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THE ROLE OF MOLECULAR MARKERS IN EMERGING ARTEMETHER-LUMEFANTRINE RESISTANT *PLASMODIUM FALCIPARUM*

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COVER PHOTO
The girl on the cover is Fatoumata Lasœur Soumah. The photo was taken in Boffa, Guinea, West Africa in 2011 by Frida Johansson. Lasœur represents one of the children that I wish the outcome of this thesis will benefit in the future.

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Till minne av Gertrud Malmberg och Niklas Lindegårdh

POPULÄRVETENSKAPLIG SAMMANFATTNING

Sjukdomen malaria orsakars av parasiten *Plasmodium falciparum* som sprids av malariamyggan *Anopheles*. Parasiten infekterar och förökar sig i de röda blodkropparna tills blodkropparna sprängs och parasiterna kan invadera nya. Om malariaparasiten inte behandlas är det stor risk att patienten dör. Varje år dör cirka 1 miljon människor av malaria. Majoriten är barn yngre än 5 år och gravida kvinnor i Afrika söder om Sahara.

Malaria går att behandla, och det finns ett antal fungerande läkemedel. Tyvärr är det oftast bara en tidsfråga innan malariaparasiten utvecklar resistens mot malarialäkemedel och detta kan få katastrofala konsekvenser när få eller inga andra fungerande läkemedel finns att tillgå. Det läkemedel som används för behandling av malaria i de flesta afrikanska länder idag heter artemether-lumefantrine (Coartem® eller Riamet®) och är en så kallad artemisininbaserad kombinationsbehandling (ACT). Det är en kombination av ett mycket aktivt artemisininderivat som snabbt reducerar mängden parasiter och ett annat läkemedel som inte är lika effektivt men är kvar länge i kroppen och dödar av de sista kvarvarande parasiterna. Denna avhandling handlar om att förstå hur parasiten utvecklar resistens mot kombinationsbehandlingen artemether-lumefantrine.

För att kunna förstå hur resistens utvecklas är det en stor fördel att veta hur läkemedlet verkar. För det allra flest malarialäkemedel är detta dock fortfarande en gåta, detta gäller även artemether-lumefantrine. Det har visats att resistens mot malarialäkemedlet klorokin beror på en förändrad aminosyra i ett transportprotein som transporterar läkemedlet ut ur parasitens inre vakuol där det annars kan påverka parasitens interaktion med hemoglobin. Den gen som denna avhandling handlar mest om heter *Plasmodium falciparum* multi-läkemedelstransportör (*pfmdr1*) och är en gen som kodar för ett protein som är en pump som kan pumpa in diverse olika läkemedel i parasitens inre vakuol. Mutationer i denna gen kan påverka transporten av läkemedel in och ut ur den inre vakuolen. Förändringar i transportkapaciteten skulle kunna leda till resistensutveckling. Till exempel om läkemedlet transporteras till ett ställe där det inte kan verka kan parasiten undkomma dess effekt.

Genom att kombinera kliniska fältstudier i Tanzania med molekylärbiologiska studier på laboratoriet har vi försökt förstå vad som påverkar resistensutvecklingen. För att förstå hur resistens utvecklas mot lumefantrine har vi jämfört parasitens DNA från insjuknade malariapatienter före behandling med de patienter som får tillbaka parasiter upp till åtta veckor efter behandling.

För att avgöra om det utvecklats resistens mot ett läkemedel behöver man kunna skilja på två olika sorters återkommande parasiter. De ena är de som faktiskt är resistenta och som varit kvar sedan påbörjad behandling, men i så små mängder att de inte har blivit upptäckta med mikroskopi. De andra är nya parasiter som patienten fått från ett nytt myggbett under pågående behandling. Det finns en molekylär metod att skilja på dessa olika sorters återkommande parasiter genom att jämföra parasiternas DNA före behandling och när parasiterna kommer tillbaka. Denna metod har dock visat sig ha ett

flertal brister vilket resulterar i att läkemedlen ofta bedöms vara bättre än vad de faktiskt är samt försvårar arbetet med förstå hur resistens utvecklas.

För att komma tillrätta med dessa problem har vi utvecklat en ny metod. Vi mätte läkemedelskoncentrationer av lumefantrine (det läkemedel som är kvar länge efter avslutad behandling), strax efter avslutad behandling och med hjälp av tidigare information om hur snabbt läkemedel försvinner ur kroppen på kunde vi uppskatta hur mycket läkemedel som fanns kvar när parasiterna kom tillbaka. Sedan jämförde vi de olika patienternas koncentrationer vid återkommande infektion med genetiska förändringar i *pfmdr1*-genen. Vi såg då att parasiter med aminosyrakombinationen NFD, hädanefter benämnda ”okänsliga”, kunde infektera patienter med 15 gånger högre lumefantrinekoncentrationer än parasiter med de så kallade ”känsliga” YYY uppsättningen.

