary of the main Malaria Key Indicator in DRC

Malaria Key Indicators	MIS 2010 (%)	MICS II 2010 (%)	DHS II 2013 (%)
Children under-five with fever	32.7	27	31
Children under-five with anaemia	69.6	-	-
Children under-five with fever who received a recommended antimalarial within 24h from onset of fever	1.6	23.8	2
Children under 5 with fever who received any anti-malarial treatment within 24h from onset of the fever	-	39	14.5
Households with at least 1 insecticide-treated mosquito net	62.4	51	70
Children under 5 who slept under an insecticide-treated mosquito net the night before the survey	55.4	38	55.9
Pregnant women with fever	34.3	-	-
Pregnant women who slept under an insecticide-treated mosquito net the night before the survey	49.1	43	60
Pregnant women who received at least two doses of SP during the current pregnancy	51.2	20.8	14

Table 1.3. Summary of efficacy studies in DRC from 1982 to 2012

Country region	Drug tested	Population	Follow-Up (days)	Treatment failure (%)	PCR	Reference
Kivu	CQ		14	9.50%	No	Delacolette *et al*.1982
Kinshasa, Mbuji Mayi	CQ	8-15 years	28	2.80%	Yes	Nguyen-Dhin *et al*. 1983
Bwamanda, Kinshasa	CQ	<5 years	7	8% Bwamanda, 56% Kinshasa	No	Paluku *et al*. 1988
Kinsuka, Kinshasa	CQ	All ages	7	5%	Yes	Ngimbi *et al*. 1985
Lubumbashi	CQ, CQ+SPIRA	<5 years	7	17% CQ, 5% CQ+ SPIRA	No	Kabeya *et al*. 1990
Kinshasa	Halofantrine	4 months to 12 years	14	2%	No	Mashako *et al* 1990
Kinshasa	CQ	All ages	7	45%	No	Garin *et al*. 1992
Goma	CQ, SP	1-55 years	7	79.5% CQ, 65% SP	No	Wolday *et al*. 1995
Seven sentinel sites	CQ, SP	<5 years	14	overall 45.4% CQ ;7.5% SP	No	Kazadi *et al*. 2003
Rutshuru	SP, AQSP, ASSP	<5 years	14	61% SP, 32% AQSP, 21% ASSP	Yes	Alker *et al*. 2008
Boende	ASSP, AQAS, SP, AQ	<5 years	28	24.6% ASSP, 15.1% AQAS, 35.9% SP, 18.3% AQ	Yes	Bonnet *et al*.2004
Kabalo	ASSP, AQAS, SP	<5 years	28	0% ASSP, 0% AQAS, 19.6% SP	Yes	Bonnet *et al*.2004
Shabunda	AQAS, ASSP	<5 years	28	6.7% AQAS, 19.7% ASSP	Yes	Swartout *et al*.2006
Kisangani	AQAS, AL	<5 years	28	3.6% AQAS, 13.8% AL	Yes	NMCP 2005
Rutshuru	AQAS, AL	<5 years	28	5.4% AQAS, 2.6% AL	Yes	NMCP 2005

Bolenge	AQAS, AL	<5 years	28	2.6% AQAS; 0% AL	Yes	NMCP 2005
Kimpese	AQAS;AL	<5 years	28	6% AQAS;6.3% AL	Yes	NMCP 2005
Kinshasa	AQAS;AL	<5 years	28	7.2% AQAS, 0% AL	Yes	NMCP 2005
Kinshasa	AQAS	≥ 16 years	28	0%	Yes	Sinou *et al*. 2009
Kinshasa (+ 9 sites)	P+AS; AL	3-60 yr	28	0.5% P+AS, 2.1% AL	Yes	Tshefu *et al*. 2010
Katanga	AQAS ; AL	>5	42	1.7% AQAS, 0.9% AL	Yes	Espie *et al*. 2012
Lwiro/East DRC	AQAS	<5	28	27% and 9%	No	Ndeba *et al*. 2012

Legend: CQ chloroquine, AQ amodiaquine, AL artemether-lumefantrine, AQAS amodiaquine-artesunate, ASSP artesunate–sulfadoxine–pyrimethamine, CQ +SPIRA chloroquine – spiramycin, P+AS pyronaridine-artesunate.

From 1982 to 2001 the efficacy of CQ was studied in different parts of the country, and throughout this period an increasing loss of efficacy was observed in all studied areas (Delacollette *et al.* 1983; Ngimbi *et al.* 1985; Nguyen-Dinh *et al.* 1985; Paluku *et al.* 1988; Kabeya *et al.* 1990; Garin *et al.* 1992; Wolday *et al.* 1995; Kazadi *et al.* 2003) with the highest rate of failure observed in the eastern region (Wolday *et al.* 1995; Kazadi *et al.* 2003).

From 1995 to 2003 a progressive lack of efficacy of SP, as monotherapy or in combination, was observed whith failure rates in some areas as high as 65% (Wolday *et al.* 1995). At the same time a high prevalence of mutations associated with SP resistance was observed in the parasite population (Kazadi *et al.* 2003; Swarthout *et al.* 2006; Bonnet *et al.* 2009).

Despite the increasing lack of efficacy of SP and chloroquine, these two drugs continue to be used across the country in the private sector as well as for the IPTp strategy (DRC-NMCP 2012b).

The efficacy of antimalarial combinations with artemisinin derivatives has been so far quite good in most studies, although reduced efficacy was observed in some cases (Swarthout *et al.* 2006; Bonnet *et al.* 2009; Sinou *et al.* 2009; Espié *et al.* 2012; Ndeba *et al.* 2012). For instance regarding AQAS, the NMCP reported a failure rate of 7.2% in Kinshasa in 2005, before its formal introduction in the country (DRC- NMCP 2007), then in 2009 a different trial conducted in the same area reported 100% efficacy (Sinou *et al.* 2009) and in 2012 a 27% failure rate was observed in the east of the country (Ndeba *et al.* 2012).

The efficacy studies presented above were conducted in different areas by different research groups and often using differing methodology and thus results might not be

comparable. This applies not only to efficacy studies but to epidemiological data as well.

Overall, the studies carried out in different areas have shown a high level of geographic variation in therapeutic efficacy and in some cases a similar variation was observed in the presence and prevalence of polymorphic alleles in *P. falciparum* associated with parasitological failure (Swarthout *et al.* 2006; Alker *et al.* 2008; Mobula *et al.* 2009).

1.2.4 Problems of treating pregnant women

The changes in maternal physiology accompanying pregnancy can alter the pharmacokinetic (PK) of drugs including some antimalarials (Dawes and Chowienczyk 2001; Frederiksen 2001; Blehar *et al.* 2014; Costantine 2014). In particular the haemodynamic and hormonal changes can alter drug absorption, and the steady increase in plasma volume affects the drug distribution in the body as well as decreasing the concentration of plasma proteins binding the drugs (Notarianni 1990). The increased plasma volume also increases the glomerular filtration rate and thus the excretion of the drug from the body as well (Little 1999). The hormonal changes, especially oestrogens and progesterone, also affect (either decrease or increase) the activity of enzymes which are important for drug metabolism (such as cytochrome CYP3A4) as well as the gastrointestinal absorption (Little 1999; Blehar *et al.* 2014; Costantine 2014).

1.2.4.1 Review of pharmacokinetics studies of AL in pregnancy.

As for many countries, DRC has adopted ACTs for use in the treatment of malaria in pregnancy without testing them specifically in this group, but rather relying on data derived from studies in the general population. Only one trial carried out in Kinshasa in 2007-2009 has studied the pharmacokinetic of an artemisinin derivative in pregnancy

(Onyamboko *et al.* 2011). The PK of artesunate/DHA was measured in 26 women with asymptomatic *falciparum* malaria in their 2nd or 3rd trimester and a control of 25 non-pregnant women and the same pregnant women three months after delivery (Onyamboko *et al.* 2011). Despite a satisfactory parasite clearance in all study subjects, pregnant subjects had a lower plasma concentration (AUC) of DHA compared to non-pregnant subjects, and following these results dose–optimization studies were recommended (Morris *et al.* 2011; Onyamboko *et al.* 2011).

Concerning AL, a limited number of studies have been conducted to characterize its pharmacokinetics in pregnant subjects over the last decade (2004 to 2014).

In the section below I review the published trials on the pharmacokinetics of AL in pregnancy and in Table 1.4, I summarize the data for the main PK parameters obtained in healthy volunteers to which most of the results in studies in pregnancy were compared.

McGready *et al.* (2006) studied the PK of artemether-lumefantrine in 13 pregnant women with malaria and compared the data with historical data in non-pregnant male adults. PK parameters were characterized by non-compartmental analysis. The findings highlighted the inadequacy of the current standard three day regimen for treating malaria in Asian women, especially in the 3rd trimester. Pregnant women had a significant reduction in the plasma levels of both artemether and dihydroartemisinin compared to the controls. The authors could not ascertain if it was due to a reduced absorption or a 'dilution' effect due to the larger volume of distribution in pregnancy. Also the exposure (AUC) to lumefantrine was significantly lower in pregnant compared to non-pregnant subjects and this was attributed to a faster lumefantrine elimination ($T_{1/2}$) in the former group. In this study smoking was a factor associated

with a lower exposure to lumefantrine (McGready, Stepniewska, Lindegardh, *et al.* 2006).

Tarning *et al*. (2009) analysed data from 103 Karen and Burmese pregnant women treated with the standard 80/480 mg AL twice daily over 3 days. Lumefantrine PK parameters were estimated by population-based modelling from capillary blood. The authors modelled lumefantrine data using a two-compartment model with first-order absorption and elimination. The main findings in this study were first the large inter-individual variability observed in plasma concentrations of lumefantrine for all PK parameters described indicating a high variability in the absorption despite the coadministration with fat. The authors also confirmed the previous findings of McGready *et al.* (2006) on the low exposure obtained with a 3-day AL regimen (McGready, Stepniewska, Lindegardh, *et al.* 2006). Low exposure for artemether, dihydroartemisinin, and lumefantrine was observed in this cohort of pregnant women compared to previous data in pregnant subjects in the same area (McGready, Stepniewska, Lindegardh, *et al.* 2006) and compared to existing data in non-pregnant adults and children from the same population. In 40% of study subjects, the plasma lumefantrine level at day 7 was below 280 ng/ml, the cut-off previously associated with increased risk of therapeutic failure in non-pregnant subjects. These low exposures had an impact on efficacy with a consequent high failure rate (17% recrudescence by day 42). Thus, different dose-regimen simulations were tested to determine the best regimen to improve drug efficacy. An increased length of treatment was suggested as more effective than increasing the mg/dose due to the dose-limited absorption of lumefantrine (Ashley, Stepniewska, Lindegårdh, McGready, *et al.* 2007). The best model was an extended 5-day regimen with twice daily AL (80/480 mg) administration, able to provide a higher exposure to lumefantrine, artemether and

dihydroartemisinin that would cover three consecutive parasite cycles instead of two as with the current 3-day treatment (Tarning *et al.* 2009).

Piola *et al.* (2010) in a non-inferiority trial conducted in 2006-2009 in Uganda compared the standard 3-day AL treatment to a 7-day oral quinine in 304 pregnant women with uncomplicated malaria. They measured the lumefantrine plasma concentration at day 7 in 92 pregnant patients amongst the 152 enrolled in the AL arm. The median level was 481 ng/ml (ranging from 15 ng/ml to 3246 ng/ml) and 32% of women had a concentration below 280 ng/ml, although the cure rate was 98.2%. A reduced lumefantrine level was associated with recurrent parasitaemia suggesting a need for dosage optimization (Piola *et al.* 2010).

Tarning *et al.* (2013) in a subsample of the same study population of Piola *et al.* (2010) evaluated the pharmacokinetic properties of quinine and artemether-lumefantrine. Lumefantrine PK parameters were compared to a small group of 17 non-pregnant subjects when parameters for artemether and DHA were compared to historical data. PK parameters of artemether, DHA and lumefantrine were estimated by non-compartmental analysis. Maximal concentration and exposure of artemether and DHA were found to be in the same range as levels previously measured by McGready *et al.* (2006) in Thai pregnant women. However, compared to data obtained by Karbwang *et al*. (1998) and Lefevre *et al.* (2001) from Thai non-pregnant adults, Ugandan pregnant women had significantly lower exposure and lower Cmax for artemether and DHA. For lumefantrine, Tmax and $T_{1/2}$ (terminal elimination half-life) were shorter in pregnant than in non-pregnant patients. The Cmax and the median lumefantrine levels at day 7 were similar between the two groups of patient. None of the non-pregnant controls had a lumefantrine concentration at day 7 below 280 ng/ml whereas 15% of pregnant subject were below this cut-off (Tarning *et al.* 2013).

Klopprogge *et al.* (2013) estimated the population pharmacokinetics of lumefantrine in a subsample of 116 pregnant from the study population of Piola *et al* (2010) and 17 non-pregnant women with uncomplicated *P. falciparum* malaria in Uganda after a standard fixed oral artemether–lumefantrine treatment. Data were well described with a transit-compartment absorption model followed by a two-compartment disposition model. The total exposure (AUC) was not affected by pregnancy but lower venous plasma lumefantrine concentrations at day 7 were observed in pregnant women compared with those in non-pregnant women. Simulation from capillary blood showed lumefantrine levels at day 7 of the studied pregnant women similar to those observed in Thai pregnant women (McGready *et al.* 2008). The authors concluded there was a need for optimization studies to avoid the development of resistance and to improve the cure rates of artemether–lumefantrine (Kloprogge *et al.* 2013).

Mosha *et al.* (2014) characterized the PK profile of AL and its metabolites by modelling data from pregnant and non-pregnant Tanzanian women treated with the standard 3-day regimen. With the final model obtained from this standard AL regimen, authors simulated the concentration-time profile of lumefantrine from 1000 subjects for two different regimens AL. The first regimen consisted of 6 doses over 3 days (at 0, 8, 24, 36, 48, and 60 h) and the second one of 6 doses over 5 days (at 0, 8, 24, 48, 72, and 96 h). Pregnant patients had lower plasma concentration of lumefantrine at day 7 compared to non-pregnant as observed in previous studies in pregnancy in Thailand (McGready, Stepniewska, Lindegardh, *et al.* 2006). The authors explained these lower levels by the reduced drug absorption, the elevated drug metabolism, and the rapid clearance rate in pregnant subjects compared to non-pregnant subjects. The median lumefantrine level at day 7 predicted from these simulations were below plasma levels predicting therapeutic failure in a substantial proportion of pregnant subjects under the

3-day AL regimen compared to the median level achieved with the 5-day regimen. However, the lumefantrine concentrations at day 7 in Tanzanian pregnant women was 2-fold higher than the levels observed in Thai pregnant subjects. Dose optimization studies aiming for higher lumefantrine levels at day 7 were suggested (Mosha, Guidi, *et al.* 2014).

Table 1.4. Summary of Artemether, DHA and lumefantrine PK parameters in healthy volunteers

	FDA Study 2102	**FDA Study 2104**	**Lefevre *et al.* 1999**	**Lefevre *et al.* 2002**
	n=50	n=48	n=16	n=14
Artemether				
Cmax (ng/ml)	60.0 ± 32.5	83.8 ± 59.7	104 ±53	30.8 ± 25.4
Tmax (h)	1.5	2	2	1.92 (1.92-3.0)
AUC_{LAST} (ng•h/ml)	146 ± 72.2	259± 150	338 ±175	61.4 ± 87.5
$T_{1/2}$ (h)	1.6 ± 0.7	2.2 ± 1.9	2.3 ±1.2	2.0 ± 1.2
DHA				
Cmax (ng/ml)	104 ± 35.3	90.4 ± 48.9	49.7 ±23.3	84.5 ± 26.5
Tmax (h)	1.76	2	2	1.92 (1.92-5.0)
AUC_{LAST} (ng•h/ml)	284 ± 83.8	285 ± 98.0	169 ±57.1	178 ± 71
$T_{1/2}$ (h)	1.6 ± 0.6	2.2 ± 1.5	3.1 ±1.6	1.2 ± 0.4
Lumefantrine				
Cmax (ng/ml)	7.38 ± 3.19	9.80 ± 4.20	5.10 ±1.90	10.0 ± 5.5
Tmax (h)	6.01	8	6	64 (38-68)
AUC_{LAST} (ng•h/ml)	158 ± 70.1	243 ± 117	108 ±47	383 ± 304
$T_{1/2}$ (h)	101 ± 35.6	119 ± 51.0	71.3 ±20.7	144 ± 31

Values are mean ± SD, except for Tmax which are median (range)

Table 1.5. Summary of pharmacokinetics studies of artemether–lumefantrine in pregnancy: Lumefantrine

Study characteristics				Lumefantrine PK parameters				
Country and year	Pregnant women (P) or non-pregnant (NP)	Drug regimen (days)	Venous (V) or capillary (C)	AUC∞ (µg*hr/mL)	Cmax (ng/mL)	Tmax (hours)	Terminal half-life (hours)	D7 (ng/ml)
Thailand 2004 (1)	13 P	3 AL *vs.* 7 AS	V (NCA)	$AUC_{60-\infty}$ 252 (51–614)	7340 (1,590–15,670)	4 (0.5–8)	68 (47–182)	384 (62–835)
Thailand 2004–2006 (2)	103 P	3 AL	C (PPK)	472 (119–1,261)	NA	NA	79.2 (36–187.2)	391 (126–1,600)
		Simulated 5 (twice daily)	C	NA	2427-17581	NA	NA	498-3180
Uganda 2006–2009 (3)	304 P	3 AL *vs.* 7 QN	V (data on 97 P from AL group)	NA	NA	NA	NA	P: 481 (15 –3,246)
Uganda 2006-2008 (4)	116 P 17 NP	3 AL	V (26 P + 17 NP) and capillary (90 P)	P: 570 (76.4–1,850) NP: 630 (285–1,240)	P: 8,400 (722–25,600); NP: 8,330 (4,360–15,000)	NA	P: 90.3 (64.3–121) NP: 69.8 (54.3–78.3)	P: 423 (45 –2,610); NP: 592 (258 –1,670)
Uganda 2006-2009 (5)	25 P 17 NP	3 AL *vs.* 7 QN	V (NCA)	P: 654 (79.4–1,870) NP: 621 (292–1,170)	P: 9,190 (485–22,400) NP: 8,880 (450–17,000)	P: 4 (0.08–12.1) NP: 6 (1.00–14.0)	P: 53.5 (28.5–79.4) NP: 65.7 (48.2–93.7)	P: 488 (30.7 –3,550) NP: 720 (339 –2,150)
Tanzania 2012 (6)	33 P 22 NP	3 AL (PK simulation 3 and 5)	V (PPK)	NA	NA	NA	NA	P-3: 908 (217 - 3,256) NP-3:1382 (386 - 5,135) P-5: 1,374 (367 - 5,536)

(1) McGready *et al,* 2006; (2) Tarning et al, 2009 ; (3) Piola *et al,* 2010, (4) Kloprogge *et al* 2013, (5) Tarning *et al* 2013 (6) Mosha *et al* 2014

Table 1.6. Summary of pharmacokinetics studies of artemether–lumefantrine in pregnancy: Artemether and DHA

Study characteristics				Artemether PK parameters			
				AUC (µg*hr/mL)	Cmax (ng/mL)	Tmax (hours)	Terminal half-life (hours)
Thailand 2004 (1)	13 P	3d AL vs. 7d AS	V (NCA)	65.6(10.5 – 280)	35 (14 -104)	1 (0.5 -2)	1.5 (1.2 -7.2)
Uganda 2006-2008 (4)	116 P; 17 NP	3 AL	V (26 P + 17 NP) and capillary (90 P	111(16.2 – 317)	32.9 (7.5 - 82.9)	1.16 (0.65 – 3.81)	NA
Uganda 2006-2009 (5)	25 P; 17 NP	3d AL vs. 7d QN	V (NCA)	104 (10.8 – 351)	35.4 (5.69 – 143)	NA	NA
Study characteristics				**DHA PK parameters**			
				AUC (µg*hr/mL)	Cmax (ng/mL)	Tmax (hours)	Terminal half-life (hours)
Thailand 2004 (1)	13 P	3d AL vs. 7d AS	V (NCA)	357 (29.8–585)	165 (72–224)	1 (0.5–2)	1.3 (0.9–8.4)
Uganda 2006-2008 (4)	116 P; 17 NP	3 AL	V (26 P + 17 NP) and capillary (90 P	167 (55.3 – 437)	45.2 (14.1 – 114)	1.37 (0.82 – 3.89)	NA
Uganda 2006-2009 (5)	25 P; 17 NP	3d AL vs. 7d QN	V (NCA)	P: 200 (55.9 – 456)	P: 83.0 (18.8 – 153)	NA	NA

In summary the findings of these pharmacokinetics studies following a 3-day regimen with artemether-lumefantrine in pregnancy, indicate that for artemether the AUC∞ ranged from 10.5 to 351 µg*hr/mL, C_{max} ranged from 5.69 to 143 ng/mL, time to reach the C_{max} after one dose ranged from 0.5 to 3.81 hours and the terminal elimination half-life ranged from 1.2 – 7.2 hours. For DHA the AUC∞ ranged from 29.8 to 585 µg*hr/mL, C_{max} ranged from 18.8 to 224 ng/mL, time to reach the C_{max} after one dose ranged from 0.5 to 4 hours andthe terminal elimination half-life ranged from 0.7 – 8.4 hours. For lumefantrine, the AUC∞ ranged from 76.5 to 1,870 µg*hr/mL, the C_{max} ranged from 485 to 25,600 ng/mL, the time to reach the C_{max} after one dose was 4 hours ranging from 0.5 to 12.1 hours, the terminal elimination half-life ranged from 28.5 to 187.2 hours and the plasma lumefantrine level at day 7 ranged from 30.7 to 3,550 ng/mL.

Most of these studies reported a modification of the main PK parameters in pregnant subjects compared to non-pregnant subjects for artemether, DHA and lumefantrine. The lower plasma concentration observed in pregnant subjects compared to non-pregnant for the different components of the drug were assumed to be the results of the physiological changes observed in pregnancy. With the standard 3-day treatment, plasma concentration of lumefantrine at day 7 was lower in pregnant compared to non-pregnant women in all studies comparing this parameter in the two groups of subjects. All these findings highlight the importance of optimising the dose of the therapy in pregnancy.

Most of these studies have compared the results with historical data from healthy volunteers (Lefevre and Thomsen 1999; Lefèvre *et al.* 2002)(Table 1.4), whereas how pregnancy modifies the pharmacokinetics of antimalarials would be better described if

comparable controls were used, i.e. controls subjects were matched by sex, age and malaria status (Ward *et al.* 2007).

Despite the importance of improving the dosage of therapies in pregnancy, no clinical trial has been conducted so far. Only one study was published reporting data obtained after mathematical simulation of a daily administration of 80/480 mg AL for 5 days (Tarning *et al.* 2009).

2 A randomized comparison of the efficacy and tolerability of three artemisinin-based combination treatments for children with acute falciparum malaria in the Democratic Republic of Congo

2.1 Introduction

Amodiaquine-artesunate (AQAS) and artemether-lumefantrine (AL) are the two recommended drugs for the treatment of uncomplicated *Plasmodium falciparum* malaria in the DRC. In the public sector AQAS is the most widely distributed whereas the availability of AL is scarce.

Antimalarial drug distribution and access in rural areas is organized through the Public Sector (Bureaux Centraux de la Zone de Santé, BCZS). In the urban setting on the other hand, the private sector plays a greater role than the public one. In Kinshasa a large number of different generic and brand antimalarials are commonly available for purchase without prescription from pharmacies and street vendors. For instance, it was possible to purchase generic formulations of AL over the counter and from street vendors before this ACT became first-line treatment and the country started importing the original product, Coartem® (Novartis, Switzerland). Similarly it has been possible to purchase generic formulations of dihydroartemisinin–piperaquine (DHA-PQ), an ACT which has never been officially imported into the country. Regarding AQAS, before the introduction of the co-formulated product the drug was sold as a co-

administered blister pack, which often resulted in patients taking only the artesunate tablets because of the low tolerability of amodiaquine.

The use of poor quality antimalarial drugs, often in monotherapy, often results in low drug exposure, leaving the infection effectively untreated or undertreated. Moreover, exposure of the parasites to sub-therapeutic antimalarial drug concentrations is the most important drug-related factor leading to the emergence and spread of antimalarial drug resistance.

The aim of this trial was to assess the efficacy of amodiaquine-artesunate for the treatment of uncomplicated *P. falciparum* malaria in children in Kinshasa, DRC, five years after its introduction as a first line treatment, and to compare this with the efficacies of potential alternatives, dihydroartemisinin-piperaquine and artemether-lumefantrine, the latter recently added to the first-line treatment policy.

The characteristics or artemether-lumefantrine have been previously discussed in Chapter 1.

Amodiaquine–artesunate is an artemisinin-based combination therapy (ACT). The fixed-dose formulation tablets contain 25/67.5 mg, 50/135 mg or 100/270 mg of artesunate and amodiaquine. Blister packs for co-administration contain separate tablets of 50 mg of artesunate and 135 mg base of amodiaquine.

Amodiaquine is a 4-aminoquinoline similar to chloroquine. It is generally effective against chloroquine-resistant *P. falciparum* infections, but efficacy varies.

The adverse effects of amodiaquine are similar to those of chloroquine. Amodiaquine is associated with much less pruritus and is more palatable than chloroquine, but is associated with a much higher risk of agranulocytosis (Booth *et al.* 1967; Hatton *et al.* 1986) and, to a lesser degree, of hepatitis (Neftel *et al.* 1986) when used for

prophylaxis. The risk of a serious adverse reaction with prophylactic use (which is no longer recommended) appears to be between 1 in 1,000 and 1 in 5,000 (Hatton *et al.* 1986). It is not clear whether the risks are lower when amodiaquine is used to treat malaria. Following overdose, cardiotoxicity appears to be much less frequent than with chloroquine (Olliaro *et al.* 1996; Olliaro and Mussano 2003; WHO 2006). Large doses of amodiaquine have been reported to cause syncope, spasticity, convulsions and involuntary movements (Radloff *et al.* 1996).

Artesunate is the sodium salt of the hemisuccinate ester of artemisinin. It is the only artemisinin derivative sufficiently water soluble, although very unstable, to allow for administration by the IV route for the treatment of severe malaria.

Dihydroartemisinin-piperaquine is an ACT with a good safety and tolerability profile which is as effective as other ACTs in endemic areas of Asia and Africa (Naing *et al.* 2013). Piperaquine is a bisquinoline with a chemical structure similar to chloroquine and amodiaquine.The long terminal elimination half-life (~23 days) provides lengthy post-treatment chemoprophylaxis and the simple once daily dosage regimen facilitates adherence (Tarning, Zongo, *et al.* 2012). Dihydroartemisinin-piperaquine efficacy in Africa has so far been effective, although no data are available for DRC. DHA-PQ could replace AQAS in the near future or be proposed as a first line treatment as part of a multiple first line treatment strategy.

2.2 Methods

2.2.1 Study area

All the research for this study was conducted in the same area, in Kinshasa, the capital city of the Democratic Republic of Congo (DRC).

The transmission of malaria in the study area is intense and perennial with two peaks in mid-October and mid-May corresponding to the rainy season. The dry season runs from mid-May to mid-September. The dry and rainy seasons are the two main seasons existing in the area. There is a short dry season from January to February and a short rainy season from October to December (Crabbé 1980). The estimated EIR ranges from 2.8 to 620.5 bites/person/year and the sporozoitic index is about 7.2% (DRC- NMCP 2007). There is considerable hydrographic coverage sustained by abundant rainfall which favors the development of suitable breeding sites for anophelines.

The research facility belonging to the Kinshasa School of Public Health is located within the compound of the Kingasani Maternity Hospital, in Kimbanseke, one of the 35 health zones (HZ) in Kinshasa. This urban and extremely poor area is densely populated and located in a suburb of the city. The facility was built in the compound of the maternity hospital and has been operational since September 2004. It is a 600 m^2 building including a clinical laboratory, waiting room, doctors' office, inpatient wards and a catering space.

Kingasani Maternity Hospital, the biggest maternity in Kinshasa, is run by the religious order Soeurs des Pauvres de Bergames. It provides antenatal and delivery care services to the population, and has been operational for over 60 years.

The Italian Catholic nuns are responsible for the functioning and administration of the setting. Congolese staffs run all medical and nursing aspects of clinical care. Overall, 7,000 deliveries occur yearly in the maternity hospital. The hospital is equipped with basic but functioning facilities for the care of low birth weight and premature babies but it has some of the best facilities for blood transfusion of the town. An outpatient unit is organized to provide antenatal care and postpartum follow up to mothers and infants.

The clinical trial was carried out in collaboration between the University of Oxford and the Kinshasa School of Public Health (KSPH).

Figure 2.1 Maps of the catchment area (from Google Map)

2.2.2 Patient population

Patients attending the health centre with suspected clinical malaria were screened and enrolled in the study if they met the following inclusion criteria: age 3 to 59 months; weight ≥ 5kg; mono-infection with *P. falciparum;* parasitaemia density between 2,000

and 200,000 asexual parasites/μL; axillary body temperature ≥37.5°C, or history of fever in the preceding 24hours; and haemoglobin ≥5.0g/dL and able to take oral medication. Patients with severe malaria (WHO 2012), mixed species malaria infection, any other significant concomitant illness, underlying disease, malnutrition, known allergy to any of the study drugs, a clear history of adequate antimalarial treatment with drugs in the previous 72 hours, or taking prophylaxis with drugs having antimalarial activity (such as cotrimoxazole for the prevention of *Pneumocisti jorovecii* pneumonia in children born to HIV+ women) were excluded. All cases excluded from the trial were referred to the hospital for diagnosis and treatment.

2.2.3 Trial design

This was an individually randomized, open label study comparing three fixed-dose oral artemisinin-based combination therapies: dihydroartemisinin-piperaquine (DHA-PQ), artemether–lumefantrine (AL) and amodiaquine-artesunate (AQAS).

2.2.4 Treatment

Patients were randomly allocated to receive one of the three study treatments.

AQAS (Sanofi, Kenya) was administered once a day for 3 days according to body weight at a mean dosage of 3.8 mg/kg/day of artesunate and 10.2 mg/kg/day of amodiaquine. Three types of fixed-dose tablets were used, containing artesunate 25 mg, 50 mg or 100 mg, plus amodiaquine 67.5 mg, 135 mg or 270 mg. Tablets were administered according to the manufacturer's instructions: 4.5 -8.9 kg, 1 tablet 25/67.5; 9 - 17.9 kg, 1 tablet 50/135; 18-35.9 kg. 2 tablets 100/270.

DHA-PQ (DARTEPP, Guilin Pharmaceutical China; 40 mg dihydroartemisinin and 320 mg piperaquine each tablet) was administered once a day for 3 days according to

body weight with the following scheme: 5 - 7.9 kg, ½ tablet; 8-9.9 kg, 0.75 tablet; 10-14.9 kg, 1 tablet; 15-20.9 kg, 1.5 tablets and 21-29.9 kg, 2.0 tablets. This dosage scheme was different from the one recommended by the manufacturer that we used at the beginning of the study (5 - 10 kg, ½ tablet; 11-20 kg, 1 tablet; and 21-35 kg, 2.0 tablets). The decision was taken following a high number of failures in this arm at the beginning of the study. It was observed that because there were only 3 age groups, children in the upper part of each interval were slightly under-dosed. These patients were receiving less than 2.5 mg/kg of DHA and less than 18 mg/kg piperaquine when the optimal dosage is 4 mg/kg (range 2-10 mg/kg) for the former and18 mg/kg (range 16 – 26 mg/kg) for the latter (WHO 2010c, 2015a; WWARN DP Study Group 2013). The adjustment consisted of using 5 intervals of weight instead of 3 to improve the therapeutic dose of dihydroartemisinin and piperaquine at the upper limit of the range for each interval; the dosage scheme was then more homogenous (mg per kg) across groups.

The mean dosage was 3.3 mg/kg/day of dihydroartemisinin and 26.6 mg/kg/day of piperaquine.

AL (Coartem^{TM}, Novartis, Switzerland) was administered in 6 doses over 3days (0, 8, 24, 36, 48, and 60 hours). Each tablet contained 20mg artemether and 120mg lumefantrine and the mean dose was 2.0mg/kg of artemether and12.7mg/kg of lumefantrine for each dose. Tablets were administered according to the manufacturer's instructions: 5-14.9 kg, 1 tablet; 15-24.9 kg, 2 tablets; 25-34.9 kg, 3 tablets; >35 kg, 4 tablets.

Tablets were administered under medical supervision and with 100 mL of milk. Patients were observed for 1 hour after drug ingestion. The full dose was repeated if the patient vomited within 30 minutes and half-dose if the patient vomited within 1 hour.

Patients who failed treatment were treated with IV quinine if severe (20 mg salt/kg body weight loading dose followed by 10 mg/kg 8 hourly) or oral quinine if uncomplicated (10 mg/kg three times daily for seven days) according to national policy. Parenteral artesunate was not available at the time this study was carried out.

All children were hospitalized for 3 days and followed-up actively once a week for 42 days after treatment. Caregivers were invited to come back to the centre or contact the study nurse in case the child was unwell. If the patient did not report for the scheduled visits, every effort was made to locate the mother at the home address. At each visit the medical history, clinical signs and symptoms, body temperature and a blood sample for parasitaemia were collected.

2.2.5 Sample size

Previously published data showed that AL efficacy by day 28 PCR-corrected was 97.9% in 2007 (Tshefu *et al.* 2010). This drug combination was however at that time already available on the market produced from a local manufacturer and imported from India.

For AQAS, there was a large variability in reported PCR-corrected efficacy at day 28, varying from 85% to 100% (DRC-NMCP 2005; Bonnet *et al.* 2009; Sinou *et al.* 2009; Ndeba *et al.* 2012).

No data were available for DHA-PQ, which was expected to be very effective. For the calculation of the sample size I assumed a cure rate of 95% with AL, 99% with DHA-PQ and 85% with AQAS. A sample size of 621 patients would have been adequate to detect a significant difference of 10% between the standard treatment (AQAS) and AL or DHA-PQ at the 5% level and with 90% power. The sample size was increased by 10% to allow for loss to follow-up and the final sample size was 684 patients.

2.2.6 Randomization, sequence generation, type, allocation concealment mechanism, implementation

The randomization sequence, in blocks of fifteen, was computer-generated and numerically sequenced. Opaque envelopes containing the study drug name were prepared at the Mahidol Oxford Tropical Medicine Research Unit (MORU) in Bangkok. Patients were enrolled by the study physician and assigned to treatment by the study nurse who opened the next consecutively numbered envelope. Once an envelope was opened, the patient was considered included in the study.

2.2.7 Outcome measurements

The primary outcome measure was the PCR-corrected cure rate at day 42.

Secondary outcome measures were parasite and fever clearance and occurrence of adverse events (AE). Treatment outcome was established according to standard WHO classification (WHO 2009). Early treatment failure (ETF) was defined as: (1) danger signs or severe malaria on day 1, 2 or 3, in the presence of parasitaemia; (2) parasitaemia on day 2 higher than on day 0, irrespective of axillary temperature; (3) parasitaemia on day 3 with axillary temperature ≥ 37.5 °C; and (4) parasitaemia on day 3 ≥ 25% of count on day 0. Late clinical failure (LCF) was defined as: (1) danger signs or severe malaria in the presence of parasitaemia on any day between day 4 and day 42 in patients who did not previously meet any of the criteria of early treatment failure; (2) axillary temperature ≥ 37.5 °C in the presence of parasitaemia on any day between day 4 and day 42 in patients who did not previously meet any of the criteria of early treatment failure. Late parasitological failure (LPF) was defined as presence of parasitaemia between day 7 and day 42 with a temperature < 37.5°C in patients who did not previously meet any of the criteria of early treatment failure or late clinical

failure. Adequate clinical and parasitological response (ACPR) was defined as absence of parasitaemia on day 42, irrespective of axillary temperature, in patients who did not previously meet any of the criteria of early treatment failure, late clinical failure or late parasitological failure.

Safety reporting was performed according to ICH Harmonized Tripartite Guideline for Good Clinical Practice (ICH-GCP 1996).

2.2.8 Laboratory Methods

Asexual and sexual malaria parasites were identified and counted on Giemsa-stained thick films and reported per 200 WBC, assuming a total WBC count of 8,000/μL (Trape 1985; Greenwood and Armstrong 1991). Slides were declared negative after examination of at least 100 high-power microscopy fields. Parasite species was determined on the thin film. The laboratory technicians were blinded to the treatment received by individual patients. The blood film prepared during the screening was considered the admission slide.

Blood films were prepared at baseline, 6 and 12 hours and then repeated every 12 hours until 2 consecutive negative blood films were observed. Parasite clearance was assessed i) as the time for the parasite count to reduce to 50% of its initial value (PC50) and ii) as parasite clearance rate derived from the log-linear section of the log parasitaemia–time curve and expressed as the parasite clearance half-life ($PCt_{½}$; $\log_e 2$/ parasite clearance rate).

To compare the $PCt_{½}$ measured in this study with the more recent data collected in 2013 during the Tracking Resistance to Artemisinin Collaboration (TRAC) project, all slides were read a second time after the study was terminated by the same microscopists using a different counting technique: if more than 20 parasites were seen

on the thick smear after 10 fields, parasitaemia per 1,000 RBC was counted on the thin smear. Below that threshold, parasites were counted on the thick smear per 500 WBC.

Indeed, below that threshold, the parasitaemia is considered low. In the thick smear constituted of several layers of hemolyzed red blood cells, parasites are concentrated in a small surface compared to that of the thin smear. Thus in low parasitaemia, the reader can quickly screen a larger number of RBC to look for the presence of parasites and then more easily estimate the parasitaemia; compared to the thin smear which is constituted of a monolayer of widely spread red blood cells. Here, in case of a low parasitaemia, the work of the reader will be fastidious as a much larger area will be needed to be examined to count the red blood cells for the presence of parasite and parasitaemia estimation (Moody 2002; Murphy *et al.* 2013).

Haemoglobin was measured on admission using a portable photometer (HemoCue Hb201+, Angelholm, Sweden). Thereafter the haematocrit was measure at baseline, daily during the hospitalization and at days 7 and 14 of the follow-up by micro-haematocrit centrifugation (Hawksley Haematospin 1400, Hawksley&Sons, Ltd.UK).

Total and differential WBC counts were assessed daily during hospitalization and at day 7 and 14 of follow-up using a Sysmex® automated haematology analyser.

Liver function tests, aspartate amino transferase (AST), alanine amino transferase (ALT) and creatinine were measured from plasma at the hospital laboratories (SEAC-Screenmaster) at baseline and 48 hours.

A dried blood spot (DBS) was prepared at admission, daily during the hospitalization and at each follow-up visit and stored for molecular analysis.

2.2.9 Drug analysis

DHA-PQ tablets selected randomly were analysed for content and quality at the Department of Pharmacology of MORU, and 2 mL of venous blood were taken at day 7 from 246 consecutive patients to measure plasma concentrations of lumefantrine and piperaquine.

2.2.10 Molecular analysis

Paired filter paper samples from enrolment and the day of treatment failure, i.e. the follow-up day on which parasites were detected by microscopy, were analysed at Sokhlo Malaria Research Unit (SMRU) in Thailand to distinguish between recrudescence and re-infection. Briefly parasite DNA was purified (QiaAmp DNA Micro kit; Qiagen, UK) and the polymorphic markers MSP-1 and MSP-2 and GLURP were genotyped. A recrudescent infection was defined as one that matched in size at least one allele of each marker between the first and second samples. If any pair of alleles of a polyclonal primary infection was detected during a second episode, this was considered a recrudescence.

2.2.11 Ethical approval

The study was approved by the Oxford University Research Ethic Committee (OXTREC), the Institutional Review Board of Kinshasa School of Public Health (KSPH) and the Ministry of Public Health of the DRC. A verbal consent was obtained from caregivers before screening children for malaria and anaemia. A written consent form was obtained from care takers whose children ful fille dall inclusion criteria before enrolling the patient in the study.

The study was monitored regularly by a qualified internal monitor (MORU Clinical Trials Support Group) for adherence to GCP regulations.

The study was registered with the International Standard Randomized Controlled Trial Number Register (www.isrctn.org), ISRCTN 20984426.

2.2.12 Statistical analysis

Data were double entered in Microsoft Access 2007 and validated using Epi Info 6.4b (CDC, Atlanta, GA, USA).

Statistical analyses were performed using STATA v.11 (College Station, TX, USA: Stata Corp LP). Descriptive statistics were used to summarize demographic data and baseline values. For the per protocol analysis, χ^2 was used to compare proportions. ANOVA was used for normally distributed continuous data and the non-parametric Kruskal-Wallis test to analyse continuous data with a non-normal distribution.

For the intention-to-treat analysis, the logrank test was used to test the equality of the survivor function across groups and Cox regression to estimate hazard ratio of infections post-treatment.

The overall fractional reduction in haematocrit was defined as the difference between the patient's lowest level of haematocrit and that at baseline (i.e., pre-treatment) divided by the haematocrit at baseline.The percentage of patients whose haematocrit fell > 20% or 25% was compared between groups.

2.3 Results

2.3.1 Intention to treat analysis, deviations from study protocol

Between September 2011 and November 2012, 684 patients were included in the study, 228 in each treatment group. Forty-two patients (6.1%) discontinued the study: 5 children were withdrawn during the hospitalization because the families changed their mind and 37 were lost to follow-up between day 7 and 42 (DHA-PQ=16; AL=10; AQAS=16). One patient, in the AQAS group, died at day 29 from causes unrelated to malaria or the study drug. These cases were not included in the per-protocol analysis (PPA), and they were censored on the last day they were visited by the doctor and tested for malaria in the intention to treat analysis (ITT). The flow of patients through the study is outlined in the patient flow diagram (Figure 2.2).

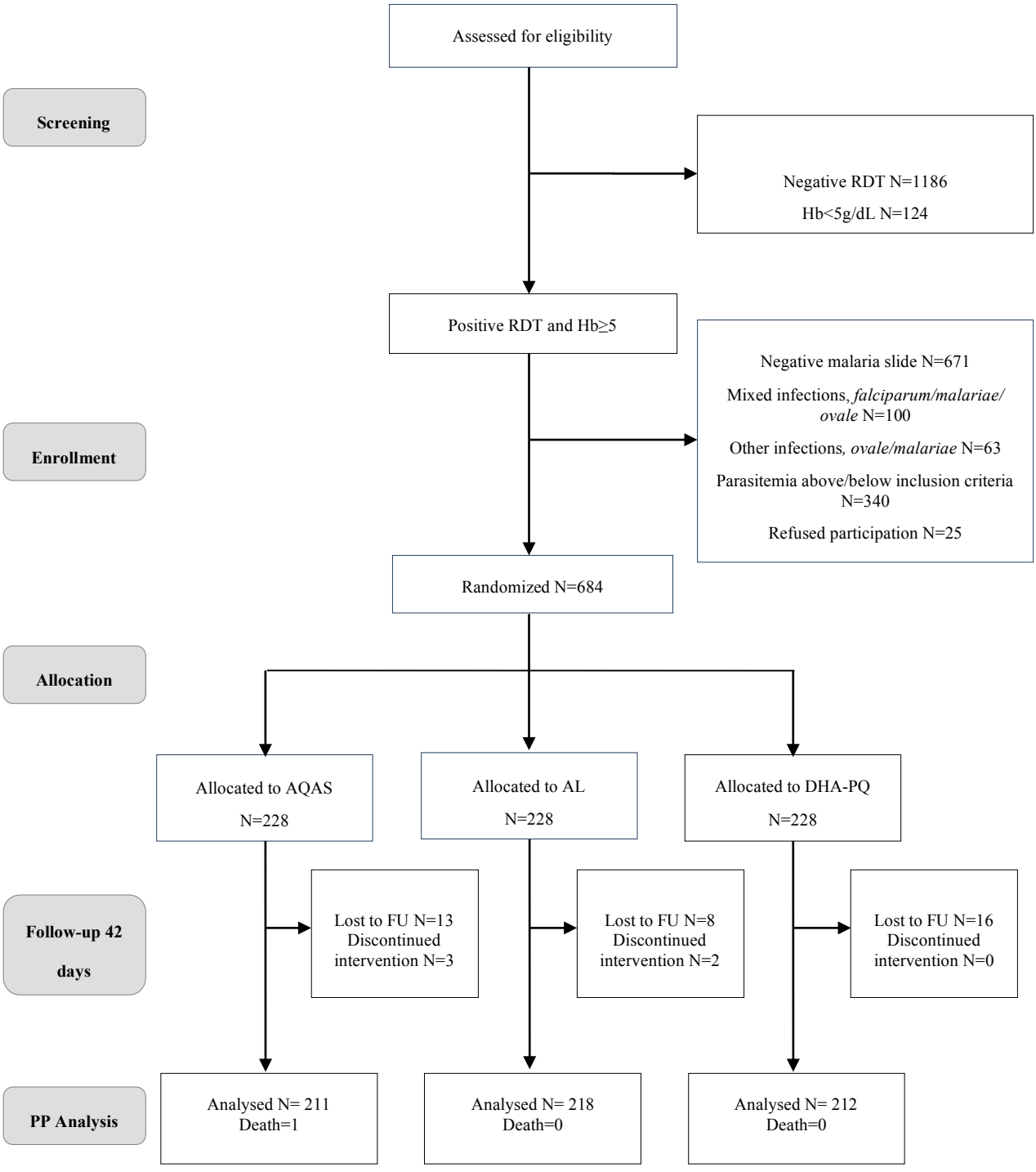


Figure 2.2. CONSORT Flow chart

2.3.2 Baseline Characteristics and treatment

At enrolment, patients had similar demographic, clinical and parasitological characteristics (Table 2.1). The tablets of DHA-PQ contained an average of 35.6 mg dihydroartemisinin (89%) and 306 mg piperaquine (95.5%). This was compared to Eurartesim (Sigma Tau) product, which contained an average of 40.7 mg dihydroartemisinin (102%) and 300 mg piperaquine (94%).

Table 2.1. Baseline characteristics of children at enrolment by treatment group

	AQAS	AL	DHA-PQ
Number of patients at admission	228	228	228
Female:Male ratio	115:113	105:123	105:123
Mean age in months (range)	35.3 (3 - 59)	33.5 (5-59)	33.7 (5-59)
Mean weight in kg (95% CI)	12.5 (12.1- 12.8)	12.5 (12.1-12.9)	12.3 (11.9- 12.7)
Axillary temperature °C (median, range)	37.5 (36.0-40.5)	37.2 (36.0-40.8)	37.1 (36.0-40.2)
Splenomegaly, %	72/226 (31.9)	84/228 (36.8)	88/228 (38.6)
Hepatomegaly, %	3/228 (1.3)	1/228 (0.44)	0/228
P. falciparum/µL Median (range)	30,066 (2,093-199,840)	30,119 (2,040-199,720)	35,207 (2,126-199,960)
P. falciparum/µL Geometric mean (95% CI)	25,179 (21,188-29,921)	25,681 (21,828-30,216)	30,403 (25,657-36,026)
Patients with >150,000/µL (%)	21/228 (9.2)	14/228 (6.1)	29/228 (12.7)
Mean haemoglobin g/dL (95%CI)	9.7 (9.4- 9.9)	9.7 (9.5-10.0)	9.6 (9.4- 9.8)

2.3.3 Drug efficacy

2.3.3.1 Per protocol analysis

The cure rate at day 42 (primary outcome), PCR uncorrected, was similar in patients treated with AQAS (73.5%) or AL (70.6%), whereas it was significantly higher in patients treated with DHA-PQ (86.8%) (p=0.001;Table 2.2). In the follow-up period, 145 children were diagnosed with a second episode of malaria (starting as early as day 16); most of these cases were new infections and only 30 (21%) were confirmed by PCR as recrudescent infections. Among the new infections there were 12 cases of *P. malariae* and 1 *P. ovale*. For 9 patients the PCR was unsuccessful; as we could not ascertain if these 9 cases were new or recrudescent infections we excluded them from the PCR-corrected analysis. After correcting the results for the new infections, the cure rates were comparable in the three groups: 93.4% for AQAS (95% CI 89.1% - 96.3%), 92.7% for AL (95% CI 88.4% - 95.7%), and 94.3% for DHA-PQ (95% CI 90.3% - 97.0%), p=0.76.

Table 2.2. Per Protocol Analysis: Efficacy by treatment at day 42 (%)

Outcome	AQAS	AL	DHA-PQ	p-value
Total allocated to treatment	228	228	228	
Withdrawn or lost to follow-up by day 42	16	10	16	
Death	1	0	0	
Evaluable	211	218	212	
Results PCR uncorrected				
Early Treatment Failure	1 (0.47)	1 (0.46)	1 (0.47)	
Late Clinical Failure	17 (8.1)	12 (5.5)	10 (4.7)	
Late Parasitological Failure	39 (18.5)	52 (23.9)	18 (8.5)	
Adequate Clinical and Parasitological Response	154 (73.0)	153 (70.2)	183 (86.3)	0.001
PCR results on recurrent episodes				
New infections *P. falciparum*	41	39	16	
New infections P. malariae /P. ovale	2	10	1	
Recrudescences *P. falciparum*	10	11	9	
Undetermined PCR result*	3	4	2	
Results PCR corrected				
Adequate Clinical and Parasitological Response	197 (93.4)	202 (92.7)	200 (94.3)	0.78

(* the samples were collected, but the PCR results were undetermined: cases were excluded from the PCR-corrected analysis)

Early treatment failure occurred in three patients (0.5%), one in each arm. Data are reported also using day 28 cure rates as the endpoint (Table 2.3).

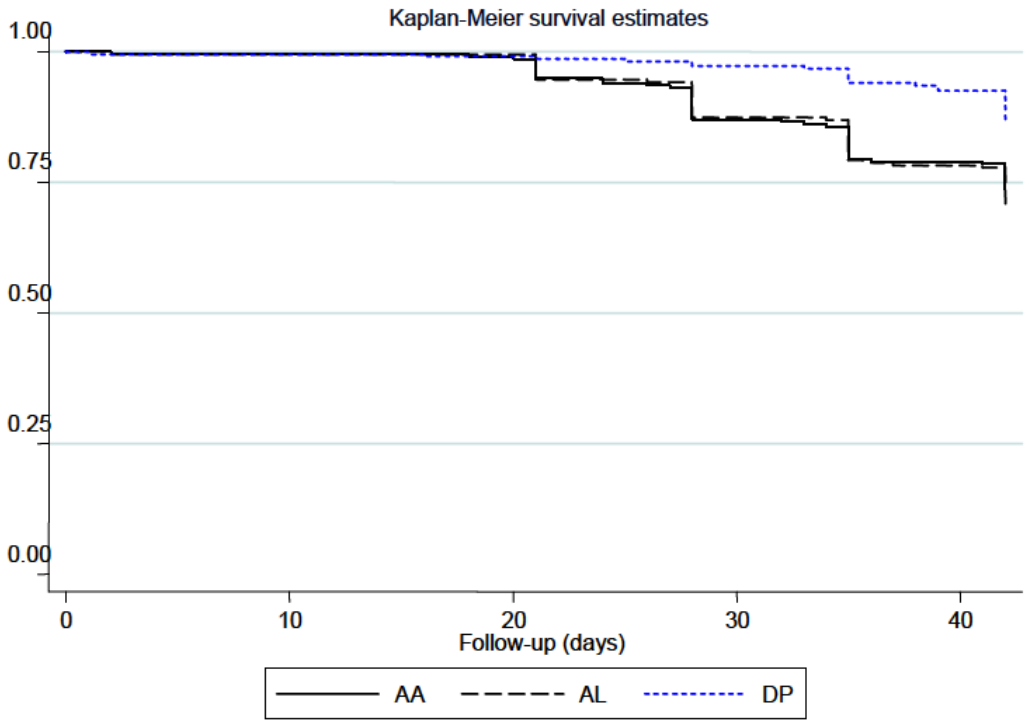
Table 2.3. Per Protocol Analysis: Efficacy by treatment at day 28 (%)

Outcome	AQAS	AL	DHA-PQ	p-value
Follow-up not completed by day 28	12	5	10	
Results PCR uncorrected				
Adequate Clinical and Parasitological Response	183 (86.7)	190 (87.1)	206 (97.2)	0.001
Results PCR corrected				
Adequate Clinical and Parasitological Response	207 (98.1)	211 (96.8)	208 (98.1)	0.58

2.3.3.2 Intention to Treat Analysis

The ITT analysis showed similar results (logrank test for equality of survivor functions PCR-uncorrected χ^2 =18.83, p = 0.0001 and PCR-corrected χ^2 =1.00, p=0.61; Figure 2.3). The risk (hazard ratio) of having a second episode of malaria (either new or recurrent) in the follow-up period was 1.5 times higher in the AQAS arm and 2.4 higher in the AL arm compared to the DHA-PQ arm (p>0.0001).The results were not affected by age or initial parasitaemia.

a)



b)

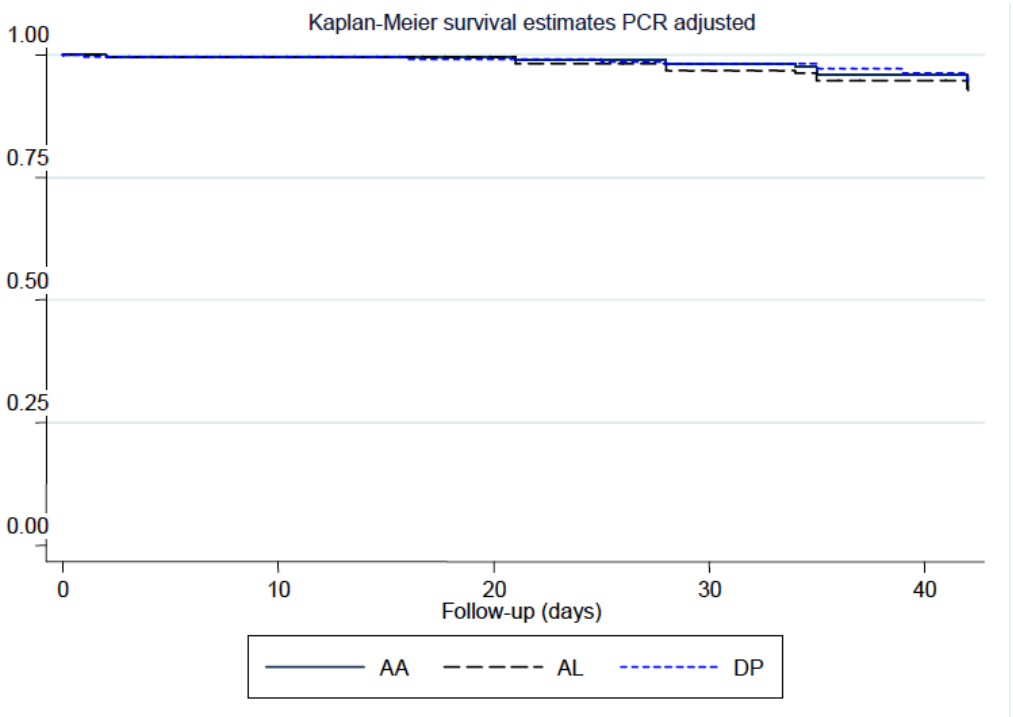


Figure 2.3. Intention to Treat Analysis: Kaplan Meier plots of failure rates a) without and b) with PCR correction

2.3.4 Fever Clearance

On admission 26.8% of patients had fever (axillary temperature ≥ 37.5°C). For the other patients the parent or guardian reported a history of fever in the preceding 24 hours as the main reason for seeking a doctor at the health centre. After 24 hours, 97% of children were afebrile and there was a significantly higher proportion of children with fever in the AL group at day 2 (p = 0.003;Table 2.4).

Table 2.4. Fever clearance: percentage still febrile on day 0 to day 3 by treatment group

Time, days	AQAS	AL	DHA-PQ	p-value
	n/N (%)	n/N (%)	n/N (%)	
0	63/228 (27.6)	65/228 (28.5)	55/228 (24.1)	0.53
1	3/226 (1.3)	11/226 (4.9)	8/228 (3.5)	0.1
2	1/226 (0.4)	9/226 (4.0)	1/228 (0.4)	0.003
3	2/226 (0.9)	3/226 (1.3)	5/227 (2.2)	0.50

2.3.5 Parasitaemia Clearance

All treatments were associated with a rapid clearance of parasitaemia. The parasite positivity rate (proportion of children with a positive slide at day 2) was significantly higher in the AL arm (p<0.001;Figure 2.4). Accordingly, the median PC50 was significantly longer for AL (8.4 hours, range 0.2 to 23.9 hours, N=214) than for AQAS (5.7 hours, range 0.1 to 24.3 hours, N=204) and DHA-PQ (6.5 hours, range 0.1 to 34.4 hours, N=212), p<0.001 (Table 2.5). The median $PCt_{½}$ was 2.2 hours (range 1.0 to 6.3 hours, N=657) with no significant differences between arms indicating a similar efficacy of the three different artemisinin derivatives, p=0.08 (Table 2.6).

Table 2.5. Time (hours) to clear 50% of parasitaemia by treatment

	AQAS	AL	DHA-PQ	Total
Observations used for the estimation	204	214	212	630
Median	5.71	8.44	6.54	7.31
Range	0.09-24.26	0.18-23.85	0.08-34.40	0.08-34.40
IQR	5.39	5.64	5.12	5.63

Table 2.6. Slope half-life (based on the slope of the log-linear portion of the parasite clearance curve)

	AQAS	AL	DHA-PQ	Total
N.Observations used for the estimation	214	223	220	657
Median	2.15	2.23	2.13	2.18
Range	1.05 -4.35	1.05-6.32	0.97-4.85	0.97-6.32
IQR	0.87	0.73	0.76	0.82

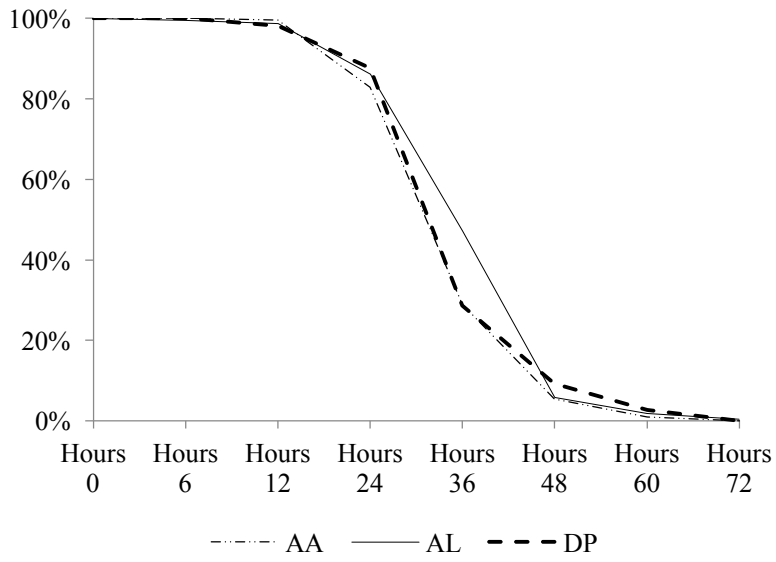


Figure 2.4.Parasite positivity rate by day and treatment group

2.3.6 Gametocytaemia

On admission 28.5% (N=195/684) of patients were gametocytaemic with no significant differences between groups. Treatment with AL resulted in lower gametocyte carriage rates than the other two treatments in the follow-up period, days 7-21 ($p<0.001$;Figure 2.5). In 7 children gametocytaemia was microscopically detectable from admission until day 35 (AQAS=4, DHA-PQ=2 and AL=1) and in 2 children treated with DHA-PQ until day 42. In a number of children gametocytes were not detected on admission blood smears, but became apparent in the first 72 hours of treatment with no significant differences between arms. New appearance of gametocytes was however uncommon from day 7 onward.

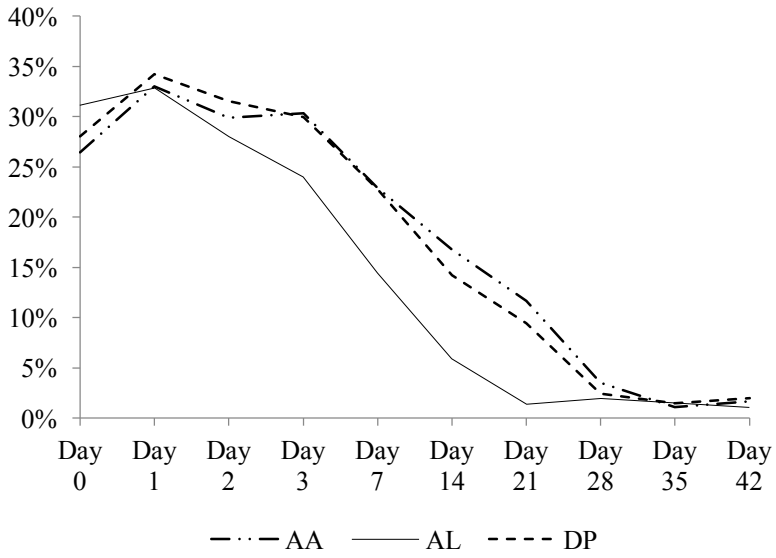


Figure 2.5 Gametocyte Positivity Rate by treatment days 0-42

2.3.7 Haematology

The mean Packed Cell Volume (PCV) at admission was 30.1% (95% CI 29.8%-30.5%; range 13%-42%). In the first week there was an overall mean fractional reduction in the PCV of 10% (SD 8.3) with no differences between arms (p=0.65; Table 2.8). Hyperparasitaemic children (≥ 150,000/μL) were those most affected, with a mean fractional reduction of 15.1% (95% CI 7.4-8.7) compared to 8.1% (95% CI 13.8-16.3) in those not hyperparasitaemic (p<0.001).

A reduction of >25% of the initial PCV value was observed in 14.9% of hyperparasitaemic children and 2.3% of non-hyperparasitaemic children (p<0.001).

Ten patients developed decompensated anaemia within 4 days of recruitment and required a blood transfusion; 3 in the AQAS group, 4 in the AL group and 3 in the DHA-PQ group (p=0.62). The risk of receiving a blood transfusion was 6.5 times higher in the hyperparasitaemic children (CI 95% 2.90 - 22.3; p=0.005). By day 14 the levels were comparable to admission in all patients.

The median WBC count was similar between the treatment groups on recruitment and at each day of follow-up with an increase from day 0 to day 7 to normal values (Table 2.7). Neutrophil counts decreased gradually from baseline values until day 14 with no differences between groups on any day. Mild neutropenia (neutrophils <1000/ μl) was observed in 2.5% of patients at enrolment and between day 1 and 7 the neutrophils count fell below 1,000/μl in 18% of patients (123/684) with no differences between groups (n=40 in AQAS, 40 in AL, and 43 in DHA-PQ; p=0.92). Fourteen of these patients developed severe neutropenia (neutrophils <500/μl) (2, 4, and 8 in the AQAS, AL and DHA-PQ groups, respectively; p=0.13).

Table 2.7. Median and interquartile range WBC μl^{-1} and differential count at days 0, 7 and 14 by treatment group

	AQAS			AL			DHA-PQ		
Day	0	7	14	0	7	14	0	7	14
N	227	216	212	228	221	217	228	219	215
WBC	6,800	8,200	7,000	6,650	7,600	6,900	6,875	7,600	7,700
IQR	4,000	4,475	3,075	4,050	4,000	2,900	5,050	3,750	3,700
Neutrophils	3,096	2,677	2,014	2,829	2,496	2,118	3,201	2,520	2,352
IQR	2,311	1,846	1,288	2,414	1,706	1,306	3,254	1,745	1,476
Lymphocytes	2,919	4,651	4,489	2,841	4,623	4,300	3,075	4,550	4,590
IQR	2,170	2,884	2,234	2,388	2,817	2,240	2,730	2,394	2,792

Table 2.8. Mean fractional reduction (SD) in PCV and number of patients whose reduction in PCV was > 20% or 25% compared to the value at admission by treatment group

		AQAS	AL	DHA-PQ	Total	p-value
Day 0-3	Mean % (SD)	10.0 (8.2)	9.0 (8.4)	9.3 (7.9)	9.4(8.1)	0.41
	>20%	12.4 (28/226)	11.8 (27/228)	9.7 (22/228)	11.3 (77/682)	0.62
	>25%	6.2 (14/226)	4.4 (10/228)	5.7 (13/228)	5.4 (37/682)	0.68
Day 0-7	Mean % (SD)	10.3 (8.2)	9.4 (8.4)	10.0 (8.2)	9.9 (8.3)	0.51
	>20%	12.8 (29/226)	11.8 (27/228)	12.7 (29/199)	12.5 (85/682)	0.94
	>25%	6.6 (15/226)	4.4 (10/228)	6.1 (14/228)	5.7 (39/682)	0.55
Day 0-14	Mean % (SD)	10.4 (8.2)	9.7 (8.7)	10.2 (8.2)	10.1(8.3)	0.65
	>20%	12.8 (29/226)	12.7 (29/228)	13.2 (30/228)	12.9 (88/682)	0.99
	>25%	6.6 (15/226)	4.8 (11/228)	6.6 (15/228)	6.0 (41/682)	0.65

2.3.8 Hepatotoxicity

Mean serum levels of aspartate aminotransferase (U/L), alanine aminotransferase (U/L) and creatinine (mg/dL) were similar at baseline among groups. With minor fluctuations (a trend toward a reduction for AST and ALT) the mean levels at day 2 remained similar to those of day 1 with no statistical differences between groups (data not shown).

2.3.9 Tolerability

During the first day, children treated with DHA-PQ vomited within 1 hour of the first dose significantly more often (N=21; 9.2%) than children treated with AQAS (N=10; 4.4%) or AL (N=5; 2.2%), p=0.03. The second dose of AL, administered after 8 hours, was vomited in seven cases, and taking this into account the overall difference between treatments during the first day was not significant (p=0.06). These children were all given a second dose of the drug. On the second day there was no difference in vomiting post-dose between children treated with AQAS (15; 6.6%) or DHA-PQ (17; 7.5%), whereas in the AL arm only 2 (0.9%) cases vomited after the first dose and none after the second dose (p=0.02).

2.3.10 Adverse Events

At least one adverse event was reported in 37.4% of patients during the post-treatment period that was not present on admission or increased in intensity and was classified as possibly or probably related to the study drug. Most AEs were graded as of minor or moderate intensity. The most frequent AEs were weakness, anorexia and gastrointestinal disorders (nausea, vomit, abdominal pain and diarrhoea). However these symptoms overlap with known malaria symptomatology. Anorexia and weakness were reported more frequently in children treated with AQAS than those treated with

DHA-PQ and AL during the second and third day of treatment. Day 1: 15.2% (34/224) for AQAS, 5.3% (12/227) for AL, and 5.8% (13/228 for DHA-PQ; p=0.0001. Day 2: 8.2% (17/207) for AQAS, 2.3% (5/220) for AL and 4.0% (9/224) for DHA-PQ; p=0.013. There were otherwise no other differences in the number of AEs between treatment groups. In 17 cases the adverse event was graded as severe or life-threatening. All cases were classified as unlikely to be related to the study treatment. Ten patients developed decompensated anaemia during the hospitalization and required a blood transfusion (described above). There was one case of severe skin eruption 18 days post-treatment (DHA-PQ), one case of chicken-pox 12 days post-treatment (AL), 1 case of abscess 11 days post-treatment (DHA-PQ), 1 case of leucocytosis 2 weeks post treatment (AL), and 3 cases of asthenia (2 AL and 1 AQAS). One child in the AQAS group died in a different hospital at day 29. The cause of death was unknown but was considered unlikely to have been caused by either malaria or the drug treatment.

2.3.11 Plasma lumefantrine levels at day 7

121 samples of venous blood were collected at day 7 from patients who received AL (Table 2.9). The median concentration of lumefantrine in the blood was 377 ng/mL (range 57.1-1150 ng/mL). Drug levels were positively correlated with body weight (r=0.22; test for trend p=0.005). The plasma level was significantly lower in children weighing < 15 kg (median 309 ng/mL; range 57.1-1080, N=87) compared to those weighing ≥ 15 kg (median473 ng/mL, range 108-1150, N=34, p=0.01). Accordingly, 43.7% of children weighing <15 kg had a plasma level ≤ 280 ng/mL considered the cut-off for therapeutic efficacy, (Ezzet and Karbwang 1998) compared to 20.6% in those weighing ≥ 15 kg (p=0.018). The 7 children in this sub-sample with a PCR-confirmed recrudescence had a median level of 429 ng/mL (range 147 to 703 ng/mL),

not significantly different from those who successfully cleared the infection (376 mg mL; range 57.1-1150, p=0.8, N=114).

Table 2.9. Lumefantrine (LM) plasma level at day 7

Body weight (kg)	Analyzed, N	Median (range) dose received mg/kg	Median day-7 LM ng/mL (range)	Samples ≤ 280 ng/mL
5-9.9	22	27.9 (24.5-45.3)	294.5 (63.4-1050)	45.5 %
10-14.9	65	20.0 (17.1-24.0)	364.0 (57.1-1080)	43.1 %
15-20.9	34	30.0 (24.0-32.0)	473 (108-1150)	20.6 %
Total	121	24.0 (17.1-45.3)	421.9 (57.1-1150)	37.2 %

2.3.12 Plasma piperaquine levels at day 7

125 samples were collected from venous blood at day 7 from patients who received DHA-PQ (Table 2.10). The median concentration of piperaquine was 31.4 ng/mL (range 10.9-189.0 ng/mL), and drug levels were positively correlated with body weight (r = 0.22; test for trend p=0.04). In 47.2% (59/125) of patients the plasma level was below 30 ng/mL, the previously published threshold associated with therapeutic efficacy (Price *et al.* 2007), and the 3 patients with a PCR-confirmed recrudescence had a piperaquine level of 16.3, 31.4 and 33.1 ng/mL, respectively.

Table 2.10. Piperaquine (PQ) plasma levels at day 7

Body weight (kg)	Analyzed, N	Median (range) dose received mg/kg	Median day-7 PQ ng/mL (range)	Samples ≤ 30 ng/mL
5-7.9	8	20.8 (20.3-33.3)	23.6 (10.9-65.2)	75 %
8-9.9	17	28.2 (17.2-30.0)	31.8 (16-70.8)	41.2%
10-14.9	65	26.7 (22.1-32.0)	28.1 (12.6-135)	52.3 %
15-20.9	35	30.0 (26.7.0-32.0)	44.3 (15.8-189)	34.3 %
Total	125	28.2 (17.2-33.3)	31.4 (10.9-189)	47.2%

2.4 Discussion

The efficacy of the three combination therapies tested in this trial was similar. The proportion of children with an Adequate Clinical and Parasitological Response was 93% for AL and 94% for DHA-PQ. These cure rates are similar to that of AQAS (93%), which has been extensively used in DRC since its introduction in 2006.

The PCR-corrected day 28 cure rate for AL in the present study was 96.8%, which is comparable to the 97.9% day 28 cure rate observed in the same area 3 years previously (Tshefu *et al.* 2010).

In the subsample analysed, the drug level of piperaquine at day 7, reflecting the concentration of drug to which residual parasites are exposed and thus predictive of outcome, was suboptimal in 47% of patients. These results are consistent with other results from paediatric patients data from Asia, Africa, and South America (Tarning, Zongo, *et al.* 2012; WWARN DP Study Group 2013) showing that as small children have a higher body-weight normalized oral clearance, they need a higher dose than the one currently recommended. Of the children who received artemether-lumefantrine

37% had a sub-optimal day 7 lumefantrine level, with the smaller children having the lowest levels. These results are comparable to those observed in Uganda (Checchi *et al.* 2006). As there was no evidence of delayed parasitaemia clearance suggestive of artemisinin resistance, the PCR-confirmed treatment failures observed in the current study are likely caused by either low drug exposure, as shown by the PK results, or to parasite resistance to the non-artemisinin partner drug. The former is much more likely. In high transmission areas, the chances that the recurrent infection contains a parasite with the same genotype as the primary infection are higher than in low transmission areas. This, along with the persistence of gametocytes can lead in some cases to a misclassification of recurrent infections as recrudescence (Greenhouse *et al.* 2007).

In this clinical trial we measured efficacy of treatments administered under supervision, with a glass of milk, and retreatment was given if the first dose was vomited. Drug exposure, in a non-trial setting (generally unsupervised) is expected to be lower (Checchi *et al.* 2006). Dose optimization and schedule changes are a priority for the ACT treatment especially in small children to ensure adequate drug exposure (Hodel *et al.* 2013; WWARN DP Study Group 2013).

Although the efficacy in terms of ACPR rates of the three ACTs was comparable, children treated with DHA-PQ were at lower risk of having a second episode of malaria during the follow-up period because of the longer post-treatment prophylactic effect of DHA-PQ related to the longer plasma half-life of piperaquine. This chemoprophylactic effect is important in endemic areas, such as the study area,and makes this drug a good candidate for replacing sulfadoxine-pyrimethamine for IPT treatment in pregnant women and children (Lwin *et al.* 2012; Nankabirwa *et al.* 2014).

This study population was characterized by hyperparasitaemia and the initial high levels of parasitaemia affected, as expected, the recovery of haematocrit after the initial

episode of malaria, but not the treatment efficacy. The initial level of gametocytaemia was also high (30%) and AL was significantly more effective in clearing the sexual stages compared to the other ACTs. Data in literature on the gametocytocidal properties of the different ACT are conflicting and the effect, if any, on malaria transmission is unclear (Price 2013).

The median $PCt_{½}$ was 2.2 hours (range 1.0 to 6.3 hours, N=657) and comparable to the results observed in 2013 during the TRAC project: 2.2 hours (range from 1.2 to 4.6 hours, N=60)(Ashley *et al.* 2014; WWARN Parasite Clearance Study Group 2015). The difference we observed between PC50 with the three therapies (but not with the $PCt_{½,}$) could be attributed to the relatively slow conversion of artemether to dihydroartemisinin (artemether half-life of 2.0 hours) compared to artesunate (artesunate half-life of 0.84 hours) and dihydroartemisinin in the acute phase of malaria (Suputtamongkol *et al.* 2001) resulting in a significantly longer lag-phase (the initial flat part of the parasite clearance profile) for AL and/or the lower dosage of artemether (Price 2013). The PC50 includes the lag phase of the parasite clearance curve, whereas the $PCt_{½}$ is based on the log-linear phase alone.

The three combinations were well tolerated and there were no significant differences in the number of adverse events between arms.

2.5 Conclusions

The three combinations tested were equally efficacious and well tolerated for the treatment of children with acute uncomplicated *falciparum* malaria.

Dihydroartemisinin-piperaquine had the longest lasting chemo-prophylactic effect which prevented repeated clinical attacks in the treated children. The recommended

dosage of DHA-PQ provides suboptimal piperaquine plasma concentrations in particular in small children.

3 Comparison of two regimens of artemether-lumefantrine (AL) for the treatment of uncomplicated *P. falciparum* malaria in pregnant women in DRC

3.1 Introduction

In Sub–Saharan Africa (SSA) malaria in pregnancy represents one of the major causes of morbidity and mortality for mother and newborn(Snow *et al.* 2001; Guyatt and Snow 2004; Hogan *et al.* 2010; WHO 2014c).While some countries are progressing toward the elimination ofmalaria, the majority are still struggling to control the disease (WHO 2013a) and in these areas almost all pregnancies can be considered at risk of malaria infection.

Effective antimalarials, used either for prevention or treatment of the disease, can significantly reduce malaria-related morbidity and mortality in the mother and the baby (WHO 2013a, 2014c; WHO and UNICEF 2013). However, the main concern is that antimalarials might be toxic to the fetus especially if the exposure takes place during the first trimester of pregnancy.For this reason pregnant women are often excluded from clinical trials, thus limited data are available on drug exposure and toxicity in this important population (Blehar *et al.* 2014).

Currently available data on the safety of some antimalarials (such as artemisinin derivatives, mefloquine, primaquine, doxycycline, azithromycin and atovaquone-proguanil) are not sufficient to exclude with certainty toxicity for the mother-fetus dyad. For instance, there are concerns that exposure to artemisinin derivatives in the

first trimester of pregnancy may increase the risk of fetal teratogenicity in human, as studies in animals have confirmed the embryotoxic effect of this class of drug when administered in early gestation (Xu and Zhang 1996; Clark *et al.* 2004, 2008; Longo *et al.* 2006; Dellicour *et al.* 2007; White and Clark 2008; Clark 2009). An association between mefloquine and stillbirth cannot be excluded with certainty, sulphonamide potentially increase the risk of kernicterus in fetus, and dapsone has been associated with neonatal hyperbilirubinemia (Phillips-Howard and Wood 1996; Nosten *et al.* 2006; Dellicour *et al.* 2007; Orton and Omari 2008).