Vi kunde också se att tiden efter en avslutad behandling som en patient är skyddad mot nya malariainfektioner kan variera med upp till tre veckor beroende på om de nya malariainfektionerna är så kallat ”okänsliga” eller ”känsliga”.

Genom att jämföra parasit-DNA hos patienter som kommit till en vårdcentral i Tanzania från 2004 till 2011 har vi kunnat se att förändringarna i *pfmdr1*-genen som resulterar i ”okänsliga” parasiter har ökat signifikant sedan artemether-lumefantrine började användas 2006.

Sammanfattningsvis har den här avhandlingen visat att trots att mycket tyder på att artemether-lumefantrine är ett effektivt läkemedel så kan det finnas anledning att vara orolig för hur länge detta kommer att fortgå. Det verkar som om genetiska förändringar i genen *pfmdr1* kan vara ett första steg mot resistensutveckling mot lumefantrine.

ABSTRACT

Malaria is a devastating disease which kills ~1 million people yearly. The vast majority of lives lost due to malaria are children and pregnant women in sub-Saharan Africa. Although malaria is a treatable disease it continues to be one of the major causes of death, especially in poor settings. Chemotherapy is the key to control the disease, decrease the burden of malaria and save lives. The malaria parasites ability to develop resistance towards antimalarial drugs is therefore a major concern. Artemether-lumefantrine (Coartem®, Novartis) is currently the most used treatment for uncomplicated *Plasmodium falciparum* malaria. The aim of this thesis was to contribute to the understanding of the role of molecular markers in emerging artemether-lumefantrine resistant *P. falciparum.*

This thesis is based on artemether-lumefantrine clinical trials designed to evaluate the efficacy and effectiveness of artemether-lumefantrine for treatment of uncomplicated *P. falciparum* malaria in children in Tanzania. We measured lumefantrine concentrations and investigated their correlation with cure rates and with tolerance/resistance associated markers within the parasite. Our focus was primarily on polymorphisms within *P. falciparum* multidrug resistance gene 1 (*pfmdr1*) and *P. falciparum* chloroquine transporter gene (*pfcrt*).

One major finding is that lumefantrine blood drug concentrations in combination with pharmacokinetic parameters can be used to assess the relative importance of different single nucleotide polymorphisms for lumefantrine drug susceptibility *in vivo*. Lumefantrine blood drug concentrations after artemether-lumefantrine treatment were correlated with selection of recurrent infections with specific *pfmdr1* N86, 184F and D1246 single nucleotide polymorphisms.

Although artemether-lumefantrine was found to have excellent efficacy and effectiveness according to PCR adjusted cure rates, the number of recurrent infections were high and we observed an up to three week difference in post-treatment prophylactic effect depending on the *pfmdr1* polymorphisms among recurrent infections. Since the introduction of artemether-lumefantrine as first line treatment for uncomplicated malaria in Tanzania in 2006, the prevalence of *pfmdr1* N86, 184F and D1246 have increased significantly up to 2011.

Overall, the results indicate that *pfmdr1* is involved in the mechanism of resistance to lumefantrine. The increased prevalence of parasites carrying the *pfmdr1* NFD haplotype could be an early warning of reduced artemether-lumefantrine efficacy.

LIST OF PUBLICATIONS

I. Billy E Ngasala, **Maja Malmberg**, Anja M Carlsson, Pedro E Ferreira, Max G Petzold, Daniel Blessborn, Yngve Bergqvist, José P Gil, Zul Premji, Anders Björkman, Andreas Mårtensson. Efficacy and effectiveness of artemether-lumefantrine after initial and repeated treatment in underfive children with acute uncomplicated *Plasmodium falciparum* malaria in rural Tanzania: a randomized trial. *Clinical Infectious Diseases* 2011 Apr 1; 52(7):873-82.

II. **Maja Malmberg***, Pedro E. Ferreira*, Joel Tarning, Johan Ursing, Billy Ngasala, Anders Björkman, Andreas Mårtensson, José P. Gil. *Plasmodium falciparum* drug resistance phenotype as assessed by patient antimalarial drug levels and its association with *pfmdr1* polymorphisms. *Journal of Infectious Diseases* Accepted 24th Sep 2012. Epub 2012 Dec 5. (*shared first authorship)

III. **Maja Malmberg**, Billy Ngasala, Pedro E. Ferreira, Erik Larsson, Irina Jovel, Angelica Hjalmarsson, Max Petzold, Zul Premji, José P. Gil, Anders Björkman, Andreas Mårtensson. Temporal trends of molecular markers associated with artemether-lumefantrine tolerance/resistance in Bagamoyo District, Tanzania. *Submitted* to *Malaria Journal* 20th Nov 2012.

IV. **Maja Malmberg**, Pedro E. Ferreira, Aminatou Kone, Berit Aydin-Schmidt, Billy Ngasala, Anders Björkman, Andreas Mårtensson, José P. Gil. Selection of *Plasmodium