The exclusion of pregnant women from clinical trials combined with the lack of adverse events (AEs) reporting system prevents further data collection on drug safety and efficacy in this vulnerable group.

Another under-evaluated but important aspect of using antimalarials in pregnancy is a possible alteration of drug pharmacokinetics (PK) caused by changes in maternal physiology (Dawes 2001; Frederiksen 2001). The haemodynamic and hormonal changes which characterise pregnancy may alter the drug absorption, distribution and elimination..As the volume of plasma increases steadily,this affects drug distribution in the body as well as the concentration of plasma proteins and therefore the binding of the drug (Notarianni 1990). The increase in glomerular filtration rate may increase drug excretion (Little 1999) and hormonal changes,particularly of oestrogens and progesterone, might also increase or decrease the activity of important enzymes linked to drug metabolism such as cytochrome CYP3A4, as well as affect gastro intestinal absorption (Little 1999; Blehar *et al.* 2014; Costantine 2014).

As data on the efficacy and safety of drugs in pregnancy are scarce, pregnant women when ill are treated with the same dose as non–pregnant adults. However taking into

account the physiological changes described above, it is likely that the standard adult dose is incorrect.

An inadequate antimalarial treatment dose will lead to under treatment of the infection in the patient, potentially exposing the mother and the fetus to sub-patent chronic infections.Women living in malaria endemic areas with moderate to high transmission maintain some level of immunity in pregnancy compared to those living in low transmission settings or epidemic areas.Therefore , the standard adult dose might still be effective in high transmission areas, as the immune system can assist in eliminating the infection (Piola *et al.* 2010). However in low transmission settings, including all areas leading toward malaria elimination, the standard adult dose is unlikely to be sufficient. Moreover, exposure to sub-therapeutic drug concentrations can select parasites for drug resistance (Barnes *et al.* 2008; White *et al.* 2009).

Artemisinin-based combination therapies (ACTs) are highly effective antimalarial drugs and since 2006 their use has been authorized in the second and third trimesters of pregnancy (WHO 2006).Artemether-lumefantrine (AL) is the most widely available, with the most available data on its use (Mosha, Mazuguni, *et al.* 2014; WHO 2015a).This ACT has been proven to be highly effective in treating *P.falciparum* malaria and is safe to use in the last two trimesters of pregnancy (McGready *et al.* 2011).

The currently recommended standard AL regimen consists of 6 doses administered over three days (at 0, 8, 24, 36, 48 and 60 hours). In children the target dose is administered according to age or body weight, whereas above 34 kilograms of body weight (in all adults) the same target dosage is given to all patients, corresponding to the maximum of 4 tablets per dose.

The two drug components, artemether and lumefantrine, have a complementary antimalarial action. Artemether absorption is fast, reaching its peak in approximately two hours. It is rapidly hydrolysed to its main active metabolite dihydroartemisinin (DHA). Both components have high antimalarial activity and reduce the asexual stage parasite biomass by approximately 10,000–fold per reproductive parasite cycle resolving consequently many clinical symptoms of malaria (Ezzet and Karbwang 1998; White *et al.* 1999). Lumefantrine is on the contrary slowly absorbed and slowly eliminated. By staying longer in the body, it clears the residual parasites thus preventing a recrudescence of the infection(White *et al.* 1999; Price *et al.* 2006).

In terms of efficacy, the lumefantrine plasma concentration at day 7 is currently considered the best proxy parameter for the area under the curve (AUC) which determines AL therapeutic response. In both pregnant and non–pregnant patients, a concentration of lumefantrine at day 7 below 280 ng/ml has been associated with an increased risk of treatment failure (Ezzet *et al.* 2000; Price *et al.* 2006; McGready *et al.* 2008; Tarning *et al.* 2009).

This cut-off was established as a result of a clinical trial where the previously recommended shorter 4-dose regimen of AL was administered. This consisted of a dose twice daily for 2 days (at 0, 8, 24 and 36 hours) After the 4-dose regimen it was found that 75% of patients with a plasma concentration of lumefantrine above the 280 ng/ml cut-off seven days after the start of drug administration were cured, whereas only half of those with a concentration below the cut-off were cured (Ezzet *et al.* 2000).

The lumefantrine level of 280 ng/ml was suggested to be a concentration close to its in vivo Minimal Inhibitory Concentration (MIC). The MIC is defined as the blood or plasma concentration of antimalarial at which the parasite multiplication factor per asexual cycle = 1(White 1997).In other terms, to effect cure, the drug concentration in

blood has to exceed the MIC for the infecting parasites in each successive cycle until either the parasites are all eradicated or sufficiently reduced to be eradicated by the host immune response (White 1997) .

Previous pharmacokinetic (PK) studies conducted in Asia showed that using the current standard 3-day treatment in pregnancy is associated with a reduced plasma concentration of artemether, DHA and lumefantrine and a faster elimination (clearance) of lumefantrine (McGready, Stepniewska, Lindegardh, *et al.* 2006), suggesting that the current standard regimen used for treatment in adults might not be sufficient to cure malaria in pregnancy.

The same altered PK parameters of artemether, DHA and lumefantrine described in pregnant Asian women were confirmed in the few studies conducted in pregnant African women (Piola *et al.* 2010; Tarning, Kloprogge, *et al.* 2012; Kloprogge *et al.* 2013; Mosha, Guidi, *et al.* 2014).

This raises the question of efficacy using the current 6-dose AL regimen to cure malaria in pregnancy. Population-based simulations suggested an increase in dose to achieve an adequate AL exposure in these patients (Tarning *et al.* 2009). However, at the current standard dosage of 6 doses twice daily over 3 days, the absorption of lumefantrine has reached its maximum and increasing the dose of AL per intake (i.e. administering more tablets at each time point) does not result necessarily in an increase in the exposure to lumefantrine (Ashley, Stepniewska, Lindegardh, *et al.* 2007). Thus,in the light ofthe dose–dependent oral absorption property of lumefantrine, extending the drug administration duration, rather than increasing the amount of drug per intake, is expected to be more effective (Ashley, Stepniewska, Lindegardh, *et al.* 2007). Mathematical simulations showed that an extended 5-day regimen would theoretically provide a plasma concentration of artemether and DHA able to cover three

consecutive asexual parasite cycles,compared to the current 3-day standard regimen covering only two consecutive asexual parasite cycle (Tarning *et al.* 2009).The proposed extended regimen would also lead to an increased plasma concentration of lumefantrine and it is likely to extend the time window for the drug level to be above the MIC (White *et al.* 1999; Tarning *et al.* 2009; Stover *et al.* 2012).

In this study I assessed the pharmacokinetics of a longer regimen of artemether-lumefantrine, 10 doses of artemether-lumefantrine over five days compared to the standard regimen, 6 doses of artemether-lumefantrine over three days, in a group of pregnant African women with uncomplicated *P. falciparum* malaria. This longer regimen was expected to improve the exposure to artemether, DHA and lumefantrine, and ensure that the curative plasma concentration of lumefantrine was sustained for a longer period of time.

Lumefantrine is a racemic fluorine derivative structurally similar to the aryl-amino alcohol group of antimalarials (mefloquine, quinine and halofantrine). These drugs are known to have adverse cardiovascular effects at therapeutic doses by prolonging ventricular repolarization which is reflected in the prolongation of the QTc interval (White 2007). Artemether as well has been reported to prolong the QTc interval when administered intramuscularly at high doses in dogs (Classen *et al.* 1999; Maude *et al.* 2009).However, evidence from large clinical trials showed that at standard dose, artemether-lumefantrine is safe and does not induce significant alteration of electrocardiograph such as a prolongation of the QTc interval in humans (Brewer *et al.* 1994; van Vugt *et al.* 1999). Increasing the dose of the combination AL however raises concerns about the potential cardiotoxicity in the exposed patients. It was therefore critical in our study, where a longer AL regimen than the one approved was

administered, to monitor the potential changes in the electrocardiographic prolongation of QTc intervals and ventricular arrhythmias in patients treated with the two regimens.

3.2 Methodology

3.2.1 Study area

The study area is described elsewhere in section 2.2 of chapter 2.

3.2.2 Patient population

Pregnant and non-pregnant women aged 18 to 45 years who presented to the research clinic through the community or via the antenatal care service of Kingasani maternity were screened and enrolled in the study if they met the following criteria: *P. falciparum* mono infection with a parasite count between 100 and 200,000 parasites/µL, haematocrit ≥21%, negative HIV test, willingness to stay in hospital for 3 to 5 days and comply with the follow-up. Pregnant women were included if they had a singleton viable fetus of Gestational Age (GA) ≥ 14 weeks confirmed by ultrasound.A negative pregnancy test was required for non-pregnant women. All enrolled women provided a written informed consent.

Women with severe malaria, a medical condition requiring concomitant drug treatment or transfer to a different hospital, an intake of artemether-lumefantrine within the two previous weeks, a known allergy to the study drug, signs of labour or a previous participation in the current trial or other studies were excluded. All women excluded from our study were referred to Kingasani Maternity Hospital for further diagnosis and treatment.

3.2.3 Aim

The primary aim of the research was to assess the pharmacokinetics of an extended regimen of artemether–lumefantrine and to compare it with the standard regimen in a group of pregnant women with uncomplicated *falciparum* malaria.

The study proposed to compare a 10-dose regimen over 5 days with the current standard 6-dose regimen over 3 days. A group of non-pregnant women treated with the two regimens represented the control. In order to assess the optimum therapy regimen data were also collected on efficacy, tolerability and safety according to WHO criteria (WHO 2009).

3.2.4 Trial design

This was an open label, two-arm, individually randomized controlled clinical trial.

There were 3 groups of women (pregnant 2nd trimester; pregnant 3rd trimester and non-pregnant) and 6 blocks (block 1: 2nd trimester, 3 day treatment; block 2: 2nd trimester, 5 day treatment; block 3: 3rd trimester, 3 day treatment; block 4: 3rd trimester, 5 day treatment; block 5: non-pregnant, 3 day treatment and block 6:non-pregnant, 5 day treatment).

The randomization lists were computer generated by an independent statistician at MORU with sampling time points randomized within each block to ensure an even distribution of random samples within each sub group. An equal number of women in their second or third trimester were chosen to avoid bias in the PK parameters measurement as the exposure to drug might change with the gestational age.

The sealed opaque brown envelopes containing the treatment regimen allocation and the PK sampling time list were prepared in Kinshasa by a person independent of the study.

The envelopes were numerically sequenced with consecutive numbers for each group. Eligible patients were randomly assigned to the 3-day or 5-day treatment regimen by study nurse and enrolled by the study doctor. The envelope was opened after a full written consent was obtained. Once an envelope was opened, the patient was considered included in the study.

All patients were followed-up once weekly for 6 weeks. They were invited to come back at the centre any time if they had complains or felt unwell. Treatment efficacy was measured according to the WHO 2009 classification (WHO 2009).

Patients who had a recurrent episode of malaria during the 6 weeks of follow-up were treated with oral quinine at a dose of 10 mg/kg three times daily for 7 days according to DRC National Guidelines. After completion of the 6 weeks follow-up, pregnant women were passively followed-up until delivery. Pregnant women who delivered before the end of the follow-up period were visited at home for the remaining visits. Those who experienced a new episode of malaria during that period were also treated with oral quinine.

We monitored patients for possible adverse events from enrolment to the end of the follow up period (42 days). Clinical symptoms were collected daily during hospitalization and at each follow-up visit. Liver and Renal Functions Tests were measured at baseline (time 0) and at the end of the treatment i.e. at 48 hours in the 3-day arm and at 96 hours in the 5-day one. An electrocardiogram was performed at the same time points.

Five additional visits after this follow up period were conducted in the pregnant group - at delivery, at 1, 3, 6 and 12 months - to assess the development of the baby.

3.2.5 Study Outcomes

The primary outcome was the characterization of drug plasma concentration profiles (pharmacokinetics) of lumefantrine, artemether and dihydroartemisinin.

The secondary outcomes were (i) to measure the tolerability and safety of the new proposed regimen through the detection and assessment of possible adverse events during the treatment and post-treatment; (ii) to assess the therapeutic efficacy assessment according to WHO protocol for the evaluation of antimalarial efficacy and (iii) to assess the development of children born from women enrolled in the study during the first year of life according to Denver developmental milestones (Frankenburg 1992).

3.2.6 Study endpoints

The primary and secondary endpoints of the study are listed below.

3.2.6.1 Primary endpoints

The PK parameters were measured for each patient according to a sampling schedule based on optimal design theory (Simpson *et al.* 2009). Ten samples for each patient were taken. Three fixed samples, at day 0 before starting the treatment, day 7 and 14 and seven sparse samples taken at random within the following time windows: 0-3 hrs, 3-6 hrs, 6-12 hrs, 12-60 hrs, 60-72 hrs, 72-144 and 192-336 hrs. The following parameters were measured:

1. Maximum concentration (Cmax) defined as the maximum plasma or serum concentration after administration of the drug;

2. Time to maximum concentration (Tmax) defined as the time to reach the Cmax;
3. The total area under the concentration-time curve from time zero to infinity (AUC0-∞) corresponding to the integral of the plasma concentration *versus* time, which allows the evaluation of total exposure to a drug;
4. Total area under the concentration-time curve from time zero to the last observed time ($AUC_{0\text{-last}}$);
5. Terminal elimination half-life (t1/2) defined as the time require for the plasma concentration of drug to decrease by 50% in the final stage of its elimination. It is estimated by the slope of the best-fit log linear regression of the observed concentrations in the terminal elimination phase;
6. Oral elimination clearance (CL/F) defined as the apparent total plasma or serum clearance of drug after oral administration;
7. Apparent volume of distribution (V/F) defined as the fictitious volume, expressed in litres or in litres per kilogram, in which the administered drug would have been distributed (AGAH working group Pharmacokinetics 2004).
8. Day 7 plasma lumefantrine levels.

3.2.6.2 Secondary endpoints

3.2.6.2.1 Treatment efficacy

Treatment efficacy was assessed according to WHO 2009 classification (WHO 2009) as described in chapter 2 (Outcome measurments).

Blood samples from recurrent episodes of malaria during the follow-up were assessed by PCR analysis to distinguish true recrudescences from new infections.

3.2.6.2.2 Parasite Clearance Time (PCT)

The PCT was assessed as i) as the time for the parasite count to decrease to half of its initial value (PC50) and ii) as the parasite clearance rate derived from the log-linear section of the log parasitaemia-time curve and expressed as the parasite clearance half-life (PCt1/2; loge2/parasite clearance rate). Blood films were prepared at H0, H6, H12 then every 12 hours until two negative consecutives slides were observed to calculate this measure.

3.2.6.2.3 Safety and tolerability

Any adverse event, either clinical or laboratory finding, from enrolment to the end of the studywas classified and reported according to the International Conference on Harmonisation Harmonised Tripartite Guidelines (ICH-GCP 2005).

Particular attention was given to the following measures:

i) comparing the electrocardiogram results at baseline and at the end of treatment to detect any abnormal results; ii) monitoring liver and kidney function at baseline and discharge and record any abnormal levels in creatinine, AST, ALT, albumin; iv) occurrence of prematurity and of congenital abnormalities at delivery; v) occurrence of any abnormality in the physical and neurological development of children born from pregnant patients and vi) deaths.

3.2.7 Definitions

a. Uncomplicated *P. falciparum* malaria was defined as a case with a microscopy-confirmed *falciparum* malaria in the absence of signs of severe malaria

b. Gametocyte clearance was defined as the number of weeks gametocytes were seen in the peripheral blood by microscopy

c. Low Birth Weight was defined as a birth weight <2500 grams and a gestational age at birth of ≥ 37.0 weeks

d. The second trimester of pregnancy was defined as a gestational age calculated by ultrasound ≥ 14 and ≤ 26 weeks.

e. The third trimester of pregnancy was defined as a gestational age calculated by ultrasound ≥ 27 and ≤ 42 weeks

f. Anaemia was defined as haematocrit <30%

g. Severe neutropenia was defined as an absolute neutrophils count lower than 500 neutrophils per microliters.

h. An adverse event was considered as any untoward medical occurrence in a patient administered a pharmaceutical product and which does not necessarily have to have a causal relationship with this treatment (ICH-GCP 2005).

i. A serious adverse event: any untoward medical occurrence that at any dose results in death, is life-threatening, requires inpatient hospitalisation or prolongation of existing hospitalisation, results in persistent or significant disability/incapacity, or is a congenital anomaly/birth defect (ICH-GCP 2005).

3.2.8 Study treatments

Patients were treated with Coartem®, Novartis, Switzerland. Each tablet contains 20 mg of artemether and 120 mg of lumefantrine.

Patients allocated to the standard 3-day regimen received 6 doses over 3-days (at 0, 8, 24, 36, 48 and 60 hours). Patients allocated to the 5–day regimen received ten doses over five days (at 0,8,24,36,48,60,72,84,96 and 108 hours).

Each dose consisted of four tablets (i.e. 80 mg artemether and 480 mg of lumefantrine) according to manufacturer recommendations for adults weighing at least 35 kg.

The total dose received differed according to treatment arm. In the 3-day regimen, patients received a total dose of 480 mg artemether (a maximum dose of 13.7 mg/kg bodyweight) and 2880 mg lumefantrine (a maximum dose of 82.3 mg/kg bodyweight). Those in the 5-day regimen received 800 mg artemether which represented a target dose of 22.9 mg/kg bodyweight and 4800 mg lumefantrine or 137.1 mg/kg bodyweight.

The manufacturer target dose range of AL is a total dose of 5–24 mg/kg bodyweight of artemether and 29–144 mg/ kg bodyweight of lumefantrine (Novartis 2015; WHO 2015a).

Tablets were taken orally under direct nurse observation with 200 mL of milk to maximize lumefantrine absorption (White *et al.* 1999; Ashley, Stepniewska, Lindegårdh, Annerberg, *et al.* 2007). Patients were observed for one hour after drug administration. If vomiting occurred within 30 minutes of drug administration a full-dose was repeated. If it occurred between 30 minutes and one hour after drug intake, half the dose was repeated.

Patients with fever (i.e. temperature > 37.5°C) were treated with 20 mg/kg bodyweight of paracetamol.

3.2.9 Study procedures and follow-up assessment.

3.2.9.1 Pregnant women

They were approached during their Antenatal Care appointment at the maternity unit of Kingasani Hospital. They underwent first an ultrasound screening to confirm the fetus viability, to exclude any congenital abnormalities and multiple pregnancies and to estimate the gestational age using the Hadlock's method.This method estimates a composite gestational age (GA) from thebiparietal diameter (BPD), the abdominal circumference (AC) and the femur length (FL), all measured in centimetres. The GA estimated with this method is accurate within 7% of the actual fetal age (Hadlock *et al.* 1981). Women with gestational age between 14 and 42 weeks (inclusive) were invited to participate to the study. At this stage, all women were screened for malaria to determine eligibility for the study.

3.2.9.2 Non-pregnant women

The community was informed about the study by community health workers. Women with fever and/or signs of malaria who were interested in participating attended the research centre where they were screened for eligibility.

3.2.10 Laboratory methods

3.2.10.1Drug analysis

Artemether and dihydroartemisinin quantification was performed by liquid chromatography tandem mass spectrometry (LC-MS/MS)(Hodel 2009; Lindegardh 2009). The quality control for artemether and DHA quantification was done by comparing the samples to three quality control samples at concentrations 3.46 ng/ml, 36 ng/ml, and 375 ng/ml analysed in triplicates within each batch of clinical study

samples. The lower limit of detection (LOD) was 0.5 ng/ml and the lower limit of quantification (LLOQ) was set to 1.4 ng/ml for both compounds.

For lumefantrine, the quantification was performed by LC-MS/MS (Kloprogge *et al.* 2015). Triplicates of quality control samples were analysed at concentrations 33.5 ng/ml, 709 ng/ml, and 150,000 ng/ml. The LOD was 2.5 ng/ml and the LLOQ was set to 9.71 ng/ml.

Concentrations below the limits of quantification were considered nil for all drugs.

3.2.10.2 Pharmacokinetics

For each sample 3 mL of whole blood was drawn using lithium heparin coated tubes at each time point and centrifuged at 1,400 g for 7 minutes at +4°C.The plasma was transferred into cryotubes and the samples stored at -80°C until shipment with cold chain to the Department of Clinical Pharmacology Laboratory, MORU, Bangkok, Thailand for drug level quantification.

Artemether and DHA were measured at each planned sampling time-point from the pre-dose (H0) to the time point within 2 hours following the last drug intake.

Lumefantrine was measured at each planned sampling time-point from the pre-dose (H0) to the last time point on day 14.

Non-compartmental pharmacokinetic analysis

Individual plasma concentration-time data were assessed using a non-compartmental analysis (NCA) in the software STATA IC 14.0 (release 14.0; STATA Corporation, college station, Texas 77845, USA). Cmax and Tmax were taken directly from the observed data. The total exposure up to the last measured drug concentration (AUC_{0-last}) was calculated using the trapezoidal method. Drug exposure was extrapolated from

the last observed concentration to time infinity for each individual subject to compute total drug exposure ($AUC_{0-\infty}$). The terminal elimination half-life ($t_{1/2}$) was estimated by the log-linear best-fit regression of the observed concentrations in the terminal elimination phase. CL/F and V/F were not estimated for any of the drug components and the terminal elimination half–life was not reported for artemether and DHA with the NCA.

Pharmacokinetic nonlinear mixed-effects modelling

Modelling and simulations were performed in NONMEM v7.3 (Icon Development Solutions, Ellicott City, Maryland, USA). Model discrimination was based on the objective function value (OFV) which is proportional to −2 times the log-likelihood of the data. A drop of at least 3.84 or 6.64 was deemed significant with significance levels of 0.05 and 0.01, respectively, when adding one parameter in a nested model. Data below the limit of quantification were omitted.

All concentration measurements were converted into their natural logarithms. Parent drug and metabolite concentration-time data were fitted simultaneously, assuming 100% in vivo conversion from parent drug to metabolite. One-, two- and three-compartment disposition models were evaluated. First-order absorption and a transit compartment absorption model with 1–10 fixed transit compartments were evaluated to describe the absorption phase of the drugs (Savic *et al.* 2007). Between-subject variability was added exponentially to all parameters. An additive residual error on log-transformed data (essentially equivalent to an exponential error on normal scale data) was used.

The impact of body weight on the pharmacokinetic parameters was evaluated by adding it as an allometric function to all clearance (power of 0.75) and volume (power of 1) parameters (Holford 1996). The potential pregnancy effect was evaluated as a

categorical covariate. The covariate was added on all parameters in a stepwise manner with a forward criterion of $P < 0.05$ and a backward criterion of $P < 0.001$. Dose regimen (3- vs 5-day treatment) was evaluated as a categorical covariate on the relative bioavailability. Model diagnostics were performed using goodness-of-fit plots and visual predictive checks (2000 simulations). Subsequent population mean simulations were performed in Berkley Madonna (v. 8.3.18).

3.2.10.3 Haematology

Haemoglobin (Hb) was measured from capillary blood at screening using a portable analyser (Hemocue Hb301; Angelholm, Sweden).

Haematocrit (Hct) was measured on admission, at 0, 6,12,24,36,48,60,72, 84 and 96 hours, and weekly at each follow-up visits using capillary tubes centrifugation (HawksleyHaematospin 1400; Hawksley&Sons, Ltd., United Kingdom).

White Blood Cells (total and differential) were measured by an automated haematology analyser (SYSMEX) at baseline and at discharge.

Tests were repeated at delivery in the mother and the newborn.

Haemoglobin S was identified using the SEBIA Hydragel Haemoglobin K20 Kit which is designed to detect the major haemoglobin variant S, D, C or E by electrophoresis on alkaline agarose gel.

3.2.10.4 Parasitology

A Rapid Diagnostic Test based on the detection of *Plasmodium*-specific antigen Histidine -Rich Protein II (HRP2) was used for the detection of *P. falciparum* malaria infection at screening (SD Bioline Malaria Ag Pf). The RDT was used at screening to detect positive cases for malaria because it can be more sensitive than malaria slide in

pregnancy as parasite could hide in the placenta(World Health Organization 2014).Indeed, by detecting the protein expressed by the parasite and circulating in the plasma, RDTs can detect a current or recent malaria infection even in case of low peripheral parasitaemia, which can occur in pregnancy when parasites are sequestered in the placenta.

The positive diagnosis was confirmed by microscopy as described below.Microscopy was used thereafter to monitor the treatment efficacy.

Blood films, think and thick smears, were prepared using standard procedures (WHO 2010b). The species identification was done on the thin smear. The parasitaemia was counted on the thin or the thick smear as follows: if more than 20 parasites were counted in 10 fields on a thick smear, the parasitaemia was estimated on the thin smear on 1000 red blood cells using the formula number of parasitized RBC x Hct x 125.6. If 20 or less parasites were counted in 10 fields on the thick smear the parasitaemia was estimated on thick smear on 500 WBC using the formula number of parasites x 16. A slide was considered negative after 500 high-power fields had been examined (Warhurst 1997; Bejon *et al.* 2006; Bowers *et al.* 2009).

If gametocytes were seen, a gametocyte count was performed against 500 leukocytes.

Blood films were prepared and read at 0, 6, 12, 24 hours and then every 12 hours thereafter until 72 hours.

To distinguish cases of recrudescence from new infections during the follow-up, Dried Blood Spots (DBS) were collected on admission then at the time of recurrent episode of malaria according to standard methodology (WHO 2005a). Polymerase-chain-reaction (PCR) genotyping was performed on the paired DBS (the one at baseline and the one on the time of failure). A recurrent infection was distinguished from a new one by

comparing selected polymorphic repetitive regions of the three markers MSP1 and MSP2 and GLURP at baseline and at the time of recurrent episode.

A DBS was also taken at delivery from peripheral blood in the mother and from blood cord and heel prick in the newborn to i) genotype the parasites in case of a malaria infection at the time of delivery and assess whether it was a new infection or a recrudescence and ii) to compare infection in the mother and the newborn in the eventuality of a malaria infection in the newborn.

3.2.10.5 Biochemistry

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin (ALB) and creatinine (CREAT) were measured at baseline in all patients, at 48 hours in the 3-day treatment group and at 96 hours in the 5-day treatment group, using automated SEAC Screen master.

3.2.10.6 Electrocardiogram

Electrocardiograms were recorded at baseline and discharge – i.e. 48 hours in the 3-day group and 96 hours in the 5-day group – using an auto electrocardiogram device (Cardiofax Electrocardiograph ECG-9620 Nihon Kohden Corporation, Japan) to monitor for abnormal QTc interval. The electrocardiography (ECG) parameters recorded were the heart rate, the PR, QRS and QT interval, and the QRS and T wave amplitudes. The electrocardiographic intervals were measured automatically by the machine and manually checked by hand. The QTc interval was calculated using Fridericia's formula: QTc= QT/ (RR^0.33).

3.2.11 Infant follow up.

The physical and neurological development of the children born from pregnant patients was assessed at 1, 3, 6 and 12 months. The physical conditions of the child were assessed by a physical exam where all the systems of the body were checked for any abnormality. The neurological development was assessed by using a specifically designed questionnaire based on Denver II test (Frankenburg 1992) and previously used in the same area in a similar follow up of infants (Onyamboko *et al.* 2011). The Denver II test is designed to monitor the development of infants and pre-schooled children. The age at which a child accomplishes a large variety of specific tasks including language skills is defined (key milestones) and a set of questions is designed to assess the achievement of these key milestones. Comparing the results of the test with the milestones the examiner is able to identify children whose development deviates significantly from others. In the study, at each follow-up visit the children were administered the test and scored accordingly.

3.2.12 Ethical approval

The study was approved by the Ethical Committee of the Kinshasa School of Public Health, the Ministry of Public Health of DRC and the Oxford University Research Ethic Committee (OXTREC). All the study documents were designed in English. The protocol, the patient information sheet (PIS) and the informed consent (IC) were translated into French before submission to the local IRB. The PIS and IC were also translated into Lingala which is the language most commonly spoken in the area. To assess the accuracy of the translation, the PIS and IC were back translated from Lingala to English. The translation and back translation were prepared by certified interpreter in accordance with GCP requirements.

The study was registered with International Standard under registration number NCT 01916954 (www.clinicalTrials.gov).

3.2.13 Data Management

The raw data were checked for accuracy and completeness comparing the data in each patient‘s Case Report Form with data from the source documents. The data entry was performed using the software OpenClinica.

3.2.14 Statistical analysis

A sample size of 48 pregnant and 48 non-pregnant patients was defined as sufficient to produce reliable data suitable for a descriptive pharmacokinetics analysis, the primary outcome of this study.

The endpoint for treatment-success or treatment-failure at day 28 and 42 was analysed per protocol and intention to-treat.

Descriptive statistics were used to summarize baseline values and demographic data. For the per-protocol analysis, Chi -Square, *z*-test and Fisher’s exact test were used to compare proportions. For the intention-to-treat analysis, the log-rank test was used (survival analysis). Values of normally distributed data were expressed as means and those non-normally distributed were expressed as median (IQR or minimum and maximum) as appropriate. ANOVA was used for normally distributed continuous data and Kruskal-Wallis test for the continuous data with a skewed distribution.

3.3 Results

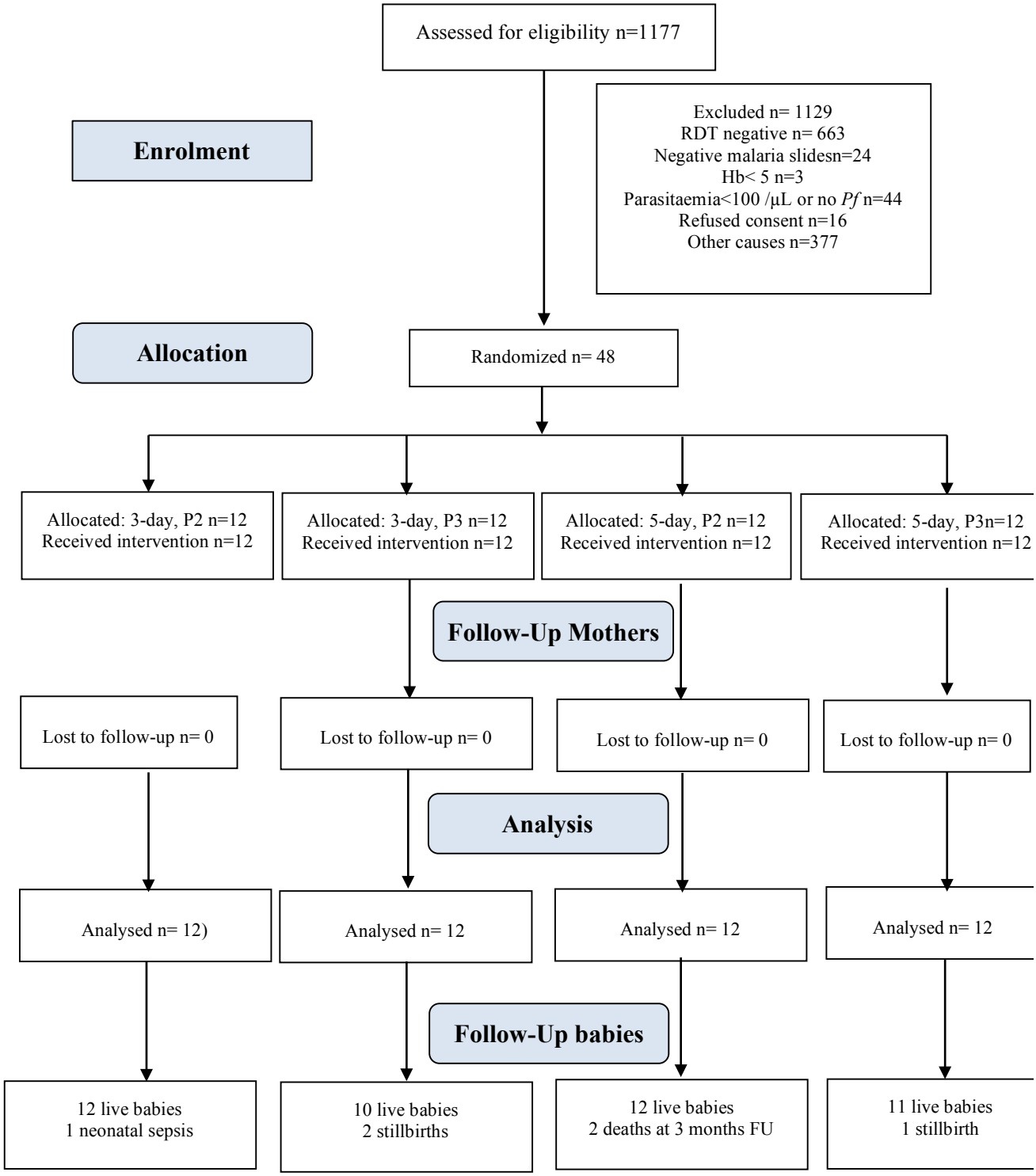


Figure 3.1. Consort 2010 flow diagram: Pregnant women

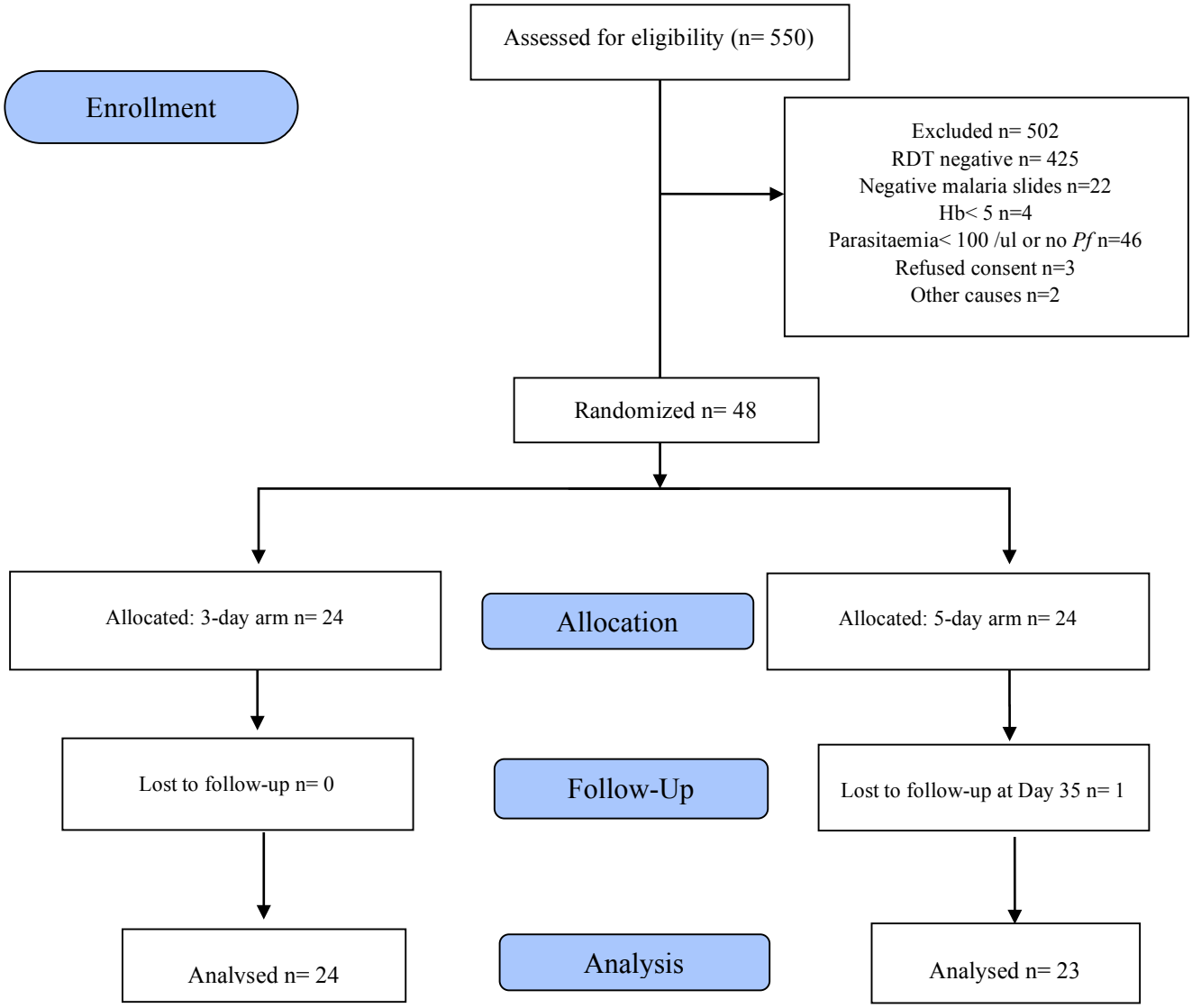


Figure 3.2. Consort 2010 Flow Diagram: Non – pregnant women

Baseline data

Demographic, parasitological and clinical data were collected at admission for all patients. Data were homogenous between arms within each group of pregnant and non-pregnant (Table 3.1;Table 3.2).

Pregnant patients had a higher body weight, a higher BMI and a lower haematocrit level than non-pregnant.The parity ranged from 1 to 5 pregnancies per study subject and 14.5% women (7/48) reported to have had a miscarriage.

Table 3.1. Baseline data in the pregnant group by treatment arm

	Treatment arm	
Characteristics	**3-day**	**5-day**
N. analysed	**24**	**24**
Age in years, mean (SD)	28.5 (6.51)	26.63 (6.18)
Body weight in kg, mean (SD)	61.7 (11.6)	63.(9.84)
BMI, kg/m^2, median (range)	25.1 (19-30.5)	24.5 (17.3-40)
Hb S trait, n (%)	4 (16.7)	6 (25)
Parity (%)		
1	6 (25.0)	10 (41.7)
2-4	12 (50.0)	6 (25.0)
≥5	6 (25.0)	8 (33.3)
Mean gestational age (weeks) 2^{nd}trimester	19.2 (3.54)	18.7 (3.11)
Mean gestational age (weeks) 3^{rd}trimester	28.9 (4.42)	24.9 (5.84)
Body temperature °C , median (min-max)	36.4 (36.0-37.7)	36.2 (36.0-37.2)
Haematocrit% , median (min- max)	29.5 (24-36)	29.5 (25-36)
Parasitaemia, parasite/µL, median (min-max)	3705 (144-70,336)	3391.5 (160-104,499)

Table 3.2. Baseline demographics in the non-pregnant group

	Treatment arm	
Characteristics	**3-day**	**5-day**
N. analysed	**24**	**24**
Age in year , mean (SD)	25.7 (7.8)	28.1 (8.52)
Body weight in kg,median (range)	52 (44-66)	54.5 (44-95)
BMI,kg/m^2, median (range)	19.6 (16.9-25.3)	21.5 (17.6-35.8)
HbS trait, n (%)	5 (20.8)	5 (20.8)
Body temperature,°C, median (range)	36.5 (36-39.2)	36.4 (36-38.9)
Haematocrit ,%, median (range)	37 (25-43)	35 (30-41)
Parasitaemia,parasite/µL , median (range)	7,097 (208-296,000)	4,898 (64-47,728)

3.3.1 Treatment administration

All women in the two treatment regimens received the planned total dose of AL except for one non pregnant patient in the 3- day arm who received an additional half–dose (i.e. 40 mg artemether and 240 mg lumefantrine) because she vomited once within one hour of drug administration and was retreated. The median dose received per kilogram of body weight is described in the Table 3.3.

The total dose administered in the extended regimen represents an increase of 67% in the total dose for both artemether and lumefantrine compared to the standard regimen.

Pregnant women in their 3rd trimester received 14% less artemether-lumefantrine per kilogram of body weight in the 3-day arm and 18% less in the 5-day arm than those in the 2nd trimester or non–pregnant women, whereas the median dose received was similar in 2nd trimester and non–pregnant groups.

Nevertheless, both treatment regimens provided a median dose per kg of bodyweight of artemether or lumefantrine higher than the minimum recommended.

Table 3.3. Median dose of artemether and lumefantrine administered by treatment arm

Treatment	Group	N. analysed	Median dose artemether mg/kg (min-max)	Median dose lumefantrine mg/kg (min-max)
Target dose according to manufacturer			5-24 mg/kg	29-144 mg/ kg
3- day	P2	12	8.35 (5.85-10.67)	50.09 (35.12-64.00)
	P3	12	7.17 (5.65-10.91)	42.99 (33.88-65.45)
	NP	24	9.23 (7.27-11.56)	55.38 (43.64-69.33)
	Total	48	8.57 (5.65-11.56)	51.43 (33.88-69.33)
5- day	P2	12	14.55 (8.89-15.38)	87.27 (53.33-92.31)
	P3	12	11.85 (10.39-16.00)	71.12 (62.34-96.00)
	NP	24	14.68 (8.42-18.18)	88.08 (50.53-109.09)
	Total	48	13.45 (8.42-18.18)	80.68 (50.53-109.09)

3.3.2 Efficacy at 42 days follow up

3.3.2.1 Pregnant group

The cure rate by day 42, PCR uncorrected, was similar for patients who received the standard 3-day treatment (20/22; 90.9%) compared to those who received 5-day treatment (18/20; 90.0%; p=0.41) (Table 3.4).

In the 3-day group there were 2 Late Parasitological Failures (day 35 and 42) and in the 5-day group, 1 Late Parasitological Failure (day 28) and 1 Late Clinical Failure (day 42).

After the PCR analysis, 2 cases were classified as new infections and 2 were indeterminate. After excluding the last two from the final per protocol analysis, the Adequate Clinical and Parasitological Response at day 42 was 100% for both treatments.

3.3.2.2 Non-pregnant group

In the non–pregnant group, the uncorrected cure rate by day 42 was 95.7% for those who received the 5-day treatment compared to 87.5% for those who received the of 3-day treatment (p=0.61).

After PCR analysis all recurrent episodes were classified as new infections and the corrected clinical and parasitological response was 100% in both arms (Table 3.5).

Table 3.4. Efficacy by treatment regimen at day 42 in the pregnant group

Treatment	**3-day**	**5-day**	**p-value[#]**
Pregnant women allocated to each group, N	24	24	
Deliveries before Day 42, N	2	4	
Evaluable, N (%)	22 (91.7)	20 (83.3)	
Results PCR uncorrected (%)			
Early Treatment Failure	0	0	
Late Clinical Failure	0	1 (5)	
Late Parasitological Failure	2 (9.1)	1 (5)	
Adequate Clinical and Parasitological Response	20 (90.9)	18 (90.0)	0.41
PCR results on recurrent episodes (n)			
New infections *P. falciparum*	0	2	
Recrudescence *P. falciparum*	0	0	
Undetermined PCR result	2	0	
Results PCR corrected (%)			
Adequate Clinical and Parasitological Response	20 (100)	20 (100)	

[#] Fisher exact test

Table 3.5. Efficacy by treatment regimen at Day 42 in the non-pregnant group

Treatment	3-day	5-day	p-value[#]
Non pregnant women allocated to each group, N	24	24	
Loss to follow-up N (%)	0	1 (4.2)	
Evaluable ,N (%)	24 (100)	23 (95.8)	
Results PCR uncorrected (%)			
Early Treatment Failure	0	0	
Late Clinical Failure	0	0	
Late Parasitological Failure	3 (12.5)	1 (4.3)	
Adequate Clinical and Parasitological Response	21 (87.5)	22 (95.7)	0.61
PCR results on recurrent episodes (%)			
New infections *P. falciparum*	3 (100)	1 (100)	
Recrudescence *P. falciparum*	0	0	
Undetermined PCR result	0	0	
Results PCR corrected (%)			
Adequate Clinical and Parasitological Response	24 (100)	23 (100)	

[#]Fisher exact test

3.3.3 Parasite clearance time and fever clearance time

3.3.3.1 Parasite Positivity Rate

There was no difference in the parasite positivity rate (defined as persistent patent parasitaemia) which decreased quickly in the two treatment arms (Table 3.6). All patients cleared their initial parasitaemia by day 3 in the 3-day regimen and by day 4 in the 5-day regimen (Figure 3.3).

Table 3.6. Parasite Positivity Rate by treatment arm

Days	3-day AL	5-day AL	p-value*
0	48/48 (100%)	48/48 (100%)	1
1	25/48 (52%)	27/48 (56%)	0.69
2	1/48 (2%)	1/48 (2%)	1
3	0	1/48 (2%)	0.5
4	0	0	

* χ^2, Fisher exact test

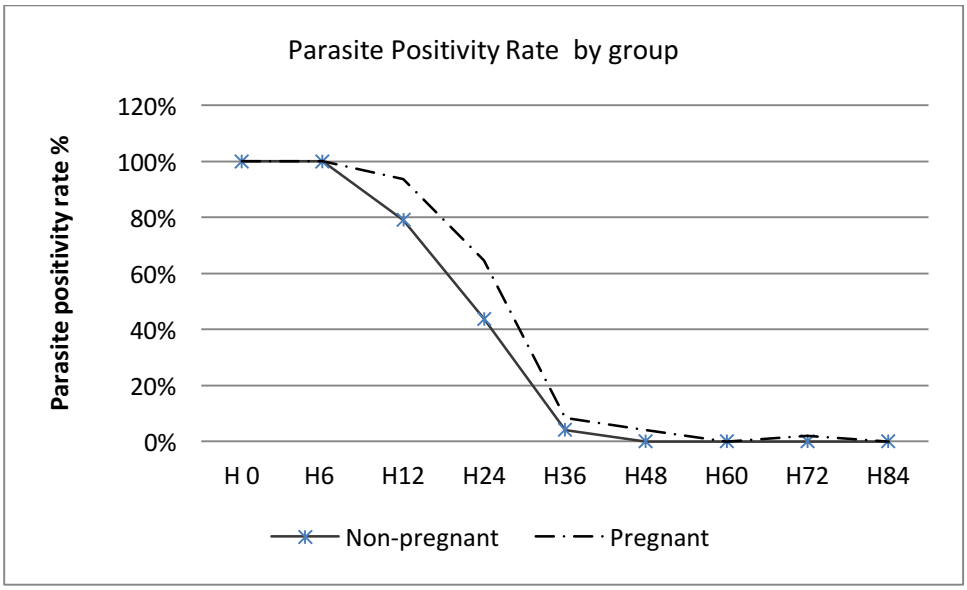


Figure 3.3. Parasite Positivity Rate in the pregnant and non-pregnant group

3.3.3.2 Parasite Clearance Rate

Parasite Clearance rate was estimated using the Parasite Clearance Estimator developed by the World Wide Antimalarial Resistance Network (WWARN) (http://www.wwarn.org/tools-resources/toolkit/analyse/parasite-clearance-estimator-pce).

Using this tool, data for 36 patients (19 in the pregnant group and 17 in the non-pregnant group) were excluded from the model because the parasitaemia was below the level of detection at the first smear after drug administration, considered 40 parasites / µL of blood. The median parasite clearance half-life ($PCT_{1/2}$) was 2.43 hours (range 1.39 - 4.32 hours) in pregnant women. This value was significantly longer than the median in non-pregnant women: $PCT_{1/2}$1.99 hours (range 1.05- 3.69 hours; p=0.004).

3.3.3.3 Gametocyte positivity rate

Initial gametocytaemia carriage was 8% (4/48) in the 3-day arm and 15% (7/48) in the 5-day one (p=0.34). Gametocytesin the peripheral blood were assessed until day 21 after treatment.

In 6 women gametocytes were observed at day 1 but not at inclusion (3 in each arm). There were no differences in gametocyte positivity rate between the two arms (Table 3.7 and **Error! Reference source not found.**).

Regardless of the treatment arm, the initial gametocytaemia was 8% (4/48) in pregnant patients and 15% (7/48) in non-pregnant. And in both groups this initial carriage persisted until the 3rd week post treatment.

Table 3.7. Gametocytes positivity rate by treatment arm

Days	3-day AL	5-day AL
0	4/48 (8%)	7/48 (15%)
2	2/48 (4%)	3/48 (6%)
3	2/48 (4%)	3/48 (6%)
7	1/48 (2%)	2/48 (4%)
14	1/48 (2%)	2/48 (4%)
21	0	0

* χ^2, Fisher exact test

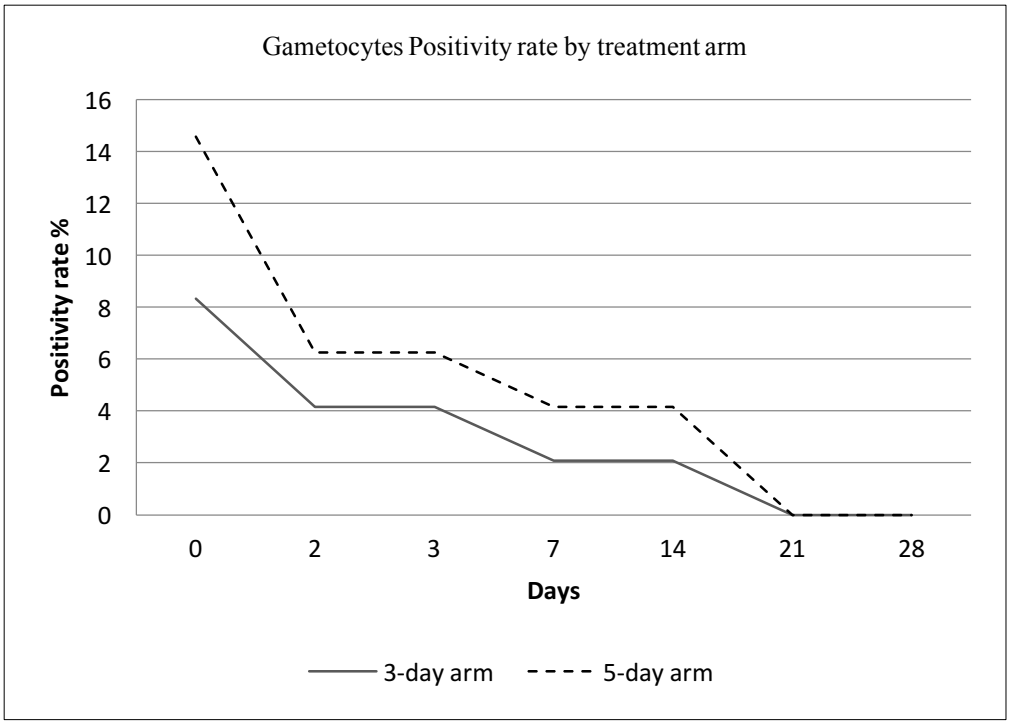


Figure 3.4. Gametocyte positivity rate by treatment arm

3.3.3.4 Fever clearance time

At admission fever was present in 12.5% (6/48) of the non-pregnant group compared to 2.1% (1/48) of the pregnant group (p=0.056). In addition, 63% (30/48) of non-pregnant patients had a history of fever prior to admission compared to 46% (22/48) of patients in the pregnant group (p=0.108).There were 12.5% (6/48) cases of fever at admission in the 3-day arm and 2.1% (1/48) in the 5-day one. Fever resolution was fast and similar in the two groups and within 24 hours all women were afebrile.

3.3.4 Haematology

The normal range for haematological values in both pregnant and non-pregnant in this study was based on international standards (Karim *et al.* 1992; Balloch and Cauchi 1993; Belo 2005; Zwang *et al.* 2012).

3.3.4.1 Pregnant group

3.3.4.1.1 Total White Blood Cells

The normal range for total White Blood Cells (WBC) count in pregnancy was taken as 5.6 to 16.9 x 10^3/µl. Six patients were excluded from the analysis because the results at discharge were not available. Four patients had leukopenia on admission and were still leukopenic at discharge with transient changes.

I included these patients then removed them from analysis and it was not different. In Table 3.8, I present the data with all the patients but the results presented below are the description of data after removing patients with leukopenia already at entry.

At baseline there was no difference between the median WBC counts between the two treatment arms.

The WBC counts increased significantly from baseline to discharge in all women regardless the treatment received. And at discharge, 72 hours for the 3-day arm and 96 hours for the 5-day arm, the total WBC count was significantly higher in the 5-day arm (7.6 x 10^3/µl, range 6.1 to 11.7) compared to the 3-day arm (6.3x 10^3/µl, range 5.4 to 8.6, $p<0.01$), (Table 3.8).

Two cases of leukopenia (defined as total WBC below 5.6 x 10^3/µl), not associated with clinical symptoms, were observed in the 3-day arm at discharge. In the first patient the median WBC count decreased from 5.7 x 10^3/µl at baseline to 5.5x 10^3/µl at discharge. In the second patient the median WBC count decreased from 6.7 x 10^3/µl at baseline to 5.4 10^3/µl at discharge.

3.3.4.1.2 Neutrophils

The normal range for absolute neutrophils count in pregnant is 3.8to 13.1 x 10^3 cells/µl. In this group, neutropenia is defined as an absolute neutrophils count below 3.8 x 10^3cells/µl.

Nineteen patients in this group were excluded from analysis because either the results at discharge were not available (6/19) or because they were already neutropenic at baseline (13/19) and still neutropenic at discharge with transient changes. The results were not different when analysing all the patients or excluding those described above. Data with all patients included are presented inTable 3.8. However, the results described below are based on data after removal of patients with abnormal values at baseline.

The median neutrophil count was comparable between the treatment arm at baseline and at discharge, respectively p=0.92 and p=0.13 (Table 3.9). Within each treatment arm, the neutrophil count was not different between entry and discharge.

Three cases of neutropenia at discharge were seen in two pregnant patients from the 3-day arm and in one patient from the 5-day arm. They were all non-clinically significant. In the 3-day arm, the count in the first patient decreased from 3,995.7 cells/ µl at baseline to 3,091 cells/ µl at discharge; In the second patient with neutropenia in this arm, the neutrophil count decreased from 3,965 cells/ µl at baseline to 3,354 cells/ µl at discharge. The count in the single patient with neutropenia from the 5-day arm decreased from 4,613 cells/ µl at baseline to 3,731 cells/ µl at discharge

3.3.4.1.3 Lymphocytes

The normal range for lymphocyte count in pregnancy is 0.9 - 3.6 X 10^3 cells/µl.

The median count of lymphocytes was comparable at baseline and discharge between the two treatment arms (Table 3.9).

Within each arm there was a significant increase inthe median lymphocytes count from baseline to discharge (baseline *versus* discharge in the 3-day arm, $p<0.01$ and in the 5-day arm, $p <0.01$). However, the increase was within the normal range in both treatment arms.

3.3.4.2 Non- pregnant group

3.3.4.2.1 Total White Blood Cells

The normal range for total WBC in non-pregnant subjects is 3.5 to 9.1 $x10^3$ cells/µl. In this group, leukopenia is defined as total WBC count < 3.5 X 10^3 cells/µl.

The median total WBC count was comparable between the two treatment arm at baseline and at discharge (p=0.52) (Table 3.11).

Within each treatment arm, the total WBC was not different between entry and discharge.

Three patients – two from the 3-day arm and one from the 5-day arm – had a leukopenia at discharge. In the 3-day arm, the first patient had a decrease of total WBC count from4.3$x10^3$ cells/µl at baseline to 2.7 $x10^3$ cells/µl at discharge. The second case of leukopenia had a decrease in WBC count from 3.7 $x10^3$ cells/µl at baseline to 3.2 $x10^3$ cells/µl at discharge. The patient from the 5-day arm had baseline WBC count of 4.9 $x10^3$ cells/µl which decreased to 3.2 X 10^3 cells/µl at discharge. All these cases of leukopenia were non clinically significant.

3.3.4.2.2 Neutrophils

The normal range for absolute neutrophils count in non-pregnant subjects is 1.4 to 4.6 $x10^3$ cells/µl. In this group, neutropenia was defined as an absolute neutrophils count < 1.4 10^3cells/µl.

Ten patients were excluded from analysis because either the results were not available at discharge (8/10) or because they had already neutropenia at baseline (2/10) and were still neutropenic at discharge.Excluding patients from the analysis did not affect the results and both tables (with and without patients described above) are presented (Table 3.10 and Table 3.11).

The median neutrophil count was comparable between the treatment arms at baseline and at discharge, respectively, p=0.35 and p=0.39. Within each treatment arm, the neutrophil count was not different between entry and discharge (respectively for the 3-day arm, p=0.10 and for the 5-day arm, p=0.42)

Three cases of neutropenia were observed, all from the 3-day arm. They were all non-clinically significant. The absolute neutrophils count in the first patient decreased from 2,244.6 cells/µl at baseline to 1,323 cells/µl; in the second patient , it decreased from 3,349.7 cells/ µl at baseline to 1,216 cells/µl at discharge. For the last patient, the count decreased from 3,477 cells/µl at baseline to 1,116 cells/µl at discharge.

3.3.4.2.3 Lymphocytes

The normal range for lymphocyte count in non-pregnant adults is 0.7 to 4.6 $x10^3$ cells/µl.

The median absolute lymphocytes count was comparable at baseline and discharge between the two treatment arms (Table 3.11).

Within the 5-day arm, the median lymphocyte count increased significantly from 1,667.4 cells/µl at baseline to 2,278.5 cells/µl at discharge ($p<0.01$). However, the increase was still within the range of normality.

Table 3.8.Total WBC, neutrophil and lymphocyte count (N, median and range) by treatment arm in the pregnant group (All patients)

Parameters		Pregnant group		p-value*
		3-day	5-day	
WBC cells/µL	Baseline	24	24	
		5,000 (2,400 - 8,700)	5,950 (3,300 - 9,800)	0.17
	Discharge[#]	19	23	
		6,200 (5,100 - 8,600)	7,200 (3,800 - 11,700)	0.01
Neutrophil cells/µL	Baseline	24	24	
		3,155 (1,324.8 - 5,220)	3,417 (983 - 6,570)	0.39
	Discharge[#]	19	23	
		3,465 (2,606 - 5,504)	4,445 (2,508 - 7,441)	<0.01
Lymphocyte cells/µL	Baseline	24	24	
		1,287 (275 - 2,871)	1,447 (638 - 2,660)	0.68
	Discharge[#]	19	23	
		1,867 (1,166 - 2,996)	2,018 (836 - 3,305)	0.64

*Kruskal-Wallis rank test

[#]72 hours for the 3-day regimen and 96 hours for the 5-day regimen

Table 3.9. Total WBC, neutrophil and lymphocyte count (N, median and range) by treatment arm in the pregnant group

Parameters		Pregnant group		p-value*
		3-day	5-day	
WBC cells/µL	Baseline	17	21	
		5,000 (3,600 - 7,200)	6,100 (3,400 - 9,800)	0.07
	Discharge#	17	21	
		6,300 (5,400 - 8,600)	7,600 (6,100 - 11,700)	<0.01
Neutrophil cells/µL	Baseline	9	20	
		3,965 (1,325 - 4,622)	3,978 (983 - 6,570)	0.92
	Discharge#	9	20	
		4,158 (3,091 - 5,504)	4,716 (3,731 - 7,441)	0.13
Lymphocyte cells/µL	Baseline	19	22	
		1,368 (275.2 - 2,275)	1,488 (638 - 2,660)	0.48
	Discharge#	19	22	
		1,867 (1,166 - 2,996)	2,054 (1258 - 3,305)	0.46

*Kruskal-Wallis rank test #72 hours for the 3-day regimen and 96 hours for the 5-day regimen

Table 3.10.Total WBC, neutrophil and lymphocyte count (N, median and range) by treatment regimen in the non-pregnant group (All patients)

Parameters		Non - Pregnant group		p-value*
		3-day	5-day	
WBC cells/µL	Baseline	24	24	
		4,900 (3,200 - 7,400)	4,700 (2,400 - 7,600)	0.21
	Discharge#	20	20	
		4,600 (2,700 - 8,000)	4,900 (3,200 - 6,500)	0.52
Neutrophil cells/µL	Baseline	24	24	
		2,462 (749 - 4,402)	2,039 (1,068 - 6,118)	0.2
	Discharge#	20	20	
		1,937 (1,116 - 5,372)	2,030 (408 - 2,836)	0.43
Lymphocyte cells/µL	Baseline	24	24	
		1,894 (563 - 3,382)	1,769 (714 - 3,538)	0.52
	Discharge#	20	20	
		2,087 (1,037 - 6,160)	2,279(1,263 - 4,248)	0.24

Table 3.11. Total WBC, neutrophil and lymphocyte count (N, median and range) by treatment regimen in the non-pregnant group

Parameters		Non - Pregnant group		p-value*
		3-day	5-day	
WBC cells/µL	Baseline	20	20	
		4,900 (3,200 - 7,400)	4,700 (3,200 - 7,600)	0.53
	Discharge#	20	20	
		4,600 (2,700 - 8000)	4,900 (3,200 - 6,500)	0.52
Neutrophil cells/µL	Baseline	19	19	
		2,474 (1,472 - 4,402)	2,069 (1,531 - 6,118)	0.35
	Discharge#	19	19	
		1,944 (1,116 - 5,372)	2,169 (1,440 - 2,836)	0.39
Lymphocyte cells/µL	Baseline	20	20	
		1,894 (563 - 3382)	1,667 (714 - 3,538)	0.59
	Discharge#	20	20	
		2,087 (1,037 - 6,160)	2,279 (1,263 - 4,248)	0.24

*Kruskal-Wallis rank test

72 hours for the 3-day regimen and 96 hours for the 5-day regimen

3.3.5 Biochemistry

In both pregnant and non-pregnant group, biochemical parameters have been assessed at baseline and in post treatment by treatment arm. In some cases explained within each section, patients have been removed from analysis. However, both tables (ie including all patients and excluding some patients) are presented. Data however are described based on the tables were patients were excluded.

3.3.5.1 Pregnant group

3.3.5.1.1 Serum Alanine Aminotransferase (ALT)

In the second and third trimester of pregnancy the normal range for ALT is 2 to 33 U/L.

Four patients were excluded from analysis because either results were not available at discharge (1/4) or they presented high ALT levels at discharge although these levels were already abnormally elevated at baseline. Analysis before and after excluding patients were not different (Table 3.12 and Table 3.13).

At baseline and discharge the median ALT level was within the normal range and comparable between the two treatment arms (Table 3.13). Within each treatment arm, the ALT level at baseline was not different from the one at discharge (respectively for 3-day arm, p=0.06 and for the 5-day arm, p=0.71).

In the 5-day arm, four women (18.2%, 4/22) had elevated ALT levelabove normal range. In the 3-day treatmentone woman had elevated ALT levels at discharge (4.5%, 1/22).

In the 5-day arm, the first case had a normal ALT plasma level of 11 U/L which increased to 37.9 U/L at discharge. In the three remaining patients, the ALT levels were respectively at 12.3 U/L, 1.7 U/L and 9.3 U/L at baseline and they increased respectively to 39 U/L, 38.4 U/L and 57.6 U/L at discharge.

In the 3-day arm, the single case with elevated ALT at discharge had a plasma level of 28.8 U/L at baseline which increased to 40.1 U/L at discharge. All cases were non-clinically significant and resumed to normal levels within a month.

3.3.5.1.2 Serum Aspartate Aminotransferase (AST)

The normal range for AST in the second and third trimester of pregnancy is 3to33 U/L.

Four patients were excluded from analysis because for three of them, AST levels were abnormally elevated at baseline and remained elevated at discharge. The fourth patient excluded from analysis had no results available at discharge.Analysis before and after excluding patients were not different.

At baseline, the median AST level in the 5-day arm was comparable to the median in the 3-day arm (p=0.18). At discharge, the median AST level in the 5-day arm was significantly lower than the one in the 3-day arm (5-day arm, median: 16 U/L (range 3.4 - 56.4) and in the 5-day arm, median: 24.6U/L (range 9 - 71), (p=0.04)(Table 3.13). However, within each treatment arm, the median AST levels at baseline and at discharge were comparable (respectively in the 3-day arm, p=0.14 and in the 5-day arm, p=0.19).

The AST level was above the normal range at discharge in five women (5/44, 11.4%), two in the 5-day arm (2/21, 9.5%) and 3 in the 3-day arm (3/23, 13%).

In the 5-day arm, the AST level at baseline in both women were 16.9 U/L and these levels increased respectively to 56.4 U/L and 37.9 U/L

In the 3-day arm, the AST levels increased from 32 U/L at baseline to 36.2 U/L at discharge in the first patient, from 23.3 U/L to 36.6 U/L in the second patient and from 22 U/L to 34.1 U/L in the third.

In both treatment regimens, the abnormal elevated values of ALT and AST at discharge were not clinically significant.

3.3.5.1.3 Creatinine

The normal range for creatinine, in the second and third trimester of pregnancy is 0.4 to 0.9 mg/dL.

Fourteen patients were excluded from analysis (13 because they had abnormally high levels of creatinine at baseline which remained elevated at discharge and one because she has no results available at discharge).Analysis before and after excluding patients were not different.

The median creatinine levels were comparable and within the normal range between the two treatment arms at baseline and at discharge (respectively p=0.53 and p=0.74) (Table 3.13). Within each treatment arm, the median levels at baseline and discharge were comparable in both treatment arm (respectively in the 3-day arm p=0.45 and in the 5-day arm p=0.79).

Although the median creatinine levels were normal in both treatment arms, five women in the 5-day arm and four in the 3-day arm had abnormally high levels at discharge.

In the 5-day arm, the levels increased in the five patients respectively from 0.5mg/dL at baseline to 1.1mg/dL at discharge; from 0.6 mg/dL to 1 mg/dL; from 0.7 mg/dL to 1.5 mg/dL; from0.8 mg/dL to 1.2 mg/dL and from 0.8 mg/dL to 1.1 mg/dL.

In the 3-day arm, the levels of creatinine in the three patients increased respectively from 0.5mg/dL at baseline to 1.2 mg/dL at discharge; from 0.7 mg/dL to 1.4 mg/dL; from 0.8 mg/dL to 1.1 mg/dL and from 0.8 mg/dL to 1.2 mg/dL.

All the cases with elevated ALT, AST and creatinine levels at discharge were followed-up weekly until resolution; the values returned to normality within a month.

3.3.5.1.4 Albumin

The normal range for albumin, in the second and third trimester of pregnancy is 2.3 to 4.5 g/dL.

The median levels for albumin were comparable and within normal range between baseline and discharge in the two treatment arm (respectively p=0.57 and p=0.68). Within each treatment arm, the median baseline level was comparable to the one at discharge).

3.3.5.2 Non – pregnant

3.3.5.2.1 Serum Alanine Aminotransferase (AST)

The normal range for AST in non-pregnant adults is 12 to 38 U/L.

Two patients were excluded from the analysis because they presented with abnormally elevated AST levels at both admission and discharge.

The median AST level was comparable and within normal range between the two treatment arms at baseline and at discharge (respectively at baseline p= 0.34 and at discharge, p=0.69) (Table 3.15). Within each treatment arm, the AST level at baseline was not different from the one at discharge (respectively for 3-day arm, p=0.47 and for the 5-day arm, p=0.88).

However, in the 5-day treatment arm, three women (3/23, 13 %) had rises in AST to levels above normal range. In the first case, AST level increased from 37.4 U/ L at baseline to 95 U/L at discharge. The two other cases had an increase respectively from 6.9 U/L to 41.9 U/L and from 3.4 U/L to 41.3 U/L.

In the 3-day treatment arm five women had elevated AST levels at discharge (5/23, 21.7%). In this arm, the five cases had an increase respectively from 20.7 U/L at baseline to 38.9 U/L at discharge; from 25 U/L to 41.3 U/L; from 27.4 U/L to 91.6 U/L; from 22 U/L to 73.3 U/L and from 18.3 U/L to 40.1 U/L.

They were all non-clinically significant and returned to withinthe normal range within a month.

3.3.5.2.2 Serum Alanine Aminotransferase (ALT)

The normal range for ALT in non-pregnant subjects is 7 to 41 U/L.

The median ALT level were comparable between the two treatment arm at baseline and at discharge (respectively p=0.56 and p=0.43) (Table 3.15). Within each treatment arm, the median at baseline and at discharge were comparable (respectively in the 3-day arm, p=0.55 and in the 5-day arm, p=0.97).

Three patients in the 5-day arm had an elevation of ALT at discharge.The ALT levels increased in these three patients respectively from 22.1 U/L, 19.2 U/L and 14 U/L at baseline to 75 U/L, 43.6U/L and 44.8 U/L at discharge.

In both treatment regimens, the abnormal elevated values of ALT and AST at discharge were not clinically significant and were followed-up weekly for a month period within which they returned to normality.

3.3.5.2.3 Creatinine

The normal range for creatinine, in non-pregnant adults, is 0.5 to 0.9 mg/dL.

Thirty patients were excluded from analysis because they had abnormally high levels of creatinine at both baseline and discharge. Results including and excluding patients are presented in Table 3.14 and Table 3.15.

The median creatinine levels were comparable between the two treatment arm at baseline and at discharge (respectively p=0.50 and p=0.65). Within each treatment arm, the median levels at baseline and discharge were comparable in both treatment arm (respectively in the 3-day arm p=0.90 and in the 5-day arm p=0.66).

3.3.5.2.4 Albumin

The normal range of albumin in non-pregnant adults is 4.1 to 5.3 g/dL.

Twenty-two patients were excluded from analysis because although they presented abnormally low levels at both baseline and discharge (Table 3.14 and Table 3.15).

The median levels for albumin were comparable and within normal range between baseline and discharge in the two treatment arm (respectively p=0.26 and p=0.63). Within each treatment arm, the median baseline level was comparable to the one at discharge; respectively in the 3-day arm, p=0.92 and in the 5-day arm, p=0.38).

Table 3.12. Liver function test, creatinine and albumin levels (N, median and range) by treatment regimen in the pregnant group (All patients)

Parameters		Pregnant group		p-value*
		3-day	5-day	
Creatinine (mg/dL)	N	24	24	
	Baseline	1 (0.5 - 2)	0.9 (0.3 - 1.8)	0.21
	N	23	24	
	Discharge#	1 (0.5 - 2)	0.8 (0.4 - 1.9)	0.28
ALT(U/L)	N	24	24	
	Baseline	16.6 (4 - 109)	20 (1.7 - 47.6)	0.93
	N	23	24	
	Discharge#	14 (4 - 42.7)	21.9 (5.2 - 57.6)	0.05
AST(U/L)	N	24	24	
	Baseline	25.6 (6.4 - 163)	24.5 (5.8 - 62)	0.59
	N	23	24	
	Discharge#	24.6 (9 - 71)	19.2 (3.4 - 56.4)	0.22
Albumin (g/dL)	N	24	24	
	Baseline	3.7 (3 - 4.8)	3.7 (2.6 - 4.9)	0.48
	N	23	24	
	Discharge#	3.6 (3 - 4.8)	3.7 (3 - 5.5)	0.68

Table 3.13.Liver function test, creatinine and albumin levels (N, median and range) by treatment regimen in the pregnant group

Parameters		Pregnant group		p-value*
		3-day	5-day	
Creatinine (mg/dL)	N	14	20	
	Baseline	0.85 (0.5 - 2)	0.8 (0.3 - 1.8)	0.53
	N	14	20	
	Discharge#	0.8 (0.5 - 1.4)	0.8 (0.4 - 1.5)	0.74
ALT(U/L)	N	22	22	
	Baseline	16.6 (6.1 - 109)	18.8 (1.7 - 41)	0.61
	N	22	22	
	Discharge#	13.6 (4 - 40.1)	21.1 (5.2 - 57.6)	0.06
AST(U/L)	N	23	21	
	Baseline	27 (11 - 163)	19.8 (5.8 - 41)	0.18
	N	23	21	
	Discharge#	24.6 (9 - 71.1)	16 (3.4 - 56.4)	0.04
Albumin (g/dL)	N	23	24	
	Baseline	3.7 (3 - 4.8)	3.7 (2.6 - 4.9)	0.57
	N	23	24	
	Discharge#	3.6 (3 - 4.8)	3.7 (3 - 5.5)	0.68

*Kruskal-Wallis rank test# 72 hours for the 3-day regimen and 96 hours for the 5-day regimen

Table 3.14. Liver function test, creatinine and albumin levels (N, median and range) by treatment regimen in the non-pregnant group (All patients).

Parameters		Non - pregnant group		p-value*
		3-day	5-day	
Creatinine (mg/dL)	N	24	24	
	Baseline	1.2 (0.6 - 2.2)	1.3 (0.5 - 2.6)	1
	N	24	24	
	Discharge#	1.5 (0.5 - 2.3)	1.3 (0.4 - 2)	0.26
ALT(U/L)	N	24	24	
	Baseline	18.1 (4.6 - 74)	18 (4 - 97)	0.56
	N	24	24	
	Discharge#	18.8 (7.5 - 36.7)	18.2 (5.2 - 75)	0.43
AST(U/L)	N	24	24	
	Baseline	26.3 (10.5 - 55.2)	27.3 (3.4 - 118)	0.63
	N	24	24	
	Discharge#	20.1 (8.7 - 91.6)	22.4 (5 - 95)	0.63
Albumin (g/dL)	N	24	24	
	Baseline	3.9 (3 - 5)	3.9 (3 - 6.1)	0.70
	N	24	24	
	Discharge#	3.8 (3 - 5.8)	3.8 (3 - 4.7)	0.91

Table 3.15. Liver function test, creatinine and albumin levels (N, median and range) by treatment regimen in the non-pregnant group

Parameters		Non - pregnant group		p-value*
		3-day	5-day	
Creatinine (mg/dL)	N	7	11	
	Baseline	0.9 (0.6 - 1.8)	0.9 (0.5 - 1.6)	0.50
	N	7	11	
	Discharge#	0.9 (0.5 - 2.3)	1 (0.4 - 2)	0.65
ALT(U/L)	N	24	24	
	Baseline	18.1 (4.6 - 74)	18 (4 - 97)	0.56
	N	24	24	
	Discharge#	18.8 (7.5 - 36.7)	18.2 (5.2 - 75)	0.43
AST(U/L)	N	23	23	
	Baseline	27 (10.5 - 55.2)	27.1 (3.4 - 49.4)	0.34
	N	23	23	
	Discharge#	21 (12.3 - 91.6)	22.1 (5 - 95)	0.69
Albumin (g/dL)	N	13	13	
	Baseline	4.1(3 - 5)	4.2(3.6 - 6.1)	0.26
	N	13	13	
	Discharge#	4.1(3.5 - 5.8)	4.1(3 - 4.7)	0.63

*Kruskal-Wallis test# 72 hours for the 3-day regimen and 96 hours for the 5-day regimen

3.3.6 Electrocardiogram

The ECG was normal at admission in all patients with a QTc interval at baseline lower than 450 milliseconds (ms). Similarly at discharge, the ECG was normal in all patients despite an increase in QTc values in some patients which still remained normal as none was greater than 60 ms.

At discharge the median value in pregnant patients who received the 3-day treatment was 399 ms (range 378 - 440) and 407 ms (range 376 - 434) in those who received the 5-day treatment. In non-pregnant patient, the median value at discharge was 407 ms (range 372 - 442) in those who received the 3-day treatment and 409.5 ms (range 372 - 463) in those who received 5-days.

The mean difference (increase) at discharge were compared with baseline values and tested with the Wilcoxon paired sample:

In the 3-day treatment arm, the QTc interval increased from baseline to 72 hours by a mean of 6.04 ms (95 % CI: -0.22–12.31) (p=0.08) in pregnant patients and 8.57 ms (95 % CI: 0.18–17.32)(p=0.07) in the non-pregnant patients.

In the 5-day treatment arm, the QTc interval increased from baseline to 96 hours by 8ms (95 % CI: 0.37–15.61) (p=0.07) in pregnant patients and 8.5ms (95 % CI: 2.86–14.12) (p=0.01) in the non-pregnant women.

In the 3-day arm, the absolute QTc measurement did not change significantly over 72 hours. In the 5-day arm, the increase in QTc duration was significant in the control group.

Table 3.16. Interval QTc measurement (N, median and range) by groups and by treatment regimen

Parameters		3-day		5-day	
		Non-pregnant	Pregnant	Non-pregnant	Pregnant
QTc	N	21	24	22	21
	Baseline	398 (374 - 423)	401 (352 - 428)	401 (369 - 448)	389 (366 - 428)
QTc	N	21	24	22	21
	Discharge[#]	407 (372 - 442)	399 (378 - 440)	410 (372 - 463)	407 (376 - 434)

[#] 72 hours for 3-day and 96 hours for 5-day

3.3.7 Non-compartmental pharmacokinetic analysis

3.3.7.1 Lumefantrine PK in the pregnant women group

As expected, pregnant women who received 5-day treatment achieved a significantly larger AUC ($AUC_{0\text{-last}}$ and $AUC_{0\text{-}\infty}$) when compared to those who received 3-day only (p=<0.01).

There was a 40 % increase of the median $AUC_{0\text{-last}}$ and $AUC_{0\text{-}\infty}$ in the 5-day arm compared to the same values in the 3-day arm.

The maximum concentration (Cmax) and time to maximum concentration (Tmax) were also higher in the 5-day regimen, but non-significantly (p=0.12 and p=0.08).

The terminal elimination half-life was significantly shorter in the 5-day regimen than in the 3-day regimen (p=<0.01) (p=<0.01) (Table 3.17).

3.3.7.2 Lumefantrine PK in the control group

In the control group of non–pregnant women the $AUC_{0\text{-last}}$, $AUC_{0\text{-}\infty}$, half-life and the Tmax were significantly higher in the 5-day regimen compared to those in the 3-day. The Cmax tended to be higher but not significantly (p=0.31) (Table 3.18).

3.3.7.3 Lumefantrine PK: Pregnant women vs. control

There were no significant differences when comparing pregnant women who received a 3-day or 5-day treatment with the control group (non-pregnant) regarding the AUCs, Cmax, Tmax, Ke and half-life parameters (Table 3.19 and Table 3.20).

Table 3.17. Lumefantrine PK parameters in pregnant women by treatment arm

LUM PK parameters, median (range)	Pregnant women group		p-value*
	3-day (n=24)	5-day(n=24)	
$AUC_{0\text{-last}}$ (µg*h/ml)	630.6 (300.4 - 1060.9)	886.4 (295.8 - 1732.2)	<0.01
$AUC_{0\text{-}\infty}$ (µg*h/ml)	652.4 (314.4 - 1097.2)	910.5 (306.7 - 1775.5)	<0.01
Cmax (µg/ml)	6.3 (3.3 - 11.8)	7.1 (3 - 20.1)	0.12
Ke (h)	0.008 (0.006 - 0.015)	0.011 (0.008 - 0.014)	<0.01
Half-life (h)	85 (60.4 - 121.6)	63.7 (49.7 - 89.1)	<0.01
Tmax (h)	63 (16 - 84)	70 (8 - 120)	0.08

*Kruskal-Wallis test

Table 3.18. Lumefantrine PK parameters in non-pregnant women by treatment arm

LUM PK parameters, median (range)	Control group		p-value
	3-day (n=24)	5-day (n=24)	
$AUC_{0\text{-last}}$ (µg*h/ml)	534.8 (272.1 - 1447.3)	912.5 (282.6 - 2170.1)	<0.01
$AUC_{0\text{-}\infty}$ (µg*h/ml)	559.7 (282.7 - 1483.9)	935.3 (294.6 - 2226.5)	<0.01
Cmax (µg/ml)	5.9 (3.5 - 16.2)	7.8 (2.5 - 19.6)	0.31
Ke (h)	0.008 (0.004 - 0.015)	0.011 (0.007 - 0.013)	<0.01
Half-life (h)	85.5 (46.2 - 159.9)	65 (52 - 95.1)	<0.01
Tmax (h)	56 (4.5 - 72)	68 (5.5 - 120)	0.03

*Kruskal-Wallis test

Table 3.19. Comparison of lumefantrine PK parameters in pregnant *versus* non-pregnant women in the 3-day arm

LUM PK parameters, median (range)	3-day treatment		p-value*
	Pregnant (n=24)	Non-pregnant (n=24)	
$AUC_{0\text{-last}}$ (µg*h/ml)	630.6 (300.4 - 1060.9)	534.8 (272.1 - 1447.3)	0.33
$AUC_{0\text{-}\infty}$ (µg*h/ml)	652.4 (314.4 - 1097.2)	559.7 (282.7 - 1483.9)	0.40
Cmax (µg/ml)	6.3 (3.3 - 11.8)	5.9 (3.5 - 16.2)	0.84
Ke (h)	0.008 (0.006 - 0.015)	0.008 (0.004 - 0.015)	0.27
Half -life (h)	85 (60.4 - 121.6)	85.5 (46.2 - 159.9)	0.27
Tmax (h)	63 (16 - 84)	56 (4.5 - 72)	0.49

*Kruskal-Wallis test

Table 3.20. Comparison of lumefantrine PK parameters in pregnant *versus* non-pregnant in the 5-day arm

LUM PK parameters, median (range)	5-day treatment		p-value*
	Pregnant (n=24)	Non-pregnant (n=24)	
$AUC_{0\text{-last}}$ (µg*h/ml)	886.4 (295.8 - 1732.2)	912.5 (282.6 - 2170.1)	0.97
$AUC_{0\text{-}\infty}$ (µg*h/ml)	910.5 (306.7 - 1775.5)	935.3 (294.6 - 2226.5)	0.93
Cmax (µg/ml)	7.1 (3 - 20.1)	7.8 (2.5 - 19.6)	0.80
Ke (h)	0.011 (0.008 - 0.014)	0.011 (0.007 - 0.013)	0.97
Half-life (h)	63.7 (49.7 - 89.1)	65 (52 - 95.1)	0.97
Tmax (h)	70 (8 - 120)	68 (5.5 - 120)	0.77

*Kruskal-Wallis test

3.3.7.4 Lumefantrine plasma concentration-time profile in patients in the two treatment regimens

The lumefantrine population concentration–time profiles were different between the two treatments and the two groups of patients (Figure 3.5).

In the 3-day arm, pregnant women achieved the maximum concentration of lumefantrine at 66 hours (median time) considering the entire treatment administration and non-pregnant women reached the maximum concentration at 52 hours (median time). In both groups the first measurement below 280 ng/ml (the cut-off associated with an increased risk of treatment failure) was measured at 240 hours (10 days after the first dose).

After 5-day treatment, in the pregnant women group the maximum concentration of lumefantrine was observed at 60 hours (median time) whereas in the control group, the

Cmax was observed at 66 hours (median time). The plasma level of lumefantrine was still above 280 ng/ml in both groups at day 14, corresponding to the last day of measurement.

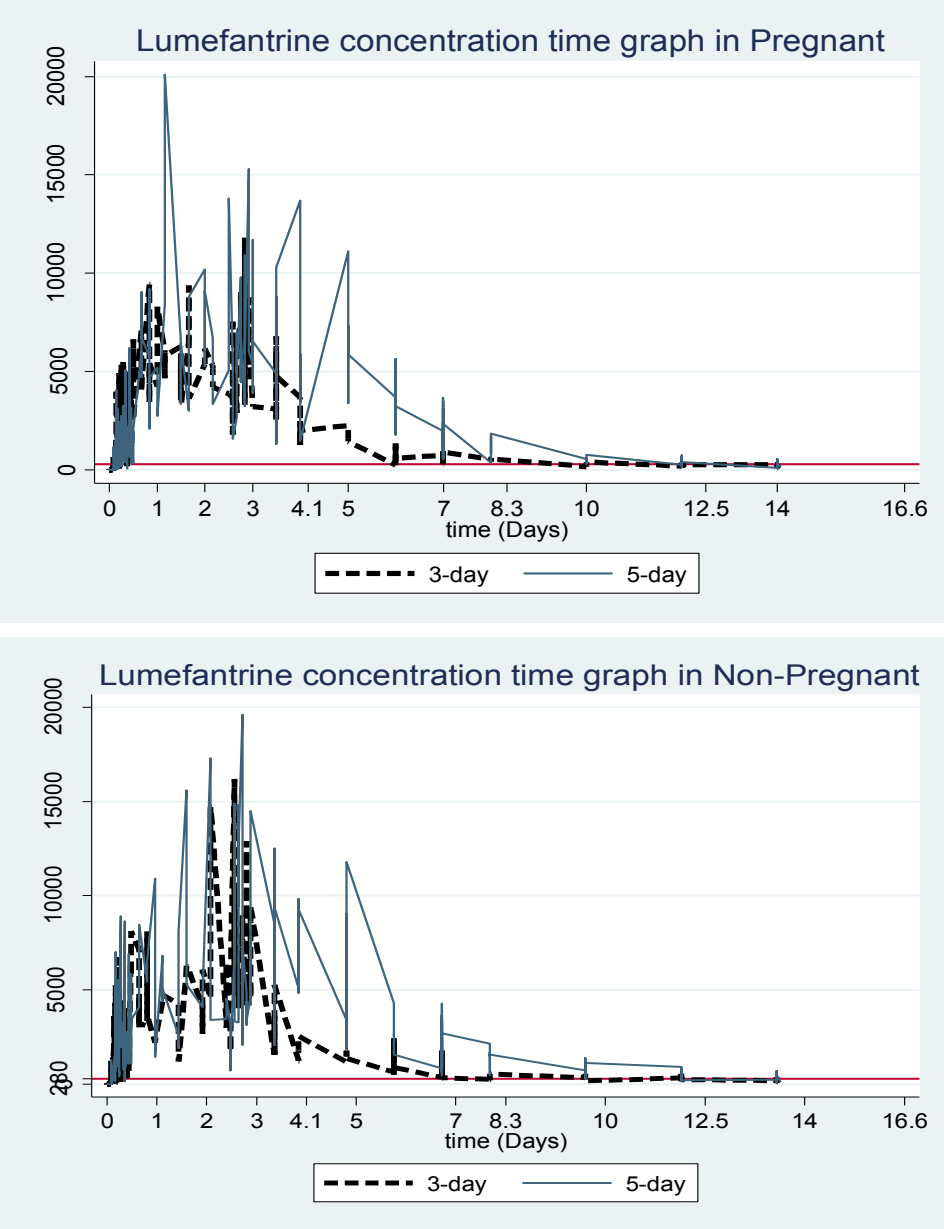


Figure 3.5. Lumefantrine concentration-time graphs in days in two groups by treatment regimen

Legend: The line in red represents the cut off of 280 ng/ml concentration of lumefantrine at day 7.

3.3.7.5 Day 7 Lumefantrine plasma concentration

The observed median lumefantrine level at day 7 in pregnant women was 596.5 ng/ml (ranging from 216 to 928 ng/ml) in the 3-day arm and 1,545 ng/ml (ranging from 537 to 3,650 ng/ml) in the 5-day arm ($p<0.01$).

In the control group of non-pregnant women the median lumefantrine level at day 7 was 541 ng/ml (range 315 to 1,780 ng/ml) in the 3-day arm and 1,995 ng/ml (range 457 to 4,270 ng/ml) in the 5-day arm ($p<0.01$).

All study subjects had a lumefantrine plasma level at day 7 above the 280 ng/ml cut-off except for one pregnant woman in the 3-day arm (**Error! Reference source not found.** and Figure 3.6)

There were no significant differences between pregnant women and their control within each arm (**Error! Reference source not found.**). However, a trend of decreased day 7 concentrations can be seen in pregnant women after a 5-day treatment compared to non-pregnant women. The high variability between patients makes this data difficult to interpret.

Table 3.21. Comparison of plasma day 7 lumefantrine levels by treatment arm in two groups

	Day 7 lumefantrine concentration, ng/ml, median (min-max)		**p-value***
Group	**3-day**	**5-day**	
Pregnant	597 (216 to 928)	1,545 (537 to 3,650)	0.001
Non-Pregnant	541 (315 to 1,780)	1,995 (457 to 4,270)	0.001
p-value*	0.90	0.44	

*Kruskal-Wallis test

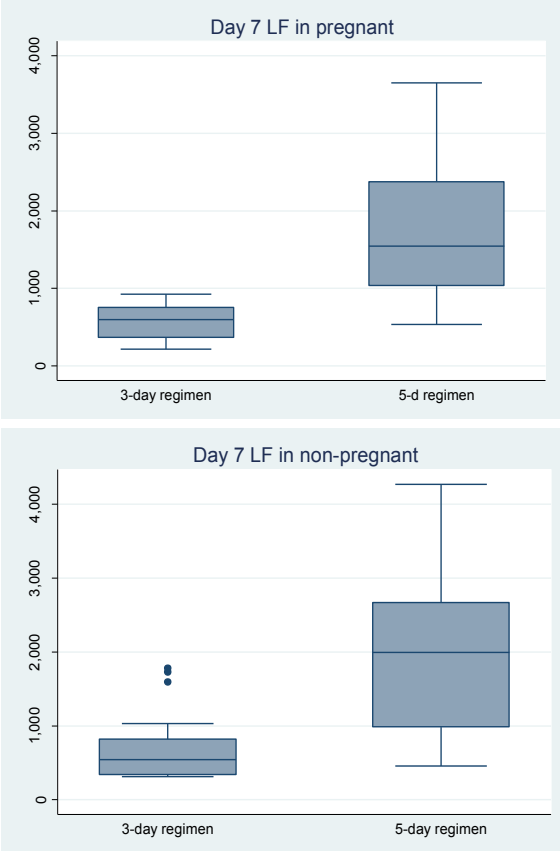


Figure 3.6. Day 7 plasma levels of lumefantrine in pregnant and non-pregnant subjects by treatment arm

3.3.7.6 Artemether PK

In the pregnant group $AUC_{0\text{-last}}$, $AUC_{0\text{-}\infty}$, Cmax, and Tmax were comparable for artemether in the two treatment arms (Table 3.22).

Similarly in the non-pregnant group $AUC_{0\text{-last}}$, $AUC_{0\text{-}\infty}$, Cmax, and Tmax were comparable between the two treatment arms (Table 3.23).

In the 3-day arm the $AUC_{0\text{-last}}$ was significantly lower in pregnant women compared to non-pregnant women. A trend of lower $AUC_{0\text{-}\infty}$ and Cmax were also observed in the pregnant group compared to the non-pregnant group, although the difference was non-significant (Table 3.24).

In the 5-day arm, pregnant women had significantly lower $AUC_{0-\infty}$ than non-pregnant women, but comparable levels for $AUC_{0-last,}$ Cmax and Tmax (Table 3.25)

Table 3.22. Artemether PK parameters in pregnant by treatment arm

ARM PK parameters, median (range)	Pregnant women		p-value*
	3-day (n=24)	5-day (n=24)	
AUC_{0-last} (ng*h/ml)	930.9 (115.2 - 4190.69)	855.7 (27.7 - 3205.1)	0.85
$AUC_{0-\infty}$ (ng*h/ml)	1332.5 (251.1 - 19140.1)	1171.2 (31.7 - 4145)	0.57
Cmax (ng/ml)	62.3 (10.3 - 285)	62.4 (11.4 - 169)	0.75
Ke (h)	0.03 (0.002 - 0.115)	0.02 (0.001 - 1.6)	0.92
Tmax (h)	2.5 (0.5 - 12)	3 (0.5 - 12)	0.78

*Kruskal-Wallis test

Table 3.23. Artemether pk parameters in non-pregnant by treatment arm

ARM PK parameters, median (range)	Control Groups		p-value*
	3-day (n=24)	5-day (n=24)	
AUC_{0-last} (ng*h/ml)	1492.6 (149.9 - 4196.7)	1428.9 (92.6 - 6277.4)	0.76
$AUC_{0-\infty}$ (ng*h/ml)	2013.3 (312.6 - 4273.7)	1702.1 (478 - 8194.1)	0.75
Cmax (ng/ml)	100.5 (12.6 - 365)	75.9 (10.6 - 430)	0.30
Ke (h)	0.022 (0.008 - 0.063)	0.02 (0.002 - 0.061)	0.37
Tmax (h)	2.25 (0.5 - 12)	3 (0.5 - 12)	0.39

*Kruskal-Wallis test

Table 3.24. Comparison of artemether pk parameters in pregnant *versus* non-pregnant in the 3-day arm

ARM PK parameters, median (range)	3-day treatment		p-value*
	Pregnant (n=24)	Non-pregnant (n=24)	
$AUC_{0\text{-last}}$ (ng*h/ml)	930.9 (115.2 - 4190.7)	1492.6 (149.9 - 4196.7)	0.01
$AUC_{0\text{-}\infty}$ (ng*h/ml)	1332.5 (251.1 - 19140.1)	2013.3 (312.6 - 4273.7)	0.25
Cmax (ng/ml)	62.3 (10.3 - 285)	100.5 (12.6 - 365)	0.06
Ke (h)	0.03 (0.002 - 0.12)	0.022 (0.007 - 0.06)	0.49
Tmax (h)	2.5 (0.5 - 12)	2.25 (0.5 - 12)	0.93

*Kruskal-Wallis test

Table 3.25. Comparison of artemether pk parameters in pregnant *versus* non-pregnant in the 5-day arm

ARM PK parameters, median (range)	5-day treatment		p-value*
	Pregnant (n=24)	Non-pregnant (n=24)	
$AUC_{0\text{-last}}$ (ng*h/ml)	855.7 (27.7 - 3205.1)	1428.9 (92.6 - 6277.4)	0.1802
$AUC_{0\text{-}\infty}$ (ng*h/ml)	1171.2 (31.7 - 4145)	1702.1 (478 - 8194.1)	0.0394
Cmax (ng/ml)	62.4 (11.4 - 169)	75.9 (10.6 - 430)	0.2482
Ke (h)	0.02 (0.001 - 1.6)	0.02 (0.002 - 0.061)	0.9169
Tmax (h)	3 (0.5 - 12)	3 (0.5 - 12)	0.5499

*Kruskal-Wallis test

3.3.7.7 Dihydroartemisinin (DHA) PK

There were no significant differences in the $AUC_{0\text{-last}}$, $AUC_{0\text{-}\infty}$, Cmax or Tmax between the two treatment arms in the group of pregnant women (Table 3.26).

Similarly there were no differences in the $AUC_{0\text{-last}}$, $AUC_{0\text{-}\infty}$, Cmax or Tmax between the two treatment arms in the group of non-pregnant women (Table 3.27).

If we consider the 3-day arm, all PK parameters were comparable between pregnant and non-pregnant women, whereas in the 5-day arm, however, the AUC (either 0 to last or infinite) and the Cmax were significantly higher in non-pregnant women. There were no differences in the Tmax (p=0.14) (Table 3.28 andTable 3.29).

Table 3.26. DHA PK parameters in pregnant by treatment arm

DHA PK parameters, median (range)	Pregnant women		p-value*
	3-day (n=24)	5-day (n=24)	
$AUC_{0\text{-last}}$ (ng*h/ml)	777.9 (221.6 - 3339.7)	926.4 (142.4 - 2959.5)	0.70
$AUC_{0\text{-}\infty}$ (ng*h/ml)	1043.5 (515.1 - 3684.6)	1185.8 (151.5 - 2085.7)	0.61
Cmax (ng/ml)	56.2 (9.6 - 165)	47.2 (11.3 - 110)	0.32
Ke (h)	0.021 (0.008 - 0.081)	0.027 (0.004 - 0.693)	0.72
Tmax (h)	3 (1 - 62)	3 (0.5 - 64)	0.45

*Kruskal-Wallis test

Table 3.27. DHA PK parameters in non-pregnant by treatment arm

DHA PK parameters, median (range)	Non Pregnant		p-value*
	3-day (n=24)	5-day (n=24)	
$AUC_{0\text{-last}}$ (ng*h/ml)	1524 (237.8 - 4609.3)	2102.2 (341.6 - 6652.9)	0.06
$AUC_{0\text{-}\infty}$ (ng*h/ml)	1915.5(616.2 - 4726.3)	2466.2 (805.04 - 6869.5)	0.19
Cmax (ng/ml)	70 (10.4 - 198)	97.7 (19.5 - 213)	0.24
Ke (h)	0.03 (0.009 - 0.119)	0.02 (0.003 - 0.084)	0.41
Tmax (h)	4.8 (0.5 - 62)	9 (0.5 - 64)	0.93

*Kruskal-Wallis test

Table 3.28. Comparison of DHA PK parameters in pregnant *versus* non-pregnant in the 3-day arm

DHA PK parameters, median (range)	3-day treatment		p-value*
	Pregnant (n=24)	Non pregnant (n=24)	
$AUC_{0\text{-last}}$ (ng*h/ml)	777.9 (221.6 - 3339.7)	1524 (237.8 - 4609.3)	0.28
$AUC_{0\text{-}\infty}$ (ng*h/ml)	1043.5 (515.1 - 3684.6)	1915.5 (616.2 - 4726.3)	0.24
Cmax (ng/ml)	56.2 (9.6 - 165)	70 (10.4 - 198)	0.21
Ke (h)	0.021 (0.008 - 0.081)	0.032 (0.009 - 0.119)	0.24
Tmax (h)	3 (1 - 62)	4.8 (0.5 - 62)	0.40

*Kruskal-Wallis test

Table 3.29. Comparison of DHA PK parameters in pregnant *versus* non-pregnant in the 5-day arm

DHA PK parameters, median (range)	5-day treatment		p-value*
	Pregnant (n=24)	Non-pregnant (n=24)	
$AUC_{0\text{-last}}$ (ng*h/ml)	926.4 (142.4 - 2959.5)	2102.2 (341.6 - 6652.9)	<0.01
$AUC_{0\text{-}\infty}$ (ng*h/ml)	1185.8 (151.5 - 2085.7)	2466.2 (805.04 - 6869.5)	<0.01
Cmax (ng/ml)	47.2 (11.3 - 110)	97.7 (19.5 - 213)	<0.01
Ke (h)	0.027 (0.004 - 0.693)	0.02 (0.003 - 0.084)	0.95
Tmax (h)	3 (0.5 - 64)	9 (0.5 - 64)	0.14

*Kruskal-Wallis test

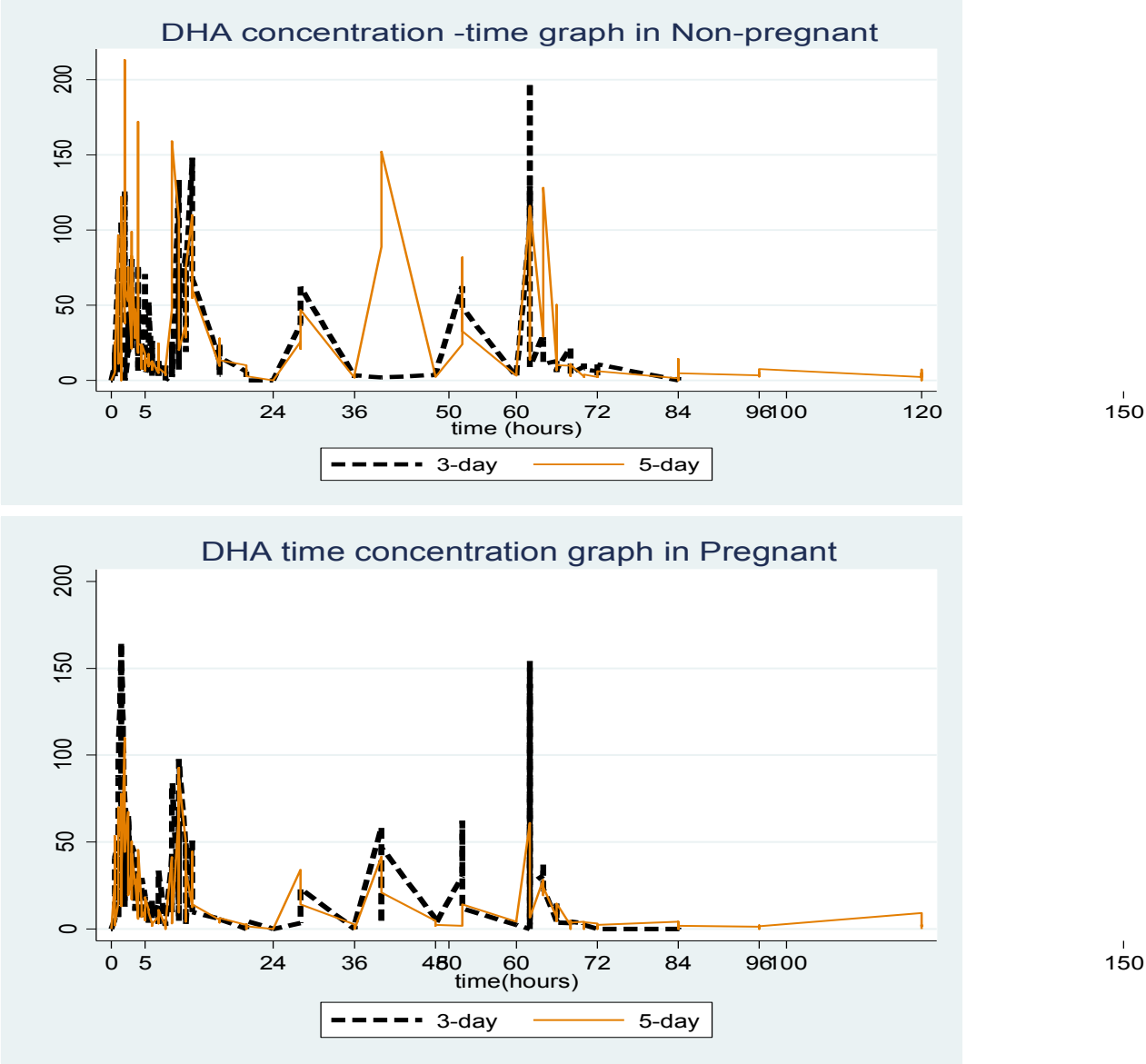


Figure 3.7. DHA plasma time concentration profile in pregnant and non-pregnant subjects in the two treatment regimens

3.3.7.8 Correlation between Day 7 plasma lumefantrine levels and weight

In the linear regression using day 7 lumefantrine as dependent variable and age, weight, height, haematocrit, Body Mass Index (BMI) and parasitaemia at admission as independent variables, a positive association was found between BMI and the day 7 lumefantrine concentration in both pregnant and non-pregnant women irrespective of

the treatment received: in pregnant slope 0.063, p= <0.01 and in non-pregnant slope 0.088, p<0.01) (Table 3.30).

The parasitaemia at admission and age were also found to be positively associated with the plasma lumefantrine concentration in non-pregnant women treated with the 5-day arm, respectively; slope 718.76, p<0.01 and slope 85.87 and p<0.01 (Table 3.30).

Table 3.30. Linear regression of lumefantrine concentration at day 7

Lumefantrine concentration at day 7	Group	Coefficient	Standard Error	t	P>|t|	95% CI
BMI	Non-pregnant	0.088	0.03	3.24	0.002	0.03 - 0.14
BMI	Pregnant	0.063	0.02	2.75	0.009	0.02 - 0.11
Parasitaemia at admission	Non-pregnant (5-day arm)	718.76	206.5	3.48	0.002	290.62 - 1146.92
Age	Non-pregnant (5-day arm)	85.87	26.68	3.22	0.004	30.54 - 141.20

3.3.8 Pharmacokinetic nonlinear mixed-effects modelling

3.3.8.1 Lumefantrine/desbutyl-lumefantrine pharmacokinetics

A total of 816 samples were analysed for lumefantrine and 712 samples were analysed for desbutyl-lumefantrine. A two-compartment disposition model were used to describe the pharmacokinetics for both lumefantrine and desbutyl-lumefantrine and were superior to other investigated disposition models (p<0.05). A transit absorption model with four transit compartments were found superior to all other absorption models (p<0.05). The addition of relative bioavailability improved the model fit significantly

(p<0.05) and was therefore retained in the final model. A graphical illustration of the model is presented in Figure 3.8.

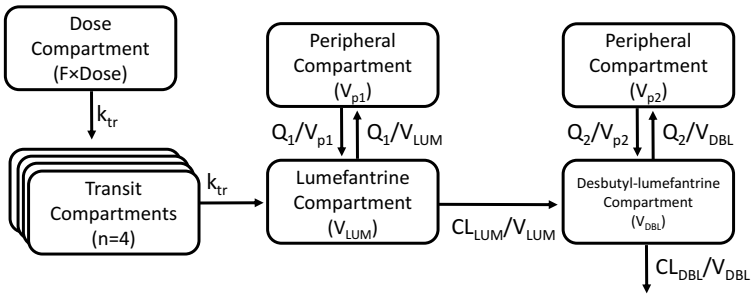


Figure 3.8. Graphical representation of the pharmacokinetic model for lumefantrine and desbutyl-lumefantrine.

Pregnancy was found significant on both the elimination clearance of lumefantrine and on the elimination clearance of desbutyl-lumefantrine, resulting in an increase of lumefantrine elimination clearance (16.3%) and a decrease of desbutyl-lumefantrine elimination clearance (19.2%). The relative bioavailability was not different between the two dose regimens, suggesting that the dose-dependent absorption is not affected by an extended treatment regimen.

The goodness-of-fit plots (Figure 3.9) and the visual predictive check (Figure 3.10) of the final model showed a good description of the observed data. The final population pharmacokinetic parameters are summarised in Table 3.31. Secondary parameters (i.e. Cmax, Tmax, $AUC_{0-\infty}$, elimination half-life, and concentrations at day 7) are presented in Table 3.32.

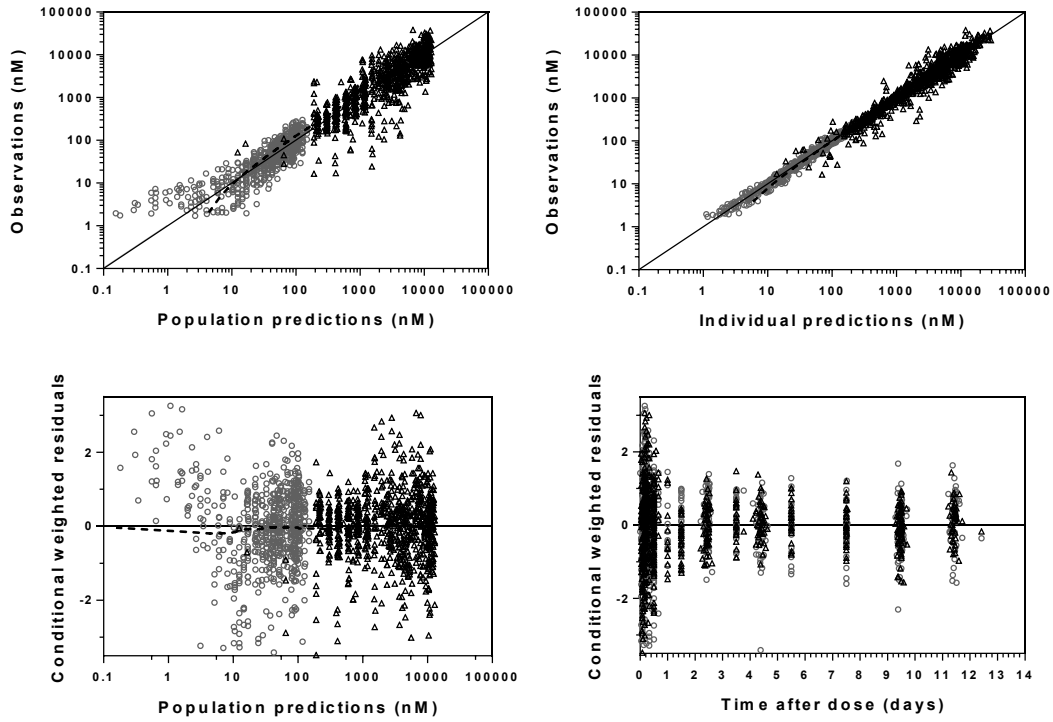


Figure 3.9. Goodness-of fit plots of the final model, triangles represents lumefantrine data and circles desbutyl-lumefantrine data.

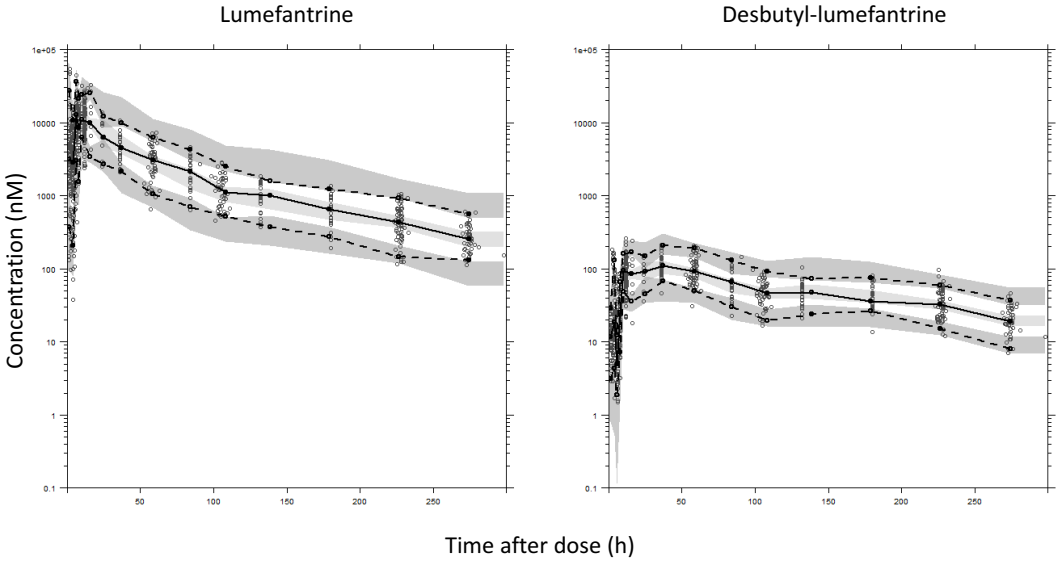


Figure 3.10. Visual predictive checks of the final pharmacokinetic model.

Table 3.31. Pharmacokinetic population model parameters for lumefantrine and desbutyl-lumefantrine.

Population parameters	Population estimates [%RSE]	%CV for BSV [%RSE]
Lumefantrine		
CL/F (L/h)	4.90 [5.00]	11.5 [17.2]
V_c/F (L)	166 [6.40]	25.1 [15.7]
Q/F (L/h)	1.24 [8.10]	-
V_p/F (L)	144 [6.20]	-
MTT (h)	5.6 [5.80]	60.7 [7.10]
No. of trans comp	4 *fix*	-
F (%)	100 *fix*	42.2 [6.80]
RUV	0.183 [10.2]	-
Desbutyl-lumefantrine		
CL/F (L/h)	318 [7.90]	-
V_c/F (L)	7,910 [14.3]	72.2 [14.8]
Q/F (L/h)	476 [12.5]	52.9 [14.4]
V_p/F (L)	25,200 [5.40]	-
RUV	0.0353 [13.2]	-
Covariates relationships		
$Pregnancy_{CL\text{-}LUM}$ (%)	16.3 [26.7]	-
$Pregnancy_{CL\text{-}DBL}$ (%)	-19.2 [17.3]	-

Legend: *CL/F* is the apparent elimination clearance. *Vc/F* is the apparent volume of distribution of the central compartment. *Q/F* is the inter-compartment clearance. V_P/F is the apparent volume of distribution of the peripheral compartment. *MTT* is the mean transit time of the absorption. *No. of trans comp* is the number of transit compartments used in the absorption model. *F* is the relative bioavailability. *RUV* is the variance of the unexplained residual variability. $Pregnancy_{CL\text{-}LUM}$ and $Pregnancy_{CL\text{-}DBL}$ represent the change in apparent elimination clearance of lumefantrine and desbutyl-lumefantrine between pregnant and non-pregnant patients. Coefficients of variation (%CV) for between-subject variability (BSV) were calculated as $100 \times (e^{variance} - 1)^{1/2}$. Relative standard errors (%RSE) were derived from the covariance step in NONMEM.

Table 3.32. Secondary parameters for lumefantrine

Secondary PK parameters	3 day treatment median (range)		5 day treatment median (range)	
	Non-pregnant (n=24)	Pregnant (n=24)	Non-pregnant (n=24)	Pregnant (n=24)
C_{MAX} (nM)	5,970 (2,560-11,600)	6,390 (3,880-12,300)	7,400 (2,960-18,000)	7,170 (3,600-12,000)
T_{MAX} (h)	7.50 (3.72-15.6)	7.33 (3.87-16.7)	7.56 (4.54-11.4)	8.89 (4.32-14.2)
$AUC_{0-\infty}$ (h*uM)	954 (481-2,070)	1,070 (562-1,910)	1,880 (682-4,300)	1,710 (871-3,010)
$T_{1/2}$ (days)	4.45 (4.23-4.75)	4.26 (4.13-4.47)	4.42 (4.17-4.65)	4.23 (4.08-4.69)
Day 7 conc. (nM)	986 (549-2,540)	1,050 (405-1,720)	3,150 (980-6,890)	2,760 (913-5,970)

Legend: C_{MAX} is the maximum concentration. T_{MAX} is the time after dose to reach the maximum concentration. $T_{1/2}$ is the terminal elimination half-life. $AUC_{0-\infty}$ is the area under the concentration-time curve from time 0 to infinity. *Day 7 conc.* is the individually predicted concentrations of lumefantrine at day 7.

Figure 3.11 shows the distribution of observed day 7 and 14 concentrations of lumefantrine for non-pregnant and pregnant patients and also differentiates between three and five day treatment. Simulations of the lumefantrine population mean for a typical pregnant and non-pregnant patient receiving a typical three day treatment is presented in Figure 3.12. Figure 3.12 also presents a simulation of lumefantrine concentration for a typical pregnant patient who receives the three day treatment and the five day treatment.

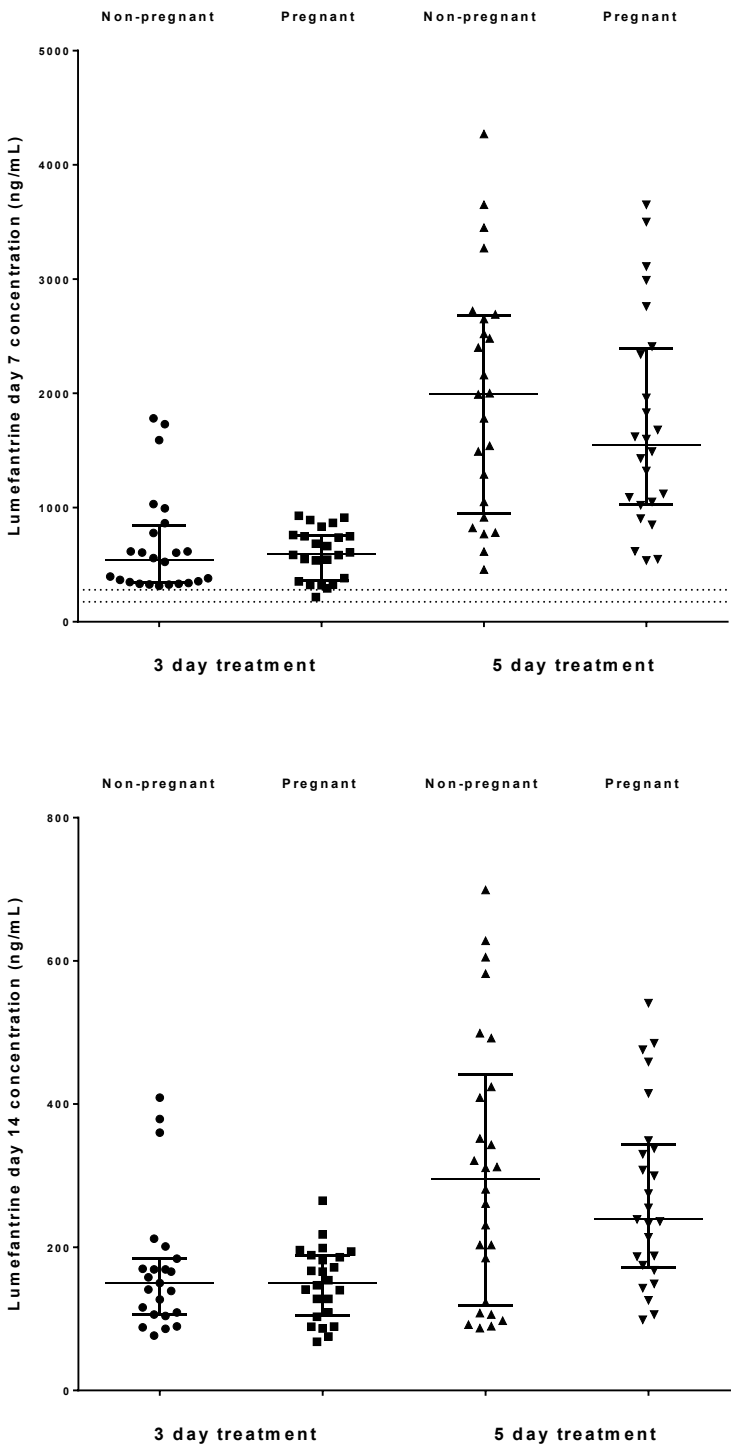


Figure 3.11. Observed individual concentrations at day 7 and 14.

Legend : The interval represents the mean standard deviation of the data. The upper dotted line represents the cut off of 280 ng/mL and the lower dotted lie represents the cut off of 175 ng/mL.

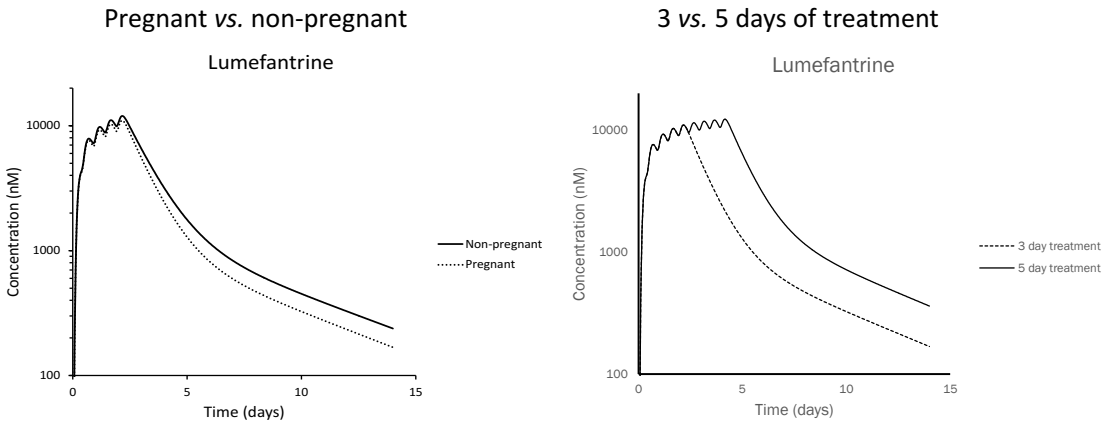


Figure 3.12. Simulations of lumefantrine concentration-time profiles for a typical pregnant and-non-pregnant patient receiving three days of artemether-lumefantrine, and simulated profiles for a typical pregnant patient receiving a three and five day treatment respectively.

3.3.8.2 Artemether and dihydroartemisinin pharmacokinetics

A total of 496 samples were analysed for artemether and 488 samples were analysed for dihydroartemisinin. A one-compartment disposition model was used to describe both the pharmacokinetics of artemether and dihydroartemisinin. A transit compartment model with six transit compartment were superior in describing the data compared to all other absorption models ($p<0.05$). The implementation of a relative bioavailability improved the model fit significantly ($p<0.05$) and was retained in the final model. A graphical representation of the model is illustrated in Figure 3.13.

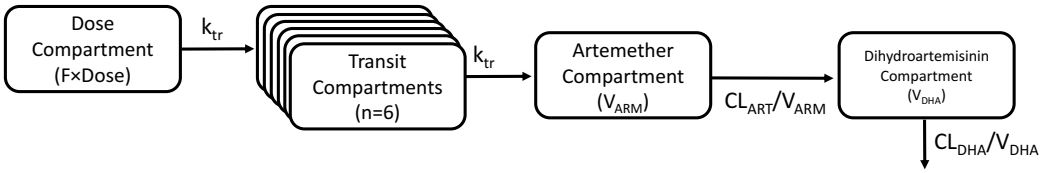


Figure 3.13. Graphical representation of the pharmacokinetic model for artemether and dihydroartemisinin.

Pregnancy was a significant categorical covariate on the relative bioavailability of artemether, resulting in a 35% decrease in relative bioavailability for pregnant patients. The impact on dihydroartemisinin exposure will be proportion to that established for artemether. Dose regimen (3 vs 5 days of dosing) was not found significant on relative bioavailability. The final model showed adequate goodness-of-fit (Figure 3.14) and visual predictive check (Figure 3.15). The final population pharmacokinetic parameters are summarised in Table 3.33. Secondary parameters (i.e. Cmax, Tmax, $AUC_{0-\infty}$, and elimination half-life) are presented in Table 3.34.

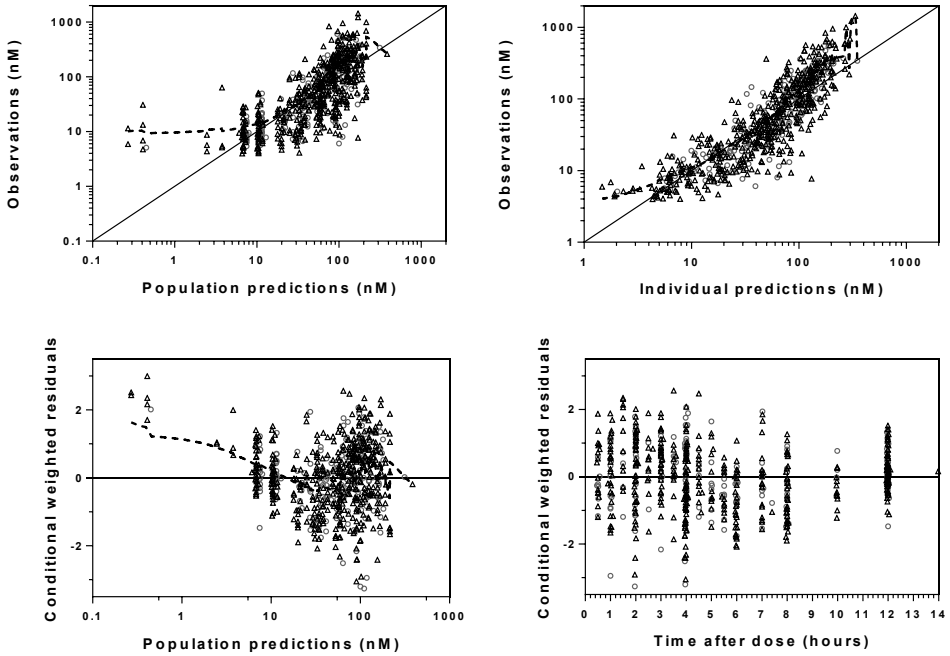


Figure 3.14. Goodness-of fit plots of the final model, triangles represents artemether data and circles dihydroartemisinin data.

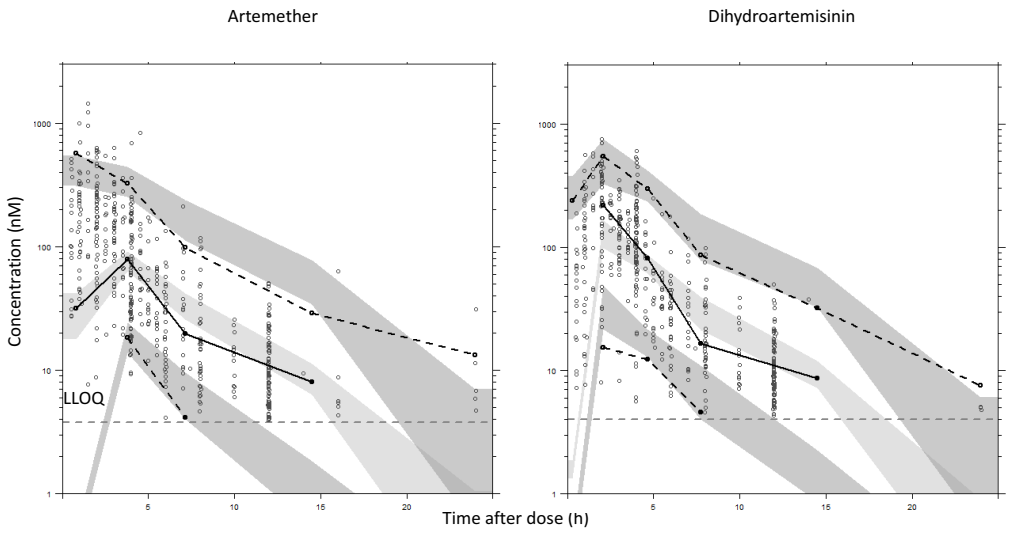


Figure 3.15. Visual predictive checks of the final pharmacokinetic model.

Table 3.33. Pharmacokinetic population model parameters for artemether and dihydroartemisinin.

Population parameters	Population estimates [%RSE]	%CV for BSV [%RSE]
Artemether		
CL/F (L/h)	324 [9.00]	21.3 [21.6]
V_c/F (L)	1,200 [9.90]	15.3 [42.0]
MTT (h)	0.487 [13.7]	67.0 [15.0]
No. of trans comp	6 *fix*	-
F (%)	100 *fix*	35.2 [13.1]
RUV	0.55 [4.10]	-
Dihydroartemisinin		
CL/F (L/h)	322 [7.90]	23.6 [33.2]
V_c/F (L)	101 [14.3]	-
RUV	0.477 [4.80]	-
Covariate relationships		
$Pregnancy_F$ (%)	-34.8 [18.0]	-

Legend: *CL/F* is the apparent elimination clearance. *Vc/F* is the apparent volume of distribution of the central compartment. *MTT* is the mean transit time of the absorption. *No. of trans comp* is the number of transit compartments used in the absorption model. *F* is the relative bioavailability. *RUV* is the variance of the unexplained residual variability. $Pregnancy_F$ represents the decrease in relative bioavailability

between pregnant and non-pregnant. Coefficients of variation (%CV) for between-subject variability (BSV) were calculated as $100 \times (e^{variance} - 1)^{1/2}$. Relative standard errors (%RSE) were derived from the covariance step in NONMEM.

Table 3.34. Secondary parameters for artemether

Secondary PK parameters	3 day treatment median (range)		5 day treatment median (range)	
	Non-pregnant (n=24)	**Pregnant (n=24)**	**Non-pregnant (n=24)**	**Pregnant (n=24)**
C_{MAX} (nM)	215 (114-410)	142 (84.1-269)	211 (117-398)	139 (95.2-212)
T_{MAX} (h)	0.87 (0.70-2.33)	0.82 (0.56-1.79)	0.82 (0.77-1.86)	0.86 (0.53-1.60)
$AUC_{0-\infty}$ (h*nM)	5,610 (2,250-12,000)	3,330 (1,870-7,880)	7,080 (4,020-15,100)	5,430 (2,980-7,540)
$T_{1/2}$ (hours)	2.71 (2.07-4.76)	2.44 (1.65-3.84)	2.35 (1.61-2.94)	2.39 (1.87-3.68)

C_{MAX} is the maximum concentration. T_{MAX} is the time after dose to reach the maximum concentration. $T_{1/2}$ is the terminal elimination half-life. $AUC_{0-\infty}$ is the area under the concentration-time curve from time 0 to infinity.

Simulations of the population mean of a typical pregnant and non-pregnant patient receiving a three-day treatment is presented in Figure 3.16. Simulations in NONMEM (n=200) to investigate the impact of pregnancy on the exposure to dihydroartemisinin can be found in Figure 3.17.

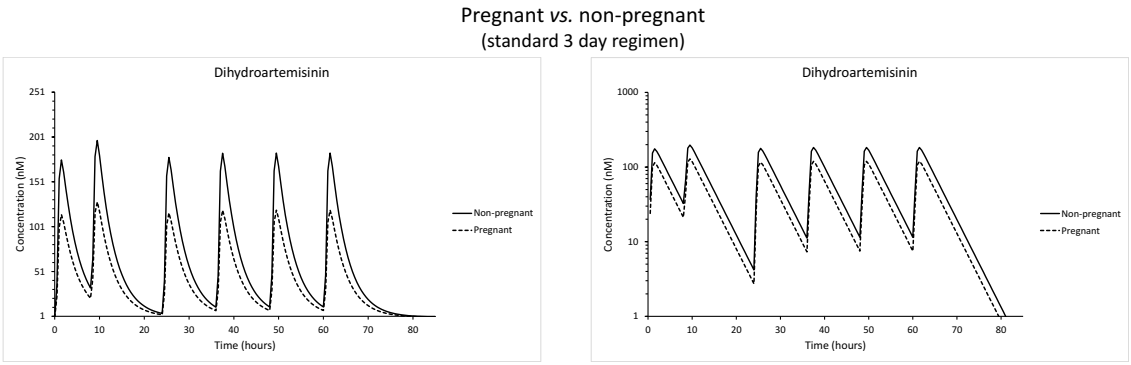


Figure 3.16. Simulations of dihydroartemisinin concentration-time profiles for a typical pregnant and-non-pregnant patient receiving artemether-lumefantrine.

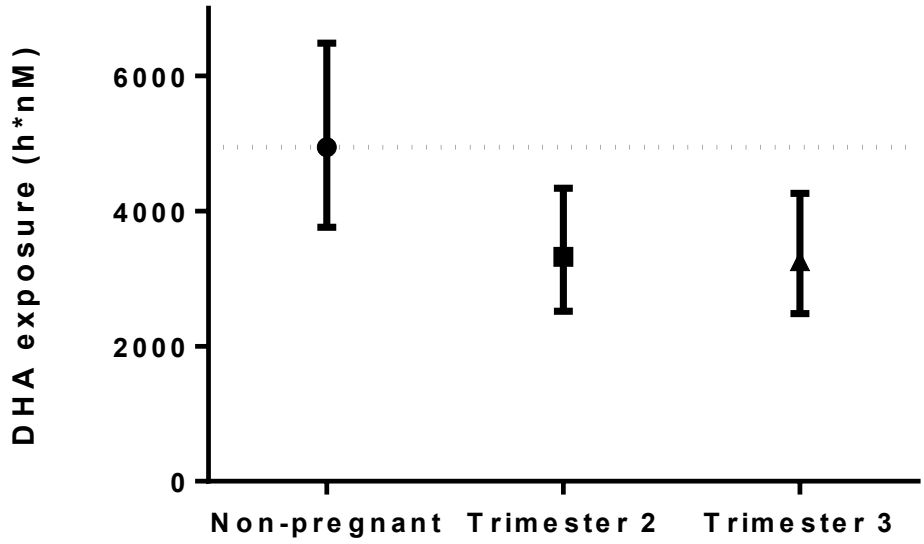


Figure 3.17. Simulated area-under the concentration-time curve (exposure) for dihydroartemisinin for non-pregnant patients and patients in their second and third trimester.

3.3.9 Safety and Tolerability

Sixty-six percentof patients (63/96) experienced at least one adverse event after drug administration. The intensity was graded as mild or moderate and they were all classified as non- related to the treatment administered.

The most common adverse events reported by the patients in the two treatment arm were headache and gastro - intestinal disorders (increased frequency abdominal pain and diarrhoea). In both treatment arms, gastro - intestinal disorders were mostly reported by pregnant subjects.

The most common adverse events in the laboratory findings were the variation of the biochemical and haematological parameters (an increase of AST, ALT and creatinine and a decrease of albumin level at discharge) (Table 3.35).

Women who received 5-day treatment had significantly more gastro-intestinal manifestations than those receiving the standard 3-day treatment (p=0.03).

Only one patient from the non-pregnant group vomited her first dose of AL within one hour and received a half dose again

There were no differences between the two treatment regimens for the other adverse events (Table 3.35).

Table 3.35. Most frequent adverse events by treatment arm and by group

	3-day			5-day			
	P	**NP**	**All**	**P**	**NP**	**All**	**p-value***
	N	**N**	**N (%)**	**N**	**N**	**N (%)**	
Gastrointestinal disorders	1	0	1 (2)	5	2	7 (14.6)	0.03
Headache	6	6	12 (25)	3	3	6 (12.5)	0.12
Influenza like syndrome	3	0	3 (6.3)	0	1	1(2)	0.31
Dizziness	3	2	5 (10.4)	1	2	3(6.3)	0.49
Back pain	1	0	1 (2)	1	0	1 (2)	1
Genital infection	0	0	0 (0)	2	0	2 (4.2)	0,16
High Blood Pressure	0	0	0 (0)	0	2	2 (4.2)	0,16
Epistaxis	1	0	1 (2)	1	0	1 (2)	1
Decreased total WBC	2	2	4(8.3)	0	1	1(2)	0.34
Decreased neutrophils	2	3	5(10.4)	1	0	1(2)	0.206
Increased ALT	1	0	1(2)	3	3	6(12.5)	0.23
Increased AST	3	5	8 (16.6)	2	3	5 (10.4)	0.55
Increased creatinine	4	3	7 (14.6)	5	4	9 (18.8)	0.60

*χ^2, Pearson

3.3.9.1 Follow-up of pregnant women until delivery and delivery outcome

Data on birth outcome were collected for all 48 pregnant women. Forty-one women (85.4%) had a full-term pregnancy (between ≥37 and 40 weeks) and 4 (8.3%) had an

early term pregnancy (between ≥ 28 to < 37 weeks). All 41 gave birth to a live baby whereas 3 women (6.3%) had a still birth (Table 3.36).

Four serious adverse events (SAE) occurred: two stillbirths and one neonatal sepsis in the group who received the 3day AL regimenand 1 stillbirth in the group who received the 5 day AL regimen.

In the first case the subject was enrolled in the 3rd trimester (GA week 28) and received 3 day of AL. The SAE occurred ten weeks after inclusion in the study. Two weeks before the delivery (at week 36), the mother had fever and headache for which she took paracetamol at home as self-medication. The day of delivery there was no audible fetal heart beat and she delivered a macerated stillborn. The event was classified as not related to the study drug and the alternative aetiology was a possible infection.

In the second case the mother was enrolled in the study in the 3rd trimester (GA week 33) and treated with 3day of AL. The SAE occurred eight weeks after inclusion in the study. The patient delivered at home a baby in breech presentation with the retention of the head in the vagina canal. By the time she was brought to hospital the baby was deceased. The event was classified as not related to the study drug.

The third case was of neonatal sepsis. The mother was enrolled in the 2nd trimester and treated with 3days of AL. The SAE occurred therefore 16 weeks after inclusion in the study. The patient had a prolonged labour complicated by peripartum asphyxia and a genital infection. The baby had a low apgar score at birth and his conditions worsened rapidly with fever and respiratory distress.The baby was monitored for ten days until complete recovery.The event was classified as not related to the study drug.

The fourth case was a stillbirth which occurred in the 5 day treatment arm. The mother was enrolled in the study at GA 34 weeks. A placental abruption occurred at 40 weeks.

The SAE occurred six weeks and two days after drug administration. The mother experienced pain and severe bleeding at home and arrived at hospital 24 hours later with no audible fetal heart-beat. The event was classified as not related to the study drug.

Only one case of neonatal malformation was observed in a baby of a woman treated with 5 days ofAL at 31 weeks of pregnancy. The child was born 8 weeks after mother's AL treatment with polydactylism of both hands. This malformation was reported as a family trait and therefore was classified as unlikely to have been caused by the treatment regimen (Table 3.36).

Table 3.36. Delivery outcome and adverse event in new-born and babies per treatment arm

Parameters	3 day AL (n=24)	5 day AL (n=24)	p-value *
Birth weight, grams, median(range)	3,040(2,300-4,190)	3,135(900-3,900)	0.75
LBW, n (%)	5 (22.7)	2 (9.1)	0.25
Premature, n (%)	3 (12.6)	1 (4.2)	0.36
Stillborn, n (%)	2 (8.3)	1 (4.2)	0.62
Male babies, n (%)	10 (41.7)	12 (50)	0.58
Malformation, n (%)	0 (0)	1 (4.2)	1
Median babiesHct ,% (range)	55 (43.8-65)	56.7 (33-74)	0.31
Head circumference, cm, median(range)	34 (31-37)	35 (31.5- 36)	0.52
Arm circumference, cm, median(range)	11 (9-13)	11.5 (9.5-14)	0.65
Length, cm, median (range)	49 (43-55)	49 (46-54)	0.89
Neonatal sepsis, n (%)	1	0	1
Baby's death (at 3 months), n (%)	0	2	0.49

*Fisher exact test

3.3.9.2 One year follow-up of infants

Babies were visited at 3, 6 and 12 months. Forty-one babies (85%) attended all visits up to 1 year and displayed normal physical and neurological development.

Two infant deaths occurred in the 5-day arm. In the first case, the baby died 3 weeks after the completion of her 3 month follow-up visit. The family reported that the baby died due complications from a severe episode of diarrhoea.In the second case, the baby died 5 weeks after his 6 month follow-up visit. The family reported that the baby presented with respiratory distress and received a blood transfusion. He died during the blood transfusion and the true aetiology was unknown

Two cases were lost to follow up at 12 months (one from each treatment arm).

3.4 Discussion

In this study I compared the pharmacokinetics, efficacy and tolerability of the extended 5-day treatment of artemether-lumefantrine to the standard 3-day treatment in a group of African pregnant womenand a control group of non-pregnant women with uncomplicated *P. falciparum* malaria. Non pregnant women were actively followed-up weekly for 42 days after treatment, whereas pregnant women were actively followed-up weekly for 42 days, then passively until delivery (if it did not occur before). The babies were followed up at 3, 6 and 12 months.

A sparse sampling approach was chosen for the measurement of PK parameters for artemether, DHA and lumefantrine.

The conventional pharmacokinetic approach (i.e. NCA) is often used when dense data are collected after the drug administration. A population modelling approach is

recommended when sparse data are collected, to allow a more comprehensive assessment of the pharmacokinetic properties of the drug (Ezzet and Karbwang 1998; Fda 1999).

In this study, the samples were collected at fixed and random time-points (seven sparse and 3 fixed time-points) and they were analysed by NCA and a population approach to maximise the information gained from the collected data. NCA has a number of limitations but provides an initial descriptive analysis of the PK properties that can be used as initial estimates in the modelling approach (Muir and Gomeni 2004). NCA is suitable to determine the degree of exposure following the administration of a drug (Gabrielsson and Weiner 2012) which was our primary aim (assessment of the exposure) in comparing the two AL regimens. NCA also allows the estimation of most basic PK parameters (AUC and Cmax) characterising the absorption , the distribution and the elimination of a drug after a single or multiple dose (Muir and Gomeni 2004).

All study subjects had an adequate clinical and parasitological response, PCR-corrected, indicating that the current standard regimen of 3-day artemether-lumefantrine is still effective in curing malaria in this study population, represented by pregnant and non-pregnant women living in a malaria endemic area.

The clinical results are supported by the pharmacokinetic data. The main PK parameters of lumefantrine i.e. AUCs, Cmax, Tmax were similar in pregnant and non-pregnant women after 3-day and 5-day treatment. However, there was a trend of decreasing exposures to lumefantrine in pregnant women compared to non-pregnant patients. This was supported by the more detailed mixed-effects analysis that demonstrated that pregnancy had a significant impact on the clearance of lumefantrine and therefore an expected lower drug exposure. Furthermore, pregnant patients showed

a substantially decreased exposure to artemether and dihydroartemisinin (35% reduction) which could potentially explain the slower parasite clearance half-life observed in these pregnant patients. This is in line with previous findings showing a reduced exposure to both artemether and dihydroartemisinin in pregnant patients (Tarning, Rijken, *et al.* 2012)(Tarning, Kloprogge, *et al.* 2012) .This is obviously a serious concern and highlights the need for dose adjustment in this group of patients. The recommended dose might be sufficient in a high transmission setting where acquired immunity is present. However, the findings in this study might explain the unacceptably low cure rates seen in low transmission areas (Tarning *et al.* 2009) since a reduced exposure to artemisinins might lead to a higher parasite burden to be eliminated by the partner drug. The trend of reduced lumefantrine exposure might then be enough to increase the clinical failure rate in this population.

The extended regimen consisted of the administration of AL for 5 days twice-daily, instead of 3-day twice-daily, thus adding 16 adult tablets to the current standard dosage, i.e. 320 mg artemether and 1920 mg lumefantrine (an increase of 67% for both artemether and lumefantrine).

The aim was to increase the dose per kilogram of body weight of both artemether and lumefantrine. This is particularly important in a population with a mean body weight above 65 kg as it has previously been reported that patients weighing more than 65 kg are at increased risk of treatment failure (Hatz 2008).

According to the recommended dosage, adults weighing more than 35 kg should receive 4 tablets of fixed AL formulation (20/120 mg) twice daily for 3 days. Considering the large variability in the BMI across different populations and in pregnancy, it is expected that subjects with a higher BMI receive less milligrams of drug per kilogram of bodyweight thus decreasing the drug exposure.

The median body weight in the study population was 53 kg, ranging from 44 kg to 95 kg, in pregnant women and 61.5, ranging from 44 to 90 kg, in non-pregnant women.

Subjects in the extended treatment received 60% more artemether and lumefantrine per kilogram of body weight than those who received the standard treatment.Regardless the length of the treatment received, pregnant women in their 3rd trimester received 14% less artemether and lumefantrine per kilogram of body weight in the 3-day arm and 18% less in the 5-day arm than those in the 2nd trimester or non–pregnant women. Whereas there was no difference between pregnant women, in their 2ndtrimester, and non-pregnant women.

Most data on efficacy and pharmacokinetic of artemisinin combination therapies in pregnancy, including artemether-lumefantrine, come from studies conducted in Asian women. These data are likely to differ from those collected in Sub Saharan Africa due to host specific differences such as the BMI and host-immunity, differences in the parasite, such as species, prevalence and the level of resistance toward specific drugs (Dellicour *et al.* 2007).

Pregnant subjects in the current study in the 3-day arm received a median dose of 51.4 mg per kg of body weight (range 33.9 - 69.3). However, because their BMI was higher than those in pregnant Asian population, they received a lower quantity of drug per kilogram of body weight compared to Asian pregnant women treated with the standard 3-day regimen (Table 3.37 and Table 3.38)(McGready, Stepniewska, Lindegardh, *et al.* 2006; McGready *et al.* 2008; Tarning *et al.* 2009; Tarning, Kloprogge, *et al.* 2012).

In another study conducted in Uganda, pregnant women treated with 3-day AL had a median bodyweight of 56.0 kg (range 44.0 – 74.0) similar to the one in our pregnant population, median bodyweight 60.5 kg (range 44 – 85). Thus, the lumefantrine dose (mg/kg) administered in ugandese women was 51.4 mg/kg ranging from 38.9 to 65.5,

similar to the lumefantrine median dose in the 3-day arm of 51.4 mg/kg (range 33.9 - 69.3) in the Congolese pregnant population (Piola *et al.* 2010)

Despite the lower median total dose of artemether –lumefantrine in this study population compared to the one in Asia, the drug at current standard dosage was still effective in the DRC. Perhaps there were other factors contributing to this good efficacy such as the background host immunity – which factor that can contribute to a good efficacy - that still exists at a certain level in this semi-immune pregnant population; or the drug administration under direct observation or the low parasitaemia. However, in the case of change in immunity level in the population (for instance a reduction of transmission, change from control to elimination phase of the disease) or a reduced parasite susceptibility to lumefantrine and artemether, attention has to be made on the total dosage of drug to be administered.

Table 3.37. Comparison of artemether and lumefantrine total dose per kg to literature value in the standard 3-day regimen in pregnant women

Country	N	Weight Kg median (range)	BMI	Total dose artemether mg/kgmedian (range)	Total dose lumefantrine mg/kg median (range)	Reference
DRC[#]	48	60.5 (44-85)	25.1 (19-30.5)	8.6 (5.7-11.6)	51.4 (33.9-69.3)	Onyamboko 2015
Thailand	13	47 (41-57)	NA	NA	NA	McGready 2006
Thailand	125	50±6 (35-65)	21.5±2.0 (17.5-26.7)	9.8 (1.5–13.7)	58.8 (8.7-82.3)	McGready 2008
Uganda	21	55 (49-88)	NA	8.7 (5.5-9.8)	NA	Tarning 2012
Uganda	152	58 (10)*	NA	NA	NA	Piola 2010
Uganda[#]	26	56.0 (44.0-74.0)	NA	NA	51.4 (38.9-65.5)	Klopoggre 2010
Tanzania	33	52 (40-80)	21.8 (16.5-30.1)	NA	NA	Mosha 2014

*Mean (IQR); [#]data on pregnant and non-pregnant with venous blood samples

Table 3.38. Comparison of artemether and lumefantrine total dose per kg to literature value in the standard 3-day regimen in non-pregnant women

Country	N	Weight Kg median (range)	BMI	Artemether mg/kg median (range)	Lumefantrine mg/kg (range)	Reference
DRC	48	52 (44-66)	19.6 (16.9-25.3)	9.2 (7.3-11.6)	55.4 (43.6-69.3)	Onyamboko 2015
Tanzania	22	48.5 (41-79)	20.3 (16.4-33.3)	NA	NA	Mosha2014
Uganda	17	49.0 (40.0-63.0)	NA	NA	58.8 (45.7-72.0)	Klopproge 2010

3.4.1 Pharmacokinetics of artemether and dihydroartemisinin

I n both treatment arms (3-day and 5-day), pregnant patient had a significantly lower artemether and dihydroartemisinin exposures (AUC) compared to non-pregnant women. The demonstrated lower bioavailability in pregnant patients might be explained by an effect on P-glycoproteins or an induction of CYP enzymes induced by pregnancy. The most likely is perhaps an altered first passage metabolism by the induction of CYP-enzymes.

Artemether and dihydroartemisinin was described by one compartment model which has previously been seen. A flexible transit compartment model was used to describe the absorption phase of the data. This has also been used previously, thought the number of transit compartments has varied.

These results (lower artemether and DHA exposure in pregnant compared to non pregnant patients) are consistent with those previously reported in pregnant and non-

pregnant Asian patients (McGready, Stepniewska, Lindegardh, *et al.* 2006; McGready, Stepniewska, Ward, *et al.* 2006; Tarning, Kloprogge, *et al.* 2012).

In a previous study conducted in Congolese women in the same area the PK parameters of artesunate/DHA were measured after a single oral dose of 200 mg artesunate in a group of pregnant women (2^{nd} and 3^{rd} trimester), the same pregnant women 3 months post-partum and a group of non-pregnant patients, all with asymptomatic *falciparum* malaria (Onyamboko *et al.* 2011).The results indicated that despite a rapid elimination of the parasite in all study subjects, pregnant subjects had a significantly lower drug exposure (AUC) of DHA compared to non-pregnant control, consistent with the results of the current study.

However, DHA levels were comparable in the pregnant subject during pregnancy and the same subject at three months post-partum.

From the concentration-time profile showing individual data at each time point, we showed that the exposure to artemether /DHA was longer with the extended regimen compared to the standard one (Figure 3.7) in both pregnant and non-pregnant, although the difference calculated was not significant.

These results are in agreement with those obtained from mathematical simulations of an extended regimen of 10-doses AL over 5 days in pregnant patients (Tarning *et al.* 2009).

The exposure provided by the standard 3-day AL regimen is assumed to cover two consecutive parasite cycles. With the extended regimen, the longer exposure should cover three consecutive *Plasmodium* cycles and therefore ensure a better cure rate.

This finding is of high value for regions where resistance to artemisinins is emerging. This is also important for populations where pregnant women achieve significantly

lower levels of artemether/DHA compared to non-pregnant with the current 3-day regimen.

From this Non Compartmental Analysis, there was no difference in exposure and maximal concentration levels for artemether and DHA between pregnant recipients of the two different treatments.

However, the same women (pregnant) compared to non-pregnant control treated for five days had a significantly lower exposure and a significantly lower $C_{max}$for artemether and DHA.

3.4.2 Pharmacokinetics of lumefantrine

Lumefantrine and desbutyl-lumefantrine were well described by two compartment model. Different compartmental structural models have been used previously to describe the pharmacokinetic properties of lumefantrine and its metabolite. One study used three compartments to describe lumefantrine pharmacokinetics and two disposition compartments for desbutyl-lumefantrine (Salman *et al.* 2011), while another used two compartments for lumefantrine and one for desbutyl-lumefantrine (Tarning *et al.* 2009). The discrepancy is probably due to different follow-up times in different studies. A flexible transit compartment model were used to describe the absorption, this has previously been used then modeling these drugs. There was a substantially lower impact of pregnancy on the lumefantrine exposure as compared to artemether/lumefantrine. This was also seen in the non-compartmental analysis and the observed day 7 concentrations were no statistical differences could be demonstrated. However, the reduced exposure to lumefantrine, probably due to an induced CYP3A4 activity in pregnant women, might be of importance in pregnant patients with no acquired immunity.

The results presented here are in agreement with those reported in Ugandans women (Kloprogge *et al.* 2013; Tarning *et al.* 2013).

The median terminal elimination half-life was 4.2 days in the pregnant and 4.4 days in non-pregnant group and similar to the results obtained by Ezzet *et al* in 266 non-pregnant Thai subjects (51 adults and 215 of all ages) of 3.2 days (Ezzet *et al.* 2000).

As expected, the plasma levels reached by women treated with a 5-day regimen were significantly higher than those achieved by women treated with 3-day regimen with an approximately 100% increase in the lumefantrine plasma AUCs. Thus, extending the current standard AL treatment from 6 doses to 10 doses would improve the lumefantrine exposure in pregnant subjects significantly. This would not be possible by increasing the daily dose as absorption of lumefantrine is dose dependent (Ezzet *et al.* 2000; Ashley, Stepniewska, Lindegardh, *et al.* 2007).

PK data obtained from Thai pregnant women treated with the standard regimen of artemether-lumefantrine (Tarning *et al.* 2009) were modelled to identify the best dosage necessary to achieve the lumefantrine target concentration at day 7 of 355 ng/ml (capillary blood) corresponding to 280 ng/ml venous blood (Tarning *et al.* 2009). The results of the modelling suggested that therapeutic exposures could be achieved by increasing the dose by 50% and extending the administration i.e. 6 tablets twice daily for 5 days instead of increasing the dose of 100% and maintaining the same length, i.e. 8 tablets twice daily for 3 days. An additional benefit of an extended regimen would be an increased exposure to the artemisinin components.

The increased length would allow exposure of three consecutives 3 parasites cycles (48 hours each) to artemether and DHA compared to only2 cycles with the standard treatment. The longer exposure to artemisinin would improve the early clinical parasitological response.

The mathematical modelling was done using data obtained from measuring drug concentration inthe capillary blood whereas in the current study the drug was measured from venous blood.

Concentration of lumefantrine in the capillary blood is known to be higher than in venous blood:

$$\ln lumefantrine\ concentration\ in\ capillary\ blood = 0.52 + 0.95 \ln lumefantrine\ concentration\ in\ venous\ blood$$

Thus the day 7 cut-off of 280 ng/ml in venous blood would correspond to <355 ng/ml in capillary blood (Van Vugt 1998; McGready *et al.* 2008).

Using this formula, the range (min–max) of lumefantrine level at day 7 from capillary blood of 498-3180 ng/mLobtained simulating10 doses for 5days would correspond to 399.5-2812.2 ng/mL in venous blood.

The values obtained with the simulation are somewhat lower than the values measured in the current clinical trial. The median lumefantrine level at day 7 in pregnant women treated with the 5-day regimen was 1,545ng/mLwith a range of 537-3650 ng/mL, thus 30 % higher than those expected from the modelling.

The difference observed could be explained by the fact that the data used for the simulations were collected in a Thai population, compared to the Congolese population studied in the current trial. Also, the conversion factor used to calculate the differences between capillary and venous concentrations might not be perfectly representative for the population studied here.

The predicted C_{max} of lumefantrine reached after administration of a 5-day regimen ranged from 3,600 to 12,000 ng/mL in pregnant patients and from 2,960 to 18,000 ng/mL in non-pregnant patients (population PK analysis). The corresponding simulated

Cmax from capillary blood ranged from 2,427 to 17,581 ng/mL, corresponding to 2,116 to 17,012 ng/mL in venous blood, show good agreement with the data collected here.

According to literature data, lumefantrine absorption is dose-limited (Ashley, Stepniewska, Lindegardh, *et al.* 2007) and reaches its maximal absorption at 5,600-9,000 ng/ml with the current standard dose of AL (Novartis 2015). By extending the AL treatment, we observed a peak of plasma concentration of 20,100 ng/mL in one pregnant women, median 14,260 ng/ml (range 8420 – 20,100) while the maximum reached with the 3-day treatment was 11,800 ng/mL in pregnant and 16,200 ng/mL in non-pregnant.These results are in agreement with the expected accumulation of lumefantrine after several days of dosing due to the long half-life. However, this raises the issue of potential toxicity of lumefantrine in the longer treatment linked to these high plasma concentrations reached. We did not observe an abnormal prolongation of QTc in the study population, which is the most likely expected adverse event of lumefantrine. However, due to the small sample size, our results are limited and further larger trials to study the effect of these high lumefantrine plasma concentrations with the extended treatment are needed.

These results therefore confirm laboratory population simulations where an increased duration of AL treatment to five days with 10 doses would allow to achieve a major impact on the lumefantrine concentrations at day 7 in pregnant patientswithout major dose increase (Tarning *et al.* 2009). However, the increase in day 7 lumefantrine concentration might be higher in this study population than that observed in pregnant Thai patients.

3.4.3 Lumefantrine concentration time profile

By extending treatment duration of AL in pregnant and non-pregnant patients, the plasma concentrations of lumefantrine in both groups were maintained above the MICfor longer –ie for nine days after the last dose - than in the 3-day regimen (Figure 3.5). This is expected as these patients have received a longer course of treatment.

This finding is of importance for areas of low transmission and for those with emerging artemisinins resistance as it can ensure an adequate cure rate of malaria episodes(Ezzet and Karbwang 1998; Ezzet *et al.* 2000).As described above,in the longer regimen, the plasma lumefantrine levels dropped below 280 ng/ml nine days after the last dose in both groups (pregnant and non- pregnant), which set the best time to evaluate the risk of treatment failure by lumefantrine plasma levels measure at Day 14.

We suggest in the light of our results to assess the risk of sub-therapeutic plasma concentrations at day 14 when administering the new extended regimen instead of using the current measurement at day 7. This needs to be explored further in future trials.

3.4.4 Day 7 lumefantrine levels

The plasma concentration of lumefantrine at day 7 has been proved to be an adequate surrogate of the plasma AUC. This parameter reflects the degree of exposure of the remained parasites to lumefantrine after surviving the drastic clearance by artemether/DHA

In our study population the venous plasma lumefantrine concentrations at day 7 were above the cut off for all patients, except for one patient, whose levels fall below the 280 ng/ml cut-off of failure risk. This unique patient cleared her parasitaemia adequately and had a treatment success raising attention on the role played by immunity on

parasite clearance in an immune population (McGready 2010). The day 7 lumefantrine concentration cut off in my study was a sensitive predictor of treatment failure as all cases primarily identified as failure had all lumefantrine levels above 280 ng/ml and were lately after PCR correction found to be new infections.

As said above, the two regimens allowed achieving median plasma lumefantrine levels adequate for effective cure rate in both pregnant and non-pregnant patients. The exposure reached with the extended regimen was significantly higher than levels reached with the standard one in both pregnant and non-pregnant subjects. However, within each treatment arm, level reached by pregnant subjects were comparable to those reached by non-pregnant subjects. So, the current standard 3-day regimen is still effective in curing malaria in pregnancy in our population.

However, these findings on plasma levels of lumefantrine at day 7 in the current trial differ from other studies. Results from modelling simulation in a cohort of 13 Thai pregnant subjects reported lower lumefantrine level at day 7 in pregnant compared to non-pregnant male adults (Tarning *et al.* 2009).Similar lower lumefantrine concentration in pregnant patients were found compared to levels in non-pregnant adult after observed AL doses (McGready, Stepniewska, Lindegardh, *et al.* 2006).Mosha *et al.*(Mosha, Guidi, *et al.* 2014) also reported lower lumefantrine level at day 7 in pregnant compared to non-pregnant Tanzanian patients after modelling but not at the level described in Thai studies inferring that AL is more efficacious in Africa than in Asia. The following explanations can address these differences: All these studies were conducted in setting with different levels of endemicity and of host immunity than ours. Patients in the current study were also heavier than those from these reports with a positive association found between BMI and day 7 lumefantrine levels in both P and NP patients. They also all received AL with milk regardless of body weight. Finally, a

difference in methodology and sample size can also explain the discrepancies: the data reported in the current trial are observed data from 96 patients and I compared pregnant women to non-pregnant controls, and not to non-pregnant adults in the general population or literature values as in many other studies.

We investigated other potential predictors of failure risk in our study population with linear regression. BMI wasfound to be associated in both groups (pregnant and non-pregnant) with plasma lumefantrine levels at day 7 and thus, significant predictor of treatment failure. Age and parasitaemia at admission were associated with lumefantrine levels at day 7 only in the non-pregnant group treated with the 5-day regimen. Tarning *et al* (Tarning *et al.* 2009) in a PK study of AL in Thai women in 2009 had found BMI and an high Estimated Gestational Age (EGA), as predictors for recrudescence.

3.4.5 Efficacy

The efficacies of the two treatment regimens compared in this study were similar with a 100 % cure rate PCR –corrected at day 42 and no early clinical failures both in pregnant and non-pregnant subjects. This is greater than the 95% target for antimalarial drug's efficacy (World Health Organization 2010; McGready *et al.* 2011). This high cure rate is observed in this specific population two years after the introduction of AL as a second first line treatment (in addition to amodiaquine – artesunate) for the treatment of uncomplicated malaria as part of the revised National Guidelines of Treatment of Malaria in the DRC (DRC-NMCP 2012a).

Our results at day 28 PCR corrected (100% efficacy) are consistent with AL reported efficacy (99.2%) in non-pregnant adults and children in the same area in 2008. They contrast however with their day 42 PCR corrected efficacy which was 88.1 % (Tshefu 2010).

More recently, in a trial conducted earlier in children from the same study area, and comparing the efficacy of three ACTs, the cure rate observed in the group randomized under AL was 96.8% (Onyamboko *et al.* 2014)

Our study patients had all a confirmed positive slides for malaria and were symptomatic or not at the time of inclusion in the trial. Despite the pregnancy status that affects the level of immunity towards malaria, the treatment success was equally observed in pregnant and non-pregnant patient from both treatment arms.

This high efficacy and the fast clearance still observed with the 3-day treatment can be explained by several factors. The background immunity developed by our patients living in a high transmission area which for pregnant still confer them a partial immunity (Nosten *et al.* 1991a). The low availability of the drug in the national territory –although officially added to national treatment guidelines two years ago –can also explain the high cure rate observed as the parasite has not been exposed to the drug to develop mutation and there is no yet confirmed resistance in the area. AL was also taken under direct observation with milk which has been demonstrated to increase the lumefantrine absorption and bio availability by 16-fold and by 2-fold for artemether (which is lipid soluble and has also an increased bioavailability when taken with food) (Wernsdorfer 2004; Stover *et al.* 2012; Novartis 2015) . Outside the setting of a trial, the unsupervised dose intake can be lower and the efficacy reduced especially in the shorter standard regimen.

Efficacy reports from other sub-Saharan countries in pregnant are comparable with our findings while our high cure rate contrast with those reported in Southest Asia. In Uganda, Piola *et al* (2010) have found a comparable high efficacy of 98.2% in the studied pregnant population partially explained by the existing premunition in this mesoendemic area. Another pregnant population in Tanzania had had lower efficacy

levels (87% in pregnant, PCR uncorrected and 95% in non-pregnant) than ours which could be explained by the difference in developed premunition due to different transmission setting between our two areas of studies (Mosha, Guidi, *et al.* 2014).

In Southest Asia, AL efficacy in pregnancy is reportedly lower than what we have observed and these reduced efficacies can be explained mainly by the resistance toward artemisinin derivatives developed in this area and the low level of immunity in population from these settings where malaria is seasonal (McGready *et al.* 2006a, 2008; Tarning *et al.* 2009; Piola *et al.* 2010; Ashley *et al.* 2014; Mosha *et al.* 2014a).

The two treatment regimens were associated with a rapid clearance of parasitemia in both pregnant and non –pregnant subjects.The background immunity in this population is probably an important parameter acting in the rapid clearance seen in this population (McGready *et al.* 2008). The extended regimen might have had an effect on any subpatent parasitaemia but this is difficult to demonstrate as the study was not powered to show such a difference.

However, the PCt1/2 was longer in the pregnant group than in the non-pregnant one. The levels of premunition and parasite resistance are two factors that potentially explain the prolonged carriage of parasites in pregnancy (McGready *et al.* 2011). In our study population, the lower immunity in pregnant women compared to non-pregnant can explain the longer PCT observed in this group.

McGready *et al.* in previous works on drug's efficacy in pregnancy has suggested that the 42d period for efficacy assessment of a drug in general population might not be adequate in pregnancy as recrudescence could occur long after the administration of a rapidly eliminated drugs (McGready *et al.* 2008).In the current study, all 48 pregnant

women were followed up to day 42 then to delivery. From the 19 positive slides observed with these two follow up, only four were observed within the 42 day follow up period. However, the 19 positive slides were new infections. In an area of high transmission as the current study area, the parasite variability is high and the chance to have a new infection in case of recurrence is very high. Thus, this suggested longer follow up for efficacy assessment although important in area of low transmission might not be useful in our setting.

Few patients had initial gametocytaemia in our population with comparable proportion of patients in the two arms (8% in the 3-day arm and 15 % in the 5-day, p=0.52). These figures are considerably lower than the 30% initial gametocytaemia observed in paediatric patients from the same area (Onyamboko *et al.* 2014). However, the gametocyte clearance rate did not differ between groups and last for 3 weeks in the two arms (p=1). This 3 weeks gametocyte carriage was either consistent or longer than many reported gametocyte carriage duration observed with AL treatment highlighting the high level of transmission in the area (Bousema *et al.* 2010; The Four Artemisinin-Based Combinations (4ABC) Study Group 2011; Price 2013; Sawa *et al.* 2013).

3.4.6 Safety

The extended regimen had a good safety profile and no dose –related effect has been defined.

There were no major changes in haematological parameters studied, i.e. total WBC count, neutrophil and lymphocytes count, and the median levels reached at discharge were comparable and within normal ranges in both groups of patients and between the two treatment arms. However, in both treatment arm and in both groups, there were few

cases of leukopenia and neutropenia at at discharge. This can indicate the need of further investigations of haematologicalparameters changes in the extended regimen.

The six individual cases of neutropenia at discharge observed in the entire cohort of patients were equally distributed between groups (3 pregnant and 3 non-pregnant) but more frequent in the 3-day arm (five cases in the 3-day arm and one in the 5-day arm). The drop in neutrophil count observed was slight and not clinically significant and fast resumed to normality within a week. The patients remained healthy until the end of their six weeks follow-up without developing any other adverse event. The absence of clinical manifestation associated to neutropenia and the rapid resolution of the condition couldn't allow ascertaining a relation with the study drug. These findings could be reassuring and suggest if any, a low level of toxicity.

In a study in Western Cambodia, 28 healthy volunteers were treated with higher dose ofartesunate (6 mg/kg per day for seven days) and compared to 115 subject treated withthe standard doses (2 or 4 mg/kg). Six subjects (19%) in the high-dose arm developed neutropenia and required the discontinuation of the treatment (Bethell *et al.* 2011). These findings were not clinically significant and the patients fully recovered 48 hours later. The authors ascertained the safety of artesunate for malaria treatment but suggested the dose –limit have been reached for artesunate (Bethell *et al.* 2010).

Lumefantrine absorption is dose-limited (Ashley 2006), but this has not been described for artemether absorption. By extending the treatment duration we have increased the total dose of both drugs.

Regarding artemether, the median dose administered in the 5-day armin our study was 13.5 mg/kg, ranging from 8.4 to 18.2 which is less than the 20 mg/kg which is considered the toxicity limit (Yin *et al.* 2014). We did not observe significant differences in the neutrophils count between arms.

Artemisinin derivatives have been also associatedto transient rise in creatinine and liver enzyme levels. In our cohort, renal and liver function was not affected by the treatments received with comparable median levels of creatinine, AST and ALT between baseline and discharge in both group and both treatment arms.

However, at individual level, we observed some case of abnormal increase in ALT, AST and creatinin concentrations in patients from both groups and from both treatment regimen.

In the extended regimen, six patients had elevated AL at discharge with three having already high levels at entry. Similarly for AST in the 5-day arm, five patients had elevated AST above the range of normality with three of them having already higher levels than normal at entry. Five pregnant women treated with the 5-day arm had also elevated creatinine level at discharge. For these three parameters, these elevated abnormal values were not clinically significant for any patient. Half of the patients affected had had a baseline level already above the upper normal limit at entry which translate a different metabolism in these patients and mitigate the potential impact seen with the extended regimen in theses parameters. The early onset of these increases – which were observed by hour 96 in the extended regimen- also, is consistent with a non-toxicity of the extended regimen as hepatotoxic drug usually causes hepatotoxicity after a longer exposition to the drug. And an increase inplasma levels for these three parameters was also seen in some patients treated with the current 3-day regimen therefore relativizing the potential impact of the extended regimen on these transient increases changes. The observed changes for the three parameters were reversible in all affected patients. And the alteration of these liver enzyme levels and creatinine could also be attributed to malaria itself (Krogstad 1995; Singh *et al.* 2015).

Lumefantrine at therapeutic dosage is known to prolonge the QTc interval in human and artemether and artemether have the same effect when used in animal model atdaily high doses of 15 to 20 mg/kg(in dogs) and 50 mg/kg/day (in rats) with an alteration of the ST segment (Brewer *et al.* 1994; Nontprasert *et al.* 2002). In our cohort, we have observed a small increase between baseline and post treatment QTc measures with both treatment regimens. And in non-pregnant women treated with the extended regimen, the difference between baseline and post treatment values was significant. However, this has no clinical significance as all QTc values at discharge in the entire study population were inferior to 450 ms and no patients had a difference between baseline and post treatment greater than 60 ms. So, the prolongation of the QTc interval was within the range of normality for all patients. Furthermore, the dose range used with the extended regimen are lower than the high toxic doses reported in different animals studies and was for both artemether and lumefantrine within the manufacturer target dose range. Finally, artemether in AL combination was administered orally with a different and lower absorption than the intramuscular formulation used in these studies.

Thus, our finding of no significant ECG alteration in post treatment is consistent with those reported byother authors who all concluded to the AL harmlessness on cardiac function at therapeutic doses (von Seidlein *et al.* 1997; van Vugt *et al.* 1999; Bakshi *et al.* 2000; Wernsdorfer 2004; Hatz 2008). However, all our patients had a normal ECG tracing at entry ie prior treatment with AL and careful monitoring is still needed if the high dosage is used in patients with altered ECG (Novartis 2015).

The unique case of congenital abnormally was observed in a baby born from a pregnant women enrolled in the 5-day regimen. It was a case of polydactyly with one extra finger on both hands. The observed proportion of congenital abnormally in our series (2%) is lower than the estimated global prevalence of 3% (Penchaszadeh 2002).

Although other anomalies have been reported to be linked to artemisinin derivatives exposures, polydactyly is more likely to be the result of genetic (gene mutation) causes rather than of AL exposure (Naruse *et al.* 2010; World Health Organization 2015).

3.5 Conclusions

The results ofthis trial showed that the standard regimen of artemether-lumefantrine is still effective in curing malaria in pregnancy in the study area.

Artemether-lumefantrine administered for 3 days provided satisfactory plasma levels for both components of the ACT, artemether and lumefantrine in the pregnant and non-pregnant groups for cure. However, pregnant patients showed a 35% reduced exposure to artemether and dihydroartemisinin as compared to non-pregnant women, and a trend of reduced exposure to lumefantrine. This could potentially increase the risk of treatment failures and the development of drug resistant parasites in a larger population. This highlights the need for dose adjustment in this vulnerable population, especially in low transmission settings were the acquired immunity is relatively low.

The extended regimen improved drug exposure to all drug components substantially and provided an extended high terminal exposure of lumefantrine for a sustained period of time.

The study was not powered to show a difference in the two arms regarding safety and tolerability. However, the results showed that both regimens were well tolerated and generally safe. The transient changes observed in some haematological, renal and liver function parameters in women who received the extended regimenwere without clinical significance. However further larger studies need to be conducted to assess this safety issue.

The follow-up of pregnant women until delivery and of the babies for one year did not raise any concerns with either regimen.

In this trial the drug was administered under direct observation. It is likely that the compliance is reduced in real life conditions, especially with the 5-days treatment, which would represent a major drawback.

In the light of these results the standard regimen would be still recommended in the country for treating pregnant women with uncomplicated malaria.However, the extended 10-doses over 5 days regimeis a promising option for populations with lower host immunity and in areas where the parasite resistance to artemisinin based combination therapies is emerging.

4 Placental histopathological changes associated with falciparum malaria infection: comparison in two different artemether-lumefantrine treatment regimens

4.1 Introduction

Placental malaria infection has deleterious consequences for the mother, the fetus and the neonate through the increased risk of intra-uterine growth restriction (IUGR), Low Birth Weight (LBW), maternal anaemia, the risk of premature birth or ultimately pregnancy loss (Menendez 1995; Menendez *et al.* 2000; Rogerson *et al.* 2003; Brabin *et al.* 2004). In sub-Saharan Africa over 60 million of pregnant women are annually at risk of developing malaria (Desai *et al.* 2007; Dellicour *et al.* 2010) and depending on transmission levels, 25.8 % (range 19.7% - 31.9%) from East and Southern Africa to 39.9 % (range 34.2% - 45.7%) from West and Central Africa will develop malaria infection of the placenta (Walter *et al.* 1982; Cot *et al.* 2002; Chico *et al.* 2012; Takem and D'Alessandro 2013).

People living in areas of stable malaria transmission are constantly exposed to *P. falciparum* infections and consequently develop partial immunity which limits the level of parasitaemia and attenuates the severity of the disease. However, in pregnancy the previously acquired immunity is partially lost due to the pregnancy-related immune suppression and the hormonal changes (Costa *et al.* 2006; Rogerson *et al.* 2007). Thus the risk of developing the disease is higher in pregnant women, especially primi and secundgravidae, compared to non-pregnant women at least in part because their

immune system cannot control the infection (Menendez 1995; Duffy and Fried 1999; Guyatt and Snow 2004; Costa *et al.* 2006).

Furthermore pregnant women, in addition to their lower immunity, also have the disadvantage that during the infection parasitized erythrocytes can become sequestered in the placenta (Walter *et al.* 1982). Placental sequestration is a process where by falciparum-infected erythrocytes bind to the surface of villi via the adhesion molecule chondroitin sulfate A (CSA) through the VAR2CSA protein that parasites express on the surface of the infected erythrocyte.

During the erythrocytic stage of the parasite life cycle, merozoites invade red blood cells (RBC), mature through different forms to the schizont stage, and then rupture, releasing multiple new merozoites that will invade other RBCs and continue the cycle. During the later trophozoite and schizont stages, the parasite exports proteins to the surface of the infected RBC (*i*RBC), causing the formation of adhesive and antigenic'knobs' on the surface membrane of the *i*RBC. These knobs become the points of contact where the *i*RBC bind with the adhesion receptors on a number of host cells, including lymphocytes, platelets, trophoblastic cells and the endothelial cells lining vessels. As a consequence, during placental malaria, the placenta can contain a large number of *i*RBC (high parasitaemia) when parasites are absent or in a limited number in the circulating peripheral blood (Blacklock and Gordon 1925; Brabin 1983). Sequestration allows the parasite to avoid the immune system's surveillance, escape splenic clearance and thus facilitate parasite replication. This in turn aids development of severe infection and progression to symptoms (Beeson and Duffy 2005).

The process of sequestration is determined both by host factors and parasite phenotype. In pregnant women, *i*RBCs express a surface protein called PfEMP-1, coded for by the *var* geneVAR2CSA. This adhesion preferentially allows binding to CSA which is

strongly expressed on the surface of both cyto and syncitiotophoblastic cells lining villi. When mature *i*RBC arrive in the maternal blood space of the placenta they bind along these cells, filling and expanding the placenta intervillious spaces. This is associated with deposition of intervillous fibrin, arrival of host leukocytes and deposition of parasite pigment.

The presence of sequestered infected erythrocytes within the placental bed has secondary effects, both directly through binding to syncitiotrophoblastic cells of the villi, and initiating signalling within them, and also through the attraction of host inflammatory cells. These increase in the maternal blood space during placental malaria, which in its most florid form causes a massive 'intervillousitis'. The leukocytes, predominantly monocytes and activated lymphocytes, release proinflammatory cytokines and chemokines (Abrams *et al.* 2003), initiation of pro-coagulatory cascades and deposition of intervillous fibrin. Even after successive waves of schizogony, and in the face of antimalarial treatment, rupture of the infected schizonts leaves behind malaria pigment (haemozoin) which is adherent to red cell ghosts or phagocytosed by monocytes. This alters syncitiotophoblast (Lucchi *et al.* 2011) and monocyte function, induces release of cytokines such as TNF, and contributes to sustaining the pathological effects of a chronic infection on placental function (Kalilani-Phiri *et al.* 2013).

The placental changes described as more frequent and more marked in primiparae (Duffy 2007) as they do not display anti-adhesion antibodies, which only develop with multiple pregnancies and which hinder the adhesion of parasites to the CSA (Fried *et al.* 1998; Duffy and Fried 1999). Placental infection during maternal malaria has a direct effect on development of the materno-placental unit, and hence birth outcomes.

The mean placental weight is reported to be lower in the presence of a placental malaria infection (Walter *et al.* 1982).

Histologically, a normal placenta consists of three portions: the membranes, the umbilical cord and the disk (parenchyma with basal plate). The membranes consist of four layers: three of fetal origin, the amnion (lining the amniotic sac), the fibrous chorion (consisting of sparse collagen cells) and the intermediate trophoblast cells and one of maternal origin, the decidual layer (containing maternal cells, vessels and fibrin). The umbilical cord has two arteries and one vein embedded in a myxoid matrix called Wharton's Jelly. The disk has two surfaces: one fetal, the chorionic plate, and one maternal, the basal plate. The chorionic plate consists of an epithelium layer surrounding a collagenous matrix (the chorion) that contains several vessels of fetal origin and the fibrinoid layer. The two plates are separated by the intervillous space (villus parenchyma). The villus parenchyma makes up most of the placenta and consists of 40–60 trophoblast villi. The trophoblastic villus is the functional unit of placenta where diffusion and active transport of nutrients and waste products takes place. An outer layer of syncitiotrophoblast (the fetal villi) is in immediate contact with maternal blood in the intervillous space. The basal plate consists of two fibrinoid layers (the Rhor'sadjacent to intervillous space and Nitabuch layers) and vessels (where fibrinoid necrosis is often seen)(Benirschke 1998).

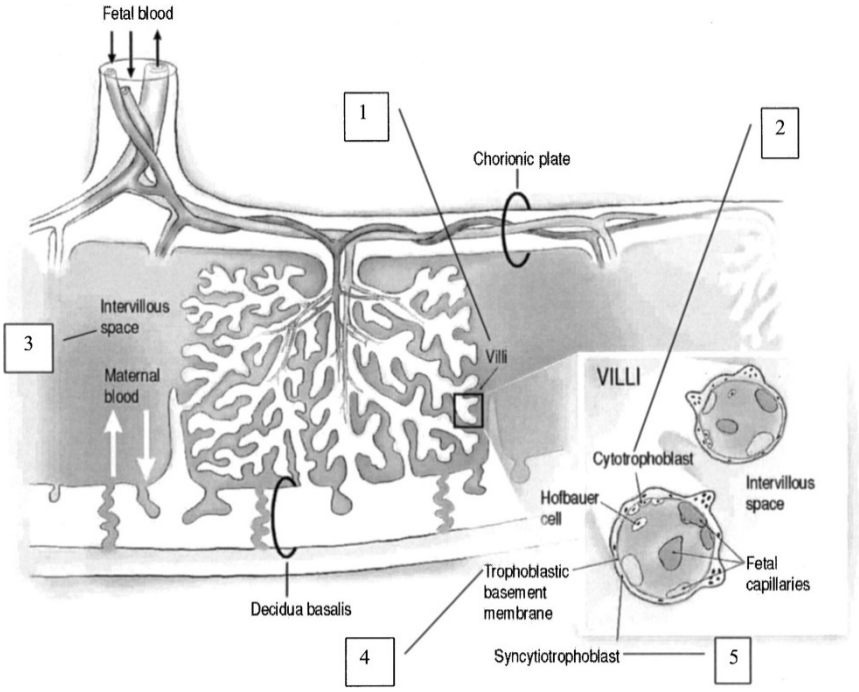


Image source:(McGready *et al.* 2004)

Figure 4.1. Schematic representation of the human placenta, with the inset showing a cross-section of terminal villi.

Legend : 1 = The villi are composed of fetal connective tissue and are covered by trophoblasts. **2** = The cytotrophoblast (Langhan's layer) is made up of distinctly separate cells that form a continuous layer in early pregnancy. **3** = Intervillous space (IVS). The severity of polymorphonuclear and mononuclear cellin filtratesise valuated within the IVS. Most villi are free in the IVS and are bathed in blood from the maternal vessels. **4** = Area of the trophoblastic membrane, **5** = The syncytiotrophoblast, the outer layer covering of the villi

Placental malaria (PM) is defined as the presence of *Plasmodium* infected RBCs in the maternal vascular regions of the placenta and the diagnosis can be only confirmed at delivery through the histological examination of the placental tissue (Bulmer *et al.* 1993).

Placenta integrity is vital for a healthy fetal development. However, sequestration of parasitized erythrocytes in the intervillous spaces of the placenta alters the dynamics of maternal-fetal gas and nutrient exchange. In response to sequestration, placental cells

secrete substances that recruit inflammatory cells that damage the placenta. Parasites and inflammatory cells recruited (such as macrophages), fibrin deposition and malaria pigment accumulate in the intervillious space. Relative hypoxia can exacerbate syncytial knotting of the villi, and cause thickening of the basement membrane. These pathological changes impair placental development and growth and the vascularization/nutrient transport function of the organ leading to fetal hypoxia, IUGR and LBW with a reduced survival of the neonate.

However, if a malaria episode is promptly diagnosed and correctly treated in the course of a pregnancy this precludes the occurrence of histological placental changes detectable at delivery. It is thought that if the peripheral infection is treated rapidly after its onset and with an adequate antimalarial, placenta integrity can be preserved, or, if it is already affected, it can return to normal functionality with few residual changes (Ismail *et al.* 2000; McGready *et al.* 2004; Muehlenbachs *et al.* 2012; Fried *et al.* 2013; Chaikitgosiyakul *et al.* 2014) with little impact on subsequent fetal development. Other factors also appear to affect the pathological changes of the placenta such as the frequency of malaria episodes and the time at which the episode occurs during the course of a pregnancy (McGready *et al.* 2004; Muehlenbachs *et al.* 2012).

The latter has an important impact on the reversibility of placental malaria lesions. The earlier the infection occurs in the course of a pregnancy, the greater the chance of developing placental malaria (Brabin 1983). This can be partially explained by a lower immune protection in early pregnancy or by the persistence of parasitaemia from the beginning of pregnancy throughout the course of pregnancy with more damage to the placenta than what could be observed with an infection in late pregnancy (Uneke 2008). The time of treatment initiation after the onset of the infection is also relevant as parasites might already be sequestered in the placenta (Kalilani-Phiri *et al.* 2013).

The pathological features of placental malaria at different stages of the disease have been histologically characterised using different systems. In the most commonly used classification of Bulmer *et al* (1993), PM is defined as 1) active if parasites are seen in the placenta, 2) active chronic if parasites and pigment are present, 3) past if only pigment are present and 4) no infection in the absence of both parasites and pigment. This classification is based on the observations on the progression of the disease from the early stage of the infection to the chronic stage post parasite clearance (Bulmer *et al.* 1993; Benirschke 1998). However, due to its limitation in capturing the complexity of acute histological changes, this classification was later revisited by Muehlenbachs and colleagues, who suggested a new scoring system which included a semi-quantitative analysis of the degree of inflammation and of the hemozoin deposition in fibrin (Muehlenbachs *et al.* 2010). According to this classification, inflammatory infiltrates and hemozoin deposition are the two parameters relevant to clinical outcomes and are both independently linked to poor birth outcomes. The classification is also suitable for use across areas of different endemicity and will allow in the future standardization and comparability of results from clinical trials. This scoring system was also applied to the current trial.

The classification system of Muehlenbachs is a semi-quantitative scoring method based on: 1) the assessment of the level of inflammation 2) the assessment of pigment deposition.

The presence of inflammatory infiltrates in placenta is linked to active malaria infection (and clinically, this presence is strongly associated with LBW and to a less extend to gravidity - particularly primigravidae). The inflammation score is qualitative and consists of 3 categories of increasing inflammatory infiltration of the intervillous space (Table 4.1).

Table 4.1. Histological classification of Placental Malaria 1. The inflammation score (Muehlenbachs' *et al* 2010)

Score	Description
Score I (Minimal inflammation)	Describes cases with no appreciable intervillous inflammation. Pigmented monocytes are rare and the white cell density in intervillous space is not increased above the level of peripheral white blood cells.
Score II (Inflammation present)	Describes cases with mononuclear cells sequestered in the intervillous space, particularly pigment-laden macrophages. This is a broad category describing the intermediate stage between no inflammation and massive intervillositis
Score III (Massive intervillositis)	Is a distinct entity where the intervillous space contains sheets of densely packed mononuclear cells

Haemozoin (malaria pigment) deposition in fibrin is taken as implying a pattern of a treated *falciparum* infection (past infection) and this pigment can persist long after a treated episode of malaria (McGready *et al.* 2002). The level of the deposition is correlated with repeated exposure, gravidity, the parasite density level at the time of delivery and the time of infection before delivery (Muehlenbachs *et al.* 2012). The pigment deposition score is a semi-quantitative method assessing the percentage of high power fields (light microscopy) positive for malaria pigment/hemozoin - in fibrin clot within the intervillous spaces (Table 4.2). This scoring method excludes pigment in erythrocytes or monocytes. Formalin pigment can be confused with malaria pigment and bias the estimation (Bulmer *et al.* 1993; Muehlenbachs *et al.* 2010). To avoid this artefact, biopsies initially fixed with the formalin should be rapidly transferred in ethanol 70% for longer term storage before they can be processed and embedded in paraffin.

Table 4.2. Histological classification of Placental Malaria 2. The pigment score (Muehlenbachs' *et al* 2010)

Score	Description
Score I	<10% of fields positive
Score II	10-40% of fields positive
Score III	>40% of fields positive

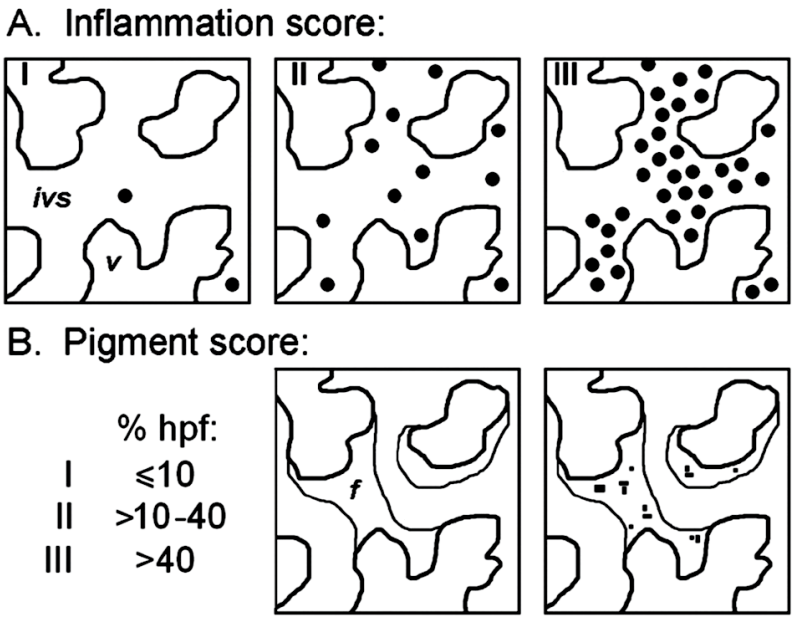


Figure 4.2. Schematic description of inflammation and pigment deposition score grading

Legend: Schematic demonstrating placental villi (v) for grading the histologic features of PM. A) Categories of maternal inflammation in the intervillous spaces (ivs): I- minimal; II-present; III- massive. B) Cut off values for categorizing malarial pigment deposition within intervillous fibrin (f). (% 60× high power fields). Image Source: Muehlenbachs 2010

As the histopathological features of placental malaria correlate with poor clinical outcomes such as reduced birth weight and maternal anaemia, they can be used as an

efficacy endpoint in clinical trials on drug efficacy (Parise *et al.* 1998; Cot *et al.* 2002; Muehlenbachs *et al.* 2010).

In this study, a placental biopsy was collected at delivery in 44 women. In the current chapter, I describe the histopathological features of these samples and correlate them with the clinical outcome.

4.2 Materials and methods

4.2.1 Sample collection and processing

Biopsies were performed on the placenta collected at delivery from 44 pregnant women enrolled in the main study, which compared two regimen of artemether-lumefantrine for treatment of malaria in uncomplicated malaria in pregnancy. In four cases the placenta was not available, in three cases because the delivery occurred outside the hospital and in one case because of the adverse pregnancy outcome.

4.2.1.1 Placental biopsy

A biopsy of the tissue was collected from the fresh placenta within four hours after delivery. For each placenta, the collection area was located midway between the insertion of the cord and the placenta edge where the membranes were inserted. A rectangular sample was cut out with a sterile scalpel, approximately 1 cm x 2 cm x 3 cm encompassing the maternal and fetal sides (Figure 4.3 and Figure 4.4). Each sample was transferred into a histological cassette identified with the patient's ID, date and time of collection (Figure 4.5). The sample was placed in a 10% formalin solution for a minimum 48 hours and a maximum of two weeks, at a temperature of 4°C. Thereafter, the formalin solution was replaced by a fresh solution of ethanol 70% and stored at 4°C until shipment to laboratory for further processing and histopathological examination at

MORU, Bangkok. The 10% neutral buffered formalin solution was chosen due to its optimal preservation of tissue morphology. This common fixative also allows the subsequent use of other fixatives and extended storage periods without major deleterious effects on the tissues.

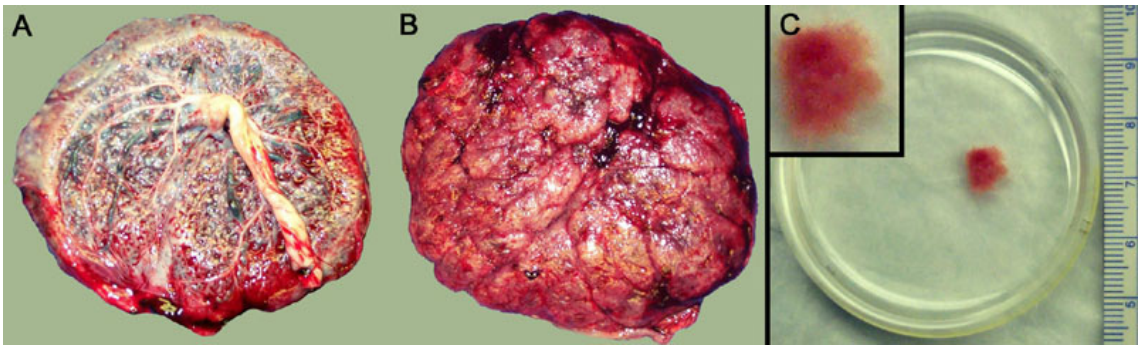


Figure 4.3. Maternal and fetal side of the placenta

Legend: A = fetal side with membranes up B= maternal sideC = rectangular sample measuring 1 x 2 x 3 cm

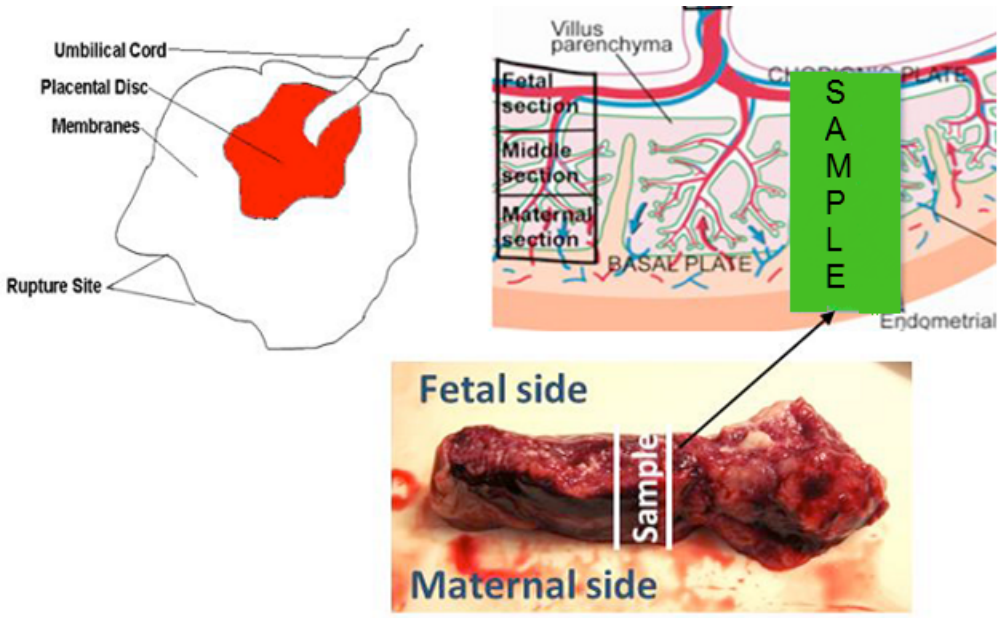


Figure 4.4. Placenta samples with maternal and fetal side encompassed

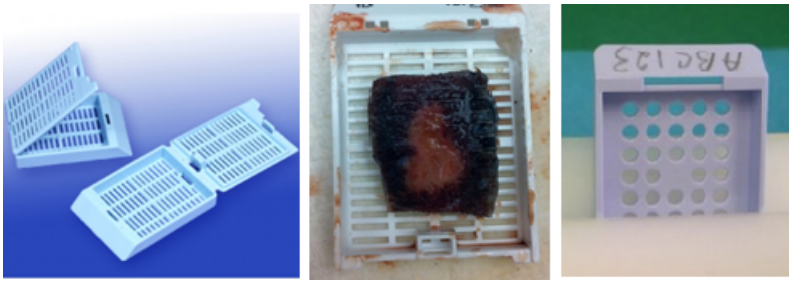


Figure 4.5. Placenta biopsy in a cassette

Source Figures 4.3; 4.4 and 4.5: Placental SOPs, Gareth Turner.

4.2.1.2 Preparation of specimens

The biopsies fixed in 10% formalin were embedded in wax and processed to produce paraffin embedded blocks by standard histopathological techniques. These blocks were subsequently cut on a microtome to make histological sections of 4 µm. The thin sections were mounted on slides, dried and stained with hematoxylin and eosin (H&E) or Giemsa stains for subsequent reading. The placental sections stained with Giemsa allowen hanced examination for the presence of infected RBCs in the maternal and fetal blood due to staining of parasite nuclei within *i*RBC.

4.2.1.3 Histopathological examination

The biopsies were examined by light microscopy by an experienced pathologist blinded to clinical data and study treatment. The following pathological patterns were examined:

- presence of infected erythrocytes
- presence of inflammation
- presence of fibrin deposition and of malaria pigments

- Examination of any pathological changes to the villi and their developmental stage.
- Any other background pathological features (such as red cell sickling, infarction or acute neutrophilic chorioamnonitis).

The classification scheme of Muehlenbachs described earlier was used to assess the features of placental malaria in the biopsies and assign them a malaria histopathological score (Muehlenbachs *et al.* 2010). Where sequestered iRBC were seen histologically, and estimate of the degree of parasitaemia in the placenta was made by quantitating the number of iRBC / total RBC in 1,000 red cells over randomly selected high powered fields (x 400 magnification).

4.2.2 Statistical analysis

Analyses were performed using Stata IC 14 (StataCorp, College Station, TX, USA). Continuous and categorical variables were analysed with Chi-square or Fisher's exact test. A p-value of less than 0.05 was considered as significant. Associations were tested using Spearman's rank correlation.

4.3 Results

4.3.1 Demographic information

The demographic characteristics of women from which placental samples were collected are presented in Table 4.3. They were comparable in the two treatment arms, with no statistically significant differences between the two groups except for the

proportion of previous abortion which was significantly greater in the 5-day treatment group (p=0.042).

Table 4.3. Demographic information of pregnant women from the placenta study

Characteristics	3-day Treatment arm	5-day Treatment arm
N	22	22
Age, years, median (range)	30.5 (20 - 40)	26 (20 - 41)
Weight, kg, median (range)	61.5 (47 - 85)	62.3 (50 - 90)
BMI, kg/m^2,median (range)	25.3 (19.4 - 30.5)	24.9 (17.3 - 40)
Estimated GA at inclusion, wks, median (range)	24.5 (13 - 34)	20 (14 - 36)
Primigravidae, n (%)	5 (22.7)	9 (41)
Previous abortion, n (%)	1 (4.5)	6 (27.3)
Sickle cell trait, n (%)	4 (18.2)	6 (27.3)
Hct at inclusion, %, median (range)	29.5 (24 - 36)	30 (25 - 36)
Peripheral parasitaemia at inclusion, /µl, median (range)	3,705 (144 - 70,336)	3,391.5 (160 - 104,499)
Hct at delivery, %, median (range)	35 (28 - 40)	36 (28 - 42)
Birthweight, gr, median (range)	3,040 (2,300 - 4,190)	3,230 (2,170 - 4,300)
Proportion of LBW, n (%)	5/22 (22.7)	2/22 (9.1)

4.3.2 Summary of results

Women either in the 2nd or 3rd trimester had *P. falciparum* malaria at the time of enrolment in the study and were successfully treated with either 3 or 5 days course of

artemether–lumefantrine. Four patients had a new episode of malaria during the 42 days follow-up. The clinical and parasitological data are presented in Chapter 3.

At delivery, 22 placenta biopsies were collected from women treated with 3-day regimen of artemether-lumefantrine and 22 from those who received the 5-day treatment. A blood sample was collected from the mother to measure peripheral parasitaemia (N=48) and two blood samples were collected from the neonate, one from the cord blood (N=44) and one from the capillary blood by heel-prick to detect eventual cases of congenital malaria (N=45).

The median placental weight was similar in the two arms: 605 grams (ranging from 430 to 730 grams) in the 3-day arm and 570 grams (ranging from 430 to 900 grams) in the 5-day arm (p-value=0.55).

Parasites were detected in 18.2% (8/44) of the slides prepared with the maternal peripheral blood and 2.3% (1/44) in those prepared with the cord blood.

Parasite iRBCs were observed in 38.6% (17/44) of the placental biopsies. Eight women with a malaria positive biopsy also had a malaria positive peripheral blood slide at delivery, whereas the remaining 9 had a negative peripheral blood slide (Table 4.4).

The median parasitaemia in malaria positive biopsies, expressed by the number of parasites per 100 iRBCs ranged from 20/1,000 to 97/1,000 iRBCs in heavy infections and from 2/1,000 to 17/1,000 iRBCs in low infections.

The risk of having placental malaria was 49 times higher in women presenting a positive peripheral parasitaemia at delivery than in those with negative malaria smear at delivery (OR 49.21, 95% CI: 3.04 - 797.8) (Table 4.4).

The presence of peripheral parasitaemia at delivery was also positively associated with pigment deposition in the placenta in the entire set of placenta samples ($\chi^2 = 8.58$, p =

0.01). However, when comparing the two parameters in the group of PM and NPM, there was no longer any association seen between the two parameters (p = 0.17).

The mother of the one baby who had iRBC positive cord blood at delivery had malaria during the 2nd trimester of pregnancy, with a peripheral blood parasitaemia of 416 parasites/µL. At delivery, the maternal peripheral blood slide was also positive with parasitaemia of 24,115 parasites/µL. Infected red blood cells were observed in the placental biopsy and parasites were seen only in the maternal erythrocytes and not in the fetal erythrocytes within the villi.

Table 4.4. Parasitaemia at delivery by placental malaria status

Maternal peripheral parasitaemia at delivery	Placental malaria		Total
	Positive	Negative	
Positive	8	0	8
Negative	9	27	36
Total	17	27	44

4.3.3 Placenta histopathological scoring

4.3.3.1 Classification of placental infection according to scoring system of Bulmer.

Among the 17 malaria positive biopsies, 9 samples were classified as active infections and 8 were classified as chronic infections. Three samples (6.8%) showed evidence of a past infection. The remaining 24 (54.5%) biopsies had a normal aspect with no signs of infection. There were no differences according to treatment received (3 or 5 days of AL).

Table 4.5. Classification of placental biopsies in the 44 samples

Classification		**N (%)**
Category 1	Active infection	9 (21)
	Active chronic	8 (18)
Category 2	Past infection	3 (6.8)
Category 3	No placental infection	24 (54.5)

4.3.3.2 Classification of placental infection according to scoring system of Muehlenbachs

4.3.3.2.1 Presence of malaria pigment

Overall, 81.8 % (36/44) of the biopsies had no pigment or less than 10% of pigment deposit (score 1). Seven biopsies, 15.9% (7/44) had 10-40% of field positives for pigment deposit (score 2). Only one case (2.3%) had more than 40% of field positives for the presence of pigments translating a heavy deposition of haemozoin in fibrin and was scored 3. In Table 4.6, the results are presented according to placental malaria classification.

Table 4.6. Malaria pigment deposition score by placental malaria classification

Pigment score	**Placental Malaria positive N (%)**	**Placental Malaria negative N (%)**
1 (pigment <10%)	11(64.7)	25(92.6)
2 (pigment 10-40%)	5 (29.4)	2 (7.4)
3 (pigment >40%)	1 (5.9)	0
Total	17	27

4.3.3.2.2 Score of inflammation

Nine samples (20.5%) had signs of inflammation (Score 2) whereas the majority of samples (79.5%; 35/44) had minimal or no signs of inflammation (Score 1). In Table 4.7, the results are presented according to placental malaria status.

Table 4.7. Score of inflammation per treatment arm in cases of PM

Inflammation score	Placental Malaria positive N (%)	Placental Malaria Negative N (%)
1 (minimal inflammation) n (%)	12 (70.6)	23 (85.2)
2 (inflammation present) n (%)	5 (29.4)	4 (14.8)
Total	17	27

4.3.4 Comparison of clinical parameters and placental malaria status

The ability of the fetus to grow depends on the placental function and its weight. The normal ratio between placenta weight and birth weight is 1:6 (Panti *et al.* 2012), but in the presence of a placental malaria infection, the weight is often reduced. The gestational age at delivery tends to be lower in the presence of placental malaria. The alteration of the dynamic of exchange between mother through the placenta and fetus can lead to early delivery (Menendez *et al.* 2000). In term of fetal morbidity, these effects can be measured using the weight at birth that can be reduced as a consequence of intrauterine growth retardation (IUGR) or/and preterm deliveries.

The three variables i.e. placental weight, birth-weight and GA at delivery were compared in women with placental malaria and those without. There was no difference

in the median birth-weight and in the median weight of placenta in women who displayed an active infection of the placenta compared to those who did not. Similarly, there was no reduction of Gestational Age at delivery in the two groups (Table 4.8).

Among women with placental malaria the median placental weight was significantly higher in the primiparae (620 grams ranging from 620 to 640 grams) compared to multiparae (570 grams, ranging from 430 to 660 grams, p=0.03). The placenta weight was comparable in women without placental malaria (p=0.58).

The median Gestational Age (GA) at delivery was comparable in primiparae and multiparae regardless if the placenta showed signs of infection or not and was greater than 28 weeks, the limit defining an early delivery.

Table 4.8. Comparison of clinical and obstetrical parameters by placental malaria status

Parameters	**Positive placental malaria, n=17**	**Non-placental malaria, n=27**	**p-value***
Birth-weight, gr, median (range)	3,300 (2,170 - 3,950)	3,070 (2,300 - 4,300)	0.85
Placenta weight, gr, median (range)	610 (430 - 660)	570 (430 - 900)	0.82
Gestational age at delivery, wks, median (range)	39.4 (37.2 - 42)	38.6 (35.2 - 42.1)	0.40

*Kruskal-Wallis rank test

Table 4.9. Comparison of clinical and obstetrical parameters by placental infection status and parity

Parity	Parameters	Positive placental malaria, n=17	Non-placental malaria, n=27	p
Primiparae	Birth-weight, gr, median (range)	3,370 (2,910 - 3,600)	3,180 (2,400 - 3,900)	0.40
	Placenta weight, gr median (range)	620 (620 - 640)	605 (460 - 720)	0.57
	Gestational age at delivery, wks, median (range)	39.8 (38.6 - 42)	39.5 (35.2 - 41.4)	0.57
Multiparae	Birth-weight, gr ,median (range)	3,010 (2,170 - 3,950)	3,030 (2,300 - 4,300)	0.92
	Placenta weight, gr median (range)	570 (430 - 660)	550 (430 - 900)	0.88
	Gestational age at delivery, wks, median (range)	39.4 (37.2 - 41.2)	38.6 (35.4 - 42.1)	0.40

4.3.5 Histopathological analysis

Some histological changes were present in both acutely infected PM cases and biopsies from uninfected placentas, which were in keeping with the range of normal histological features seen in term placentas. These included syncitiotrophoblastic knotting, formation of cytotrophic debris and deposition of intervillous fibrin. The specific pathological features seen in PM cases, examples of which are shown in Figure 4.6, included sequestration of infected erythrocytes, increased intervillous fibrin deposition, the presence of inflammatory infiltration composed of leukocytes (predominantly lymphocytes and macrophages), and the presence of malaria haemozoin pigment. A total of 35/44 cases showed no evidence for acute malaria infection (example Fig 4.6A) whereas 9/44 cases (20.5%) showed acute malaria infection, with variable parasitaemia

from heavily parasitised cases (>97/100 PRBC/RBC, case FE3P-021, Fig 4.6B and C) to much lower parasitaemias of <3 PRBC/100 RBC, case FE3P-008, Fig 4.6D). Parasitised red cells were seen both within the maternal blood space, adherent to villi and within loose fibrinous and inflammatory clot. Intervillous fibrin deposition was increased in individual cases but not specifically associated with acute PM.

The degree of inflammation in association with PM was low in this series. No cases showed massive intervillositis, corresponding to score 3 in the Muehlenbachs criteria (Figure 4.2). Occasional cases with mildly increased leukocytes, corresponding to score 2, were seen (Fig 4.6D,Table 4.7) but no difference between the degree of inflammation was seen between cases with or without PM. The degree of malaria pigment deposition (Fig 4.6E) was non-significantly higher in placental malaria cases (Table 4.6), but non-PM cases were seen with pigment deposition, indicating that this pathological feature is not specific for acute malaria infection as pigment deposition can be a reflection of prior episodes of acute PM.

In a number of cases (7/44 (15.9%)) morphological evidence of red cell sickling was seen, which could be related to the genotype of the mother (Fig 4.6F). Histological evidence of red cell sickling is well described as a diagnostic finding in the placenta of patients with sickle cell crisis and sickle cell trait (Bloomfield *et al.* 1978; Pantanowitz *et al.* 2000; Bessieres 2010). Not all of these 7 cases in this series showed active PM at delivery (eg cases FE20-006 and -007) indicating that sickling seen morphologically is not solely due to co-existing acute placental malaria.

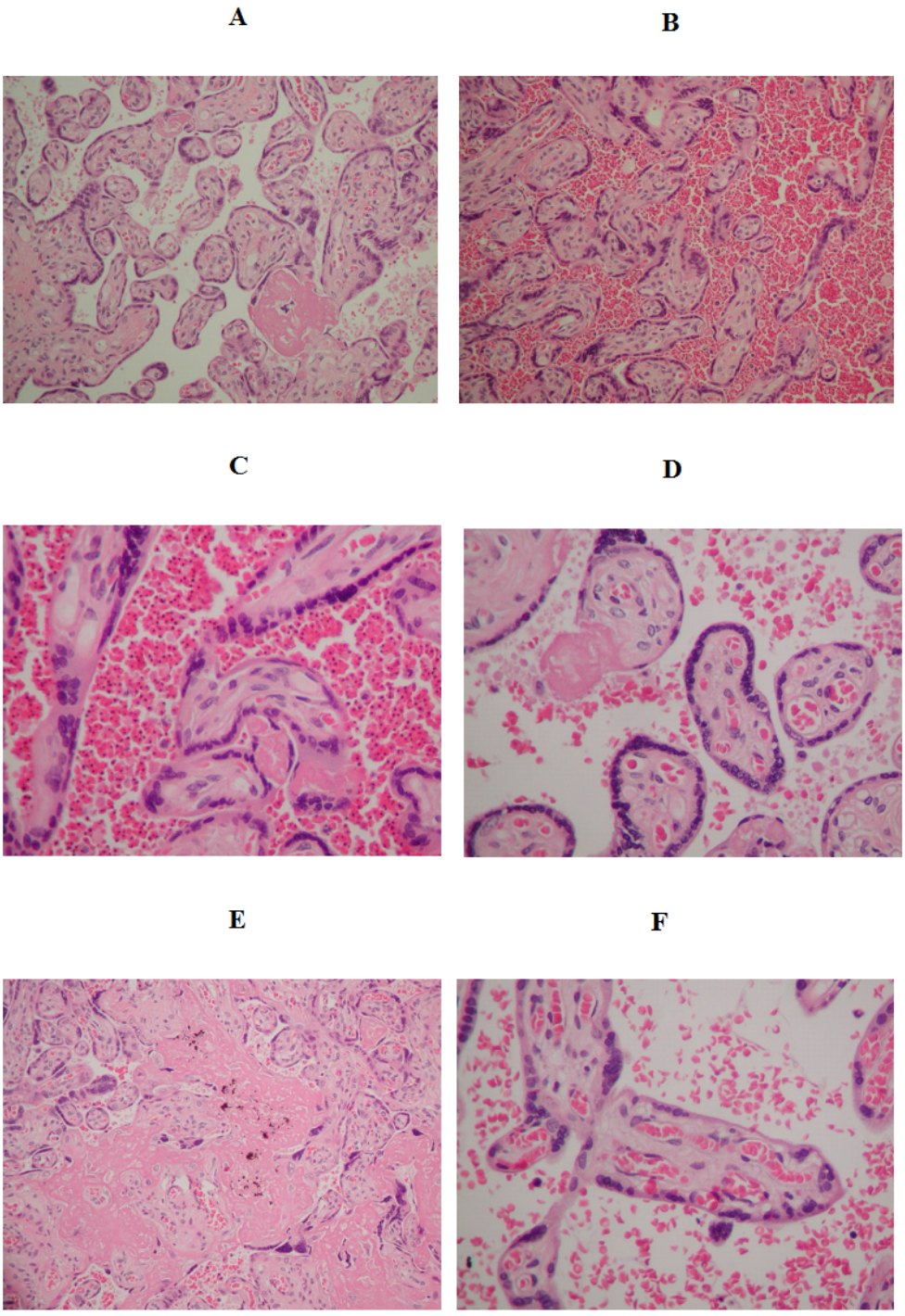


Figure 4.6. The Histopathological Features of Placental Malaria in this series

Legend: Photomicrographs of placental sections, all stained with Haematoxylin and Eosin. **A:** Placenta from an uninfected case showing normal term delivery features, including mature villi with focal syncitiotrophoblastic knots, red cells and debris within the maternal blood space, and a focus of intervillous fibrin deposition (Magnification x 400). **B:** Placental malaria infection, characterised by the presence of numerous *P. falciparum*-infected erythrocytes within the intervillous space. In this case there is little accompanying inflammation (x400). **C:** A higher power view demonstrating the very high parasitaemia amongst maternal red cells (x630). **D:** An infected case with much lower parasitaemia and occasional leukocytes in the maternal space (x 630). **E:** Heavy intervillous fibrin containing malaria pigment (haemozoin, x400). **F:** A case showing sickling of maternal red cells (x630).

4.3.6 Association between clinical parameters and histopathological features of the placenta

The relationship between observed pathological features of infected placentas (presence of pigment deposition and inflammation) with birth weight, placental weight, parity, gestational age and infection status was analysed. These relations were also assessed in the sub sample of 17 samples with PM and the one of 27 samples with no PM.

4.3.6.1 Birth weight

Overall 16% (7/44) of babies had a LBW (defined as a weight at birth less than 2,500 grams) and they were all born from patients included in the study in their 3rd trimester. Three biopsies of the placenta from these 7 cases were malaria infected and four were normal.

Amongst the three samples with placental malaria, two presented features of active malaria at the time of delivery. Both pregnant subjects cleared successfully their peripheral parasitaemia during the study. Their babies had the lowest birth weights of

the sample respectively 2,170 grams and 2,300 grams. The placental sample of the smallest baby- ie the one with 2,170 grams - had in addition to active malaria an heavy deposition of pigment (score 3).

In the third case the mother was enrolled in the 3rd trimester and treated with 3-day AL. She had a late parasitological failure at day 35 which was treated with oral quinine (10 mg per kg of body weight three times daily for seven days). This recurrent episode was later classified by PCR as a new infection. The patient also had a positive malaria smear at delivery.

There was a strong positive association between birth-weight and placental weight (rho: 0.72, p<0.01) that remained when considering samples with PM and those without (PM: rho: 0.52, p= 0.03; NPM: rho: 0.76 and p <0.01). There was no association between birth-weight and the presence of pigment, inflammation, and maternal parasitaemia at delivery, GA at the time of infection, active placental malaria and plasma level of lumefantrine at day 7 after treatment.

4.3.6.2 Placental weight

Placenta weight was not associated with parity (t: 0.89, p=0.38) or any other clinical variables except with the weight at birth as presented above.

4.3.6.3 Parity

The parity was not associated with any parameters (inflammation score, pigment deposition, status of active malaria, placental malaria, and placental weight).

4.3.6.4 Maternal haematocrit

There was a significant association between maternal haematocrit and pigment deposition score (F: 3.42, p=0.04). In the group with placental malaria, the association

was still significant between the two parameters (F: 5.8, p=0.02). In the group without placental malaria, there was no longer any association seen (F: 1.59, p = 0.24).

There was no association between maternal haematocrit and inflammation score, placental weight, maternal malaria slide positivity at delivery or with the status of active malaria at delivery.

4.4 Discussion

Timely diagnosis and treatment of malaria in pregnancy with an effective drug is critical to preserve the placental function and prevent subsequent adverse outcomes for the fetus. The pharmacokinetics of antimalarials might be modified in pregnancy leading to sub-therapeutic plasma levels which might not be sufficient to cure the infection and can select parasites for resistance.

In the present trial the pharmacokinetics, efficacy, safety and tolerability of an extended regimen of artemether-lumefantrine (10-dose over 5 days) were compared to the current 3 day regimen. To determine what effects this may have on placental malaria infection and subsequent pathology, a biopsy of the placenta was collected at birth from the women included in the study.

The objective was to describe the histopathological features of the placenta in the study population rather than to compare the effect of regimen, for which the sample size was not sufficiently powered. In fact, although extensive information on placental malaria is available in literature, a few data on this pathology are available from the Democratic Republic of Congo (Anagnos *et al.* 1986; Mbanzulu *et al.* 1998; Lukuka 2006; Modia *et al.* 2011).

We chose the classification proposed by Muehlenbachs *et al*. (Muehlenbachs *et al.* 2010) which is simple to implement, captures more information than other grading schemes and it is considered more suitable in areas of high malaria transmission.

In Sub-Saharan Africa, prevalence of placental malaria varies according to malaria endemicity and within the same geographical regions (McGregor *et al.* 1983; Shulman *et al.* 2001; N'Dao *et al.* 2006; Leke *et al.* 2010; Kattenberg *et al.* 2011; Bassey 2015).

In this study the prevalence of placental malaria was high (38.4%) and consistent with the national prevalence of peripheral malaria (DRC-NMCP 2012b) in pregnant women and in the study area, Kingasani Health Zone (CHK Annual report 2013, 2014).

Anagnos *et al*. (1986) reported a higher prevalence of placental malaria with 64 infected placenta out of 100 samples in the district of north Ubangi, which is located in Equator province in the north of the DRC and Modia *et al*.(2011) reported a prevalence of 72 % (N=295) in Kinshasa (91% in HIV-1 infected pregnant women and 54% in HIV-1 negative).

In the present study, there was histological evidence of active placental malaria at delivery in 17 cases whereas only 8 patients had malaria detected at delivery in peripheral blood.

This difference of detection with the two methods (histopathology and microscopy) might be limited and balanced by the fact that the two infections (in peripheral blood and in the placenta) could be different episodes of malaria. However, this is unlikely given that the mothers were closely monitored during the trial and treated for each malaria episode.

In our cohort, women with a positive peripheral parasitaemia at delivery were 49 times more likely to have placental malaria than women with negative peripheral

parasitaemia. These findings are consistent with those of Steketee *et al. (*1996) who reported in Malawi a five-fold higher risk of developing placental malaria in women with positive peripheral parasitaemia.

However, there was a high rate of asymptomatic infection at delivery i.e. patients with undetectable peripheral parasitaemia at delivery although having placental malaria histologically, as reported by other author (Mockenhaupt *et al.* 2002). Microscopy of peripheral thick blood films fails to detect more than half of true case of placental malaria diagnosed by histopathology. Other authors have reported the similar observations (Leke *et al.* 1999; McGready *et al.* 2004).

This absence of detectable parasite in peripheral circulation (sub patent parasitaemia) might be explained by the sequestration of iRBC in placenta, evading peripheral circulation. The peripheral parasitaemia in these women could have decreased below the threshold of microscopy detection. This problem of undetectable low peripheral parasitaemia by routine testing, accompanied by the presence of sequestered parasites in the placenta, is recognised (Shulman and Dorman 2003). It has been recently re-discussed by identifying pregnant women with such sub patent parasitaemia as the large part of the subclinical reservoir of infection sustaining malaria transmission and hindering efforts towards malaria elimination (Prof Arjen Dondorp, MORU, Bangkok, personal communication, 64th ASTMH conference 2015). This emphasises the need for implementing novel techniques such as ultrasensitive PCR,with a threshold of detection of 5-6 parasites per microliter,for detection of these low parasitemias in maternal blood, to identify cases to be treated (Poon *et al.* 2005; Imwong *et al.* 2014; Oriero *et al.* 2014; Adams *et al.* 2015).

Another alternative to histology for placental malaria detection, when the ultrasensitive PCR is not available, would be the combination of microscopy with the detection of

Histidine – rich protein 2 (HRP-2) – which antigen is produced by the whole mass of parasites, circulating or hidden in the placenta - in the peripheral blood. The two tests combined allowing for 90% of detection of placental malaria cases (Leke *et al.* 1999).

Placental weight and birth weight are easily measurable and available parameters. The normal ratio of 1:6 for the two parameters is an indicator of the fetal nutrition and of the placental function (Panti *et al.* 2012; Prabhjot 2013). In the study population, the median placenta weight was 590 grams (ranging from 430 to 900 grams) and comparable between infected 610 grams (range 430 -660grams); and non-infected 570 grams (range430 – 900 grams). This finding is similar to the weight reported in infected placenta in a study carried out in The Gambia, 650 grams (Bulmer *et al.* 1993) and Nigeria 590 ±82 grams (Panti *et al.* 2012) and higher than placental weight reported from Asian studies, respectively 487 grams in Thailand and 232 grams in India (McGready *et al.* 2004; Singh *et al.* 2014). Many factors, such as ethnicity and methodology in measuring the weight can explain this variation. Also acutely infected placentas have a mass related to the extra burden of sequestered *i*RBC, congestion with *i*RBC due to vascular pooling, and inflammatory or coagulatory infiltrates, which may explain some of the difference.

Primiparae are considered more at risk of developing placental malaria compared to multiparae (Menendez 1995; Guyatt and Snow 2004; Costa *et al.* 2006; Duffy 2007) and as a consequence, they often have lower placental weight and neonates with a mean lower birth weight (Walter *et al.* 1982). In this study, the median placental weight was significantly higher in primiparae with placental malaria compared to multiparae with placental malaria and similar results were obtained by Bulmer *et al.* (1993) in The Gambia. The suggested explanation was that the increase in placental weight in primiparae was an attempt of the organ to compensate for the damage caused by

malaria infection (Brabin 1991; Baptiste-Roberts *et al.* 2008) and has been associated also to anaemia (Beischer *et al.* 1970) and smoking (Wingerd *et al.* 1976).

The proportion of neonates with LBW was 16% (7/44), higher than the reported 10% national prevalence in the DRC, but still comparable to the meanprevalence of 16.5% in developing countries in Africa(WHO and UNICEF 2004; MICS-RDC 2011).

In the presence of placental malaria the birth weight is affected by the parasites compromising the organ circulation as well as by the presence of pathological lesions affecting the organ functions (Galbraith *et al.* 1980; Walter *et al.* 1982; Guyatt and Snow 2004). In our study the sample size was too small to show a difference in the median birth weight of neonates born from a mother with an infected placenta compared to those who had a non-infected placenta as observed in other studies (Menendez *et al.* 2000; Rijken *et al.* 2012).

A positive association was found between birth weight and placental weight at delivery delivery in women who displayed signs of placental malaria infection. Similar results were found in Tanzania in a high transmission setting area (Menendez *et al.* 2000).

A reduction of maternal haematocrit has been reported in literature associated to an increase in pigment deposition in the placenta (Rogerson *et al.* 2003, 2007). In our study, maternal haematocrit was associated with pigment deposition in the placenta (F: 3.42, p=0.04). This finding is consistent with that described by Rogerson et al (2003); indeed, one cause of maternal malarial anaemia is the destruction of infected red blood cells, which produces the malaria pigment hemozoin. The consequent accumulation of hemozoin in the placenta has been described to be associated with maternal anaemia (Rogerson *et al.* 2003).

The presence of parasitaemia at delivery was strongly associated with pigment deposition and/or inflammation of the placenta. In fact, all women with positive peripheral parasitaemia at delivery were also found to have histological evidence of placental malaria. The results observed in this study are similar to those of McGready *et al* (2004) who reported the presence of malaria-associated placental changes only in women with peripheral malaria detected at the antenatal care visit or at delivery.

The deposition of pigment within the fibrin in placental malaria has been reported to be associated to a reduction of birth weight (Muehlenbachs *et al.* 2010). In this study the fibrin deposition was in the majority of samples (82%) either absent or low, i.e. presence of fibrin in less than 10% of high powered field read by microscopy. This might be explained by the fact that the malaria episode (or episodes if more) was promptly treated with an effective antimalarial. The absence of histopathological changes when malaria episodes are correctly and promptly treated in has been previously reported by (McGready *et al.* 2004).

In this study, the presence of pigment was not associated to a reduction of birth weight. As reported by other authors, pigment deposition can be considered a marker of a recent infection but not necessarily associated to a reduction in fetal growth (Menendez *et al.* 2000; Muehlenbachs *et al.* 2012).

No case of congenital malaria was observed in the present study. Congenital malaria is a rare occurrence in neonates from malaria endemic areas where they are more likely to be protected against the infection by the presence of maternal antibodies transferred through the placenta (Snow *et al.* 1998). Moreover, the presence of fetal haemoglobin seems to inhibit the growth of *Plasmodium* within the RBCs (Pasvol *et al.* 1976; Nagel 1990) and thus infant susceptibility to infection increases with decreasing fetal hemoglobin and maternal immune IgG.

Only one neonate (1/44 = 2.2%) had a positive smear from cord blood at delivery. However peripheral blood smear from this neonate was negative and he did not present any symptoms of malaria. The biopsy of the placenta displayed signs of active malaria at the time of delivery, thus it is likely that a trans-placental transmission of *i*RBC occurred, causing congenital infection (Uneke 2007). The child resulted negative for malaria at all visit performed during the year of follow-up after birth. A contamination with the maternal blood due to birth trauma during placental separation cannot be ruled out.

4.5 Conclusions

Placental malaria was found to be high in the study population despite providing adequate and prompt treatment for malaria to the women included in the study. Lesions of the placenta due to malaria, and possibly other infections were observed and were associated with maternal anaemia and peripheral parasitaemia. More than half of the women with signs of placental infection did not have peripheral parasitaemia. This confirms the important problem of the presence of sub-patent parasitaemia in pregnancy in malaria endemic areas which leaves women often untreated with deleterious consequences for the mother and the fetus. These infections contribute also to the asymptomatic reservoir for malaria transmission.

5 Clinical implications and conclusion

Despite the considerable international efforts to control malaria worldwide, the global burden of this disease was in 2015 still very high with 3.2 billion people at risk of infection, 214 million (from 149 to 303 million) new cases and 438,000 deaths (from 236,000 to 635,000 deaths) (Murray *et al.* 2014; WHO 2015b). The majority of cases occurred in Sub-Saharan Africa with the highest rate of morbidity and mortality in children under five and pregnant women, although malaria was no longer the leading cause of death in children under five in 2015 but the fourth highest cause of death in this group (WHO 2015b). Nigeria, Mozambique and the DRC accounted for more than 5 million cases in 2013, and the same year the Democratic Republic of the Congo, Nigeria and India contributed to half of the malaria related deaths worldwide (Murray *et al.* 2014).

The emergence of *Plasmodium falciparum* resistance to the artemisinin derivatives, the most effective antimalarials available to date (Dondorp *et al.* 2009; Ashley *et al.* 2014), is alarming and reinforces the need to sustain efforts to control the disease and contain resistance. Artemisinin resistant parasites have been detected so far in five countries in the Greater Mekong sub-region: Cambodia, Lao People's Democratic Republic, Myanmar, Thailand and Viet Nam (WHO 2013b, 2015b). There are concerns about the possible spread of resistant parasite populations from Southeast Asia to Africa, as has happened with chloroquine and sulfadoxine-pyrimethamine in the past (Verdrager 1986; Roper *et al.* 2004).

Amongst existing interventions to control malaria, prevention and treatment are the most cost-effective. However prompt and effective treatment is often not available, and

WHO (WHO 2015b) estimated that in 2014 between 68 and 80 million of the 92 million children diagnosed with malaria did not receive adequate treatment.

In the DRC, 97% of the territory is considered malaria endemic and the population is at risk of being infected throughout the year. My thesis focuses on the treatment of uncomplicated falciparum malaria in the two most vulnerable groups, children under five and pregnant women.

First I tested three ACTs used for the treatment of uncomplicated malaria in children under five, amodiaquine-artesunate, artemether-lumefantrine and dihydroartemisinin-piperaquine, in order to collect baseline data on their efficacy, safety and tolerability in the study area. Although there are six ACTs recommended by WHO for the treatment of uncomplicated malaria, three were either available (amodiaquine-artesunate and artemether-lumefantrine) or were a prime candidate for deployment in the country in the near future (dihydroartemisinin-piperaquine). The other three recommended ACTs are sulfadoxine-pyrimethamine-amodiaquine, pyronaridine-artesunate and artesunate-mefloquine, but these were not studied for the following reasons: the first because sulfadoxine-pyrimethamine is no longer used in the country for treatment but only for prevention; the second because by the time I did the study, the combination was not yet produced and the last because of the mefloquine component. The use of mefloquine in highly endemic settings raises concerns regarding the possible accumulation of adverse events. As children experience approximately 6 malaria episodes per year and the half-life of mefloquine is about 3 weeks there are concerns about the possible development of neuropsychiatric reactions due to drug accumulation (Bjorkman 1989; Palmer *et al.* 1993; Hennequin *et al.* 1994; ter Kuile *et al.* 1995).

I found that the efficacy of the two ACTs currently in use in the DRC was good. The study was conducted 3 years ago and according to WHO recommendations efficacy of

the first line antimalarial drugs should be monitored at least once every 2 years to detect early changes in efficacy and help with the containment of artemisinin resistance (WHO 2005b). In 2013 our site participated in the TRAC I study (Tracking Resistance to Artemisinin Collaboration), which confirmed a good efficacy level of AL and did not detect any overt artemisinin resistance (Ashley *et al.* 2014). However the higher immunity than found in Southeast Asia contributes to rapid parasite clearance, thus potentially obscuring detection of milder degrees of artemisinin resistance. The possibility of artemisinin resistance spreading from Asia to Africa or *de novo* selection of resistant parasites in Africa are both major concerns and justify the need for regular monitoring of ACT efficacy throughout the country. We are currently participating in the multi-centre multi-country TRAC II study, the objectives of which are i. to detect further spread of artemisinin resistance beyond the GMS, and ii. to test the safety and efficacy of different triple combinations of an artemisinin derivative combined with two partner drugs for the treatment of artemisinin-resistant malaria. The combinations currently (January 2016) being tested in DRC are artemether–lumefantrine combined with amodiaquine compared to artemether–lumefantrine alone. Thus, data generated in TRAC II will be useful for monitoring the efficacy of artemether-lumefantrine in western DRC.

In my study the three ACTs tested had a similar efficacy (more than 90% cure), along with a good safety and tolerability profile. However, the study showed also that children weighing below 15 kg treated with artemether-lumefantrine and dihydroartemisinin-piperaquine had suboptimal plasma levels of lumefantrine and piperaquine, thus confirming the necessity of a higher dosage in this group. WHO has recently adapted its guidelines on dosage adjustment based on evidence from several studies (WWARN DP Study Group 2013; WWARN AL Dose Impact Study Group

2015). However, the implementation of the suggested higher dosage in children should be closely monitored in terms of safety and tolerability.

A characteristic of the paediatric population I studied was the relatively high level of parasitaemia on admission. This implies that children with uncomplicated malaria episodes in our setting are prone to progress rapidly to the severe form of the disease. This highlights the importance of early diagnosis and treatment with an effective drug to prevent complications and progression to severe malaria.

In the second part of my thesis I focused on malaria in pregnancy, which is an important problem in the DRC. Published clinical research data support the hypothesis that pregnant women treated with a standard adult dose of antimalarials are at risk of being exposed to suboptimal plasma drug concentration and therefore of not being cured. Dose-optimization studies have been recommended, however increasing the dosage raises the question of potential toxicity for the mother and their fetus as well as tolerability. Despite these recommendations, no clinical trials have been published on antimalarial dose optimization in pregnant patients. The trial confirmed that pregnancy was associated with reduced exposure to both lumefantrine and dihydroartemisinin and that the extended AL regimen improved the exposure to lumefantrine and to a less extent the exposure to artemether/DHA in both pregnant and non-pregnant patients.

There are no many safe and effective drugs for use in pregnancy and it is unlikely that new drugs will be developed as researchers and pharmaceutical companies are not eager to conduct studies in this group due to the potential toxic effect of drugs on the fetus. This narrows the choice of drugs for treatment to the few existing ones proven to be safe for use in pregnancy.

In term of safety, we did not observe serious adverse events with the longer regimen. Some changes were observed in the haematological values and liver and renal function

tests of some patients, which were however transient and not associated with clinical symptoms. Moreover, in some cases, the maximum concentration of lumefantrine reached with the extended regimen was 70% greater than that reached with the standard regimen, raising concern about the potential cardiotoxicity (White 2007). Although we did not observe any pathological increase of the QTc duration in these patients, the study was not powered for detecting infrequent AEs. These observations however warrant the need of further larger studies to assess the safety and tolerability of the extended regimen.

The efficacy of the standard regimen was high and equivalent to the extended one, and hence it can still be used in the DRC. However, the data obtained are very useful for those areas of the world affected by artemisinin resistance and characterised by a lower host–immunity background, such as South east Asia (Brabin 1991; Nosten *et al.* 1991b; Steketee *et al.* 2001b).

The pregnant women enrolled in the clinical trial had a high rate of placental malaria despite adequate treatment with an effective ACT both during the trial and in case of recurrent infection during follow-up. This could be explained by either the persistence of submicroscopic parasitaemia after the treatment of the primary episode or by recurrent episodes of asymptomatic malaria which remained undiagnosed and untreated. This highlights the importance of malaria prevention with effective chemoprophylaxis throughout pregnancy, in addition to the treatment of symptomatic malaria episodes. Currently WHO recommends the use of sulfadoxine-pyrimethamine as the drug of choice for IPTp (WHO 2015a). This strategy proved to be at least partially successful in the past but its efficacy is now questionable as SP is no longer very effective in many endemic countries due to the rising prevalence of anti-folate resistance. Additionally the pharmacokinetics of SP are suitable for intermittent

treatment but not effective continuous prophylaxis, particularly if it is given only twice during pregnancy. Thus in countries with limited resources such as the DRC, government and international donor funding is currently deployed to make widely available a drug which offers at the best partial protection and at worst no protection at all. The identification of a drug which can replace SP for IPTp, ideally one which offers complete rather than partial protection during pregnancy, is urgently needed.

Dihydroartemisinin–piperaquine offers both high treatment efficacy in Africa and, due to long the terminal elimination life of piperaquine, a long post-treatment prophylactic effect. For these reasons it is an excellent candidate to replace SP in the current IPTp strategy and for use in continuous chemoprophylaxis in pregnancy. In our ACT trial in children DHA-PQ substantially reduced the risk of a second episode of malaria during the 6 weeks follow-up when compared to AQAS and AL. Existing data on its safety in pregnancy, although scarce, indicate that the combination is safe and effective in pregnancy (Rijken *et al.* 2008, 2011; Poespoprodjo *et al.* 2014). Further studies in pregnancy on safety, tolerability and pharmacokinetics to determine the best dose and dosing interval are needed.

The clinical trials carried out for this thesis highlighted some critical issues and generated questions which could form the base for further research:

1. The importance of high-quality and regular monitoring of antimalarial drug resistance to offer the best treatment to the population and track the emergence of resistance to artemisinin and other antimalarial drugs.
2. The importance of testing drugs that can contribute to the reduction of malaria transmission, such as the addition of primaquine to the current treatment of uncomplicated malaria.

3. The importance of performing pharmacokinetic studies to optimise antimalarial regimens in both children and pregnant women. This also involves studying the safety and tolerability of these adjusted regimens.
4. The need to identify new drugs to replace SP for IPTp such as DHA-PQ. This would include studying the pharmacokinetics, dosing interval, safety, tolerability and acceptability.

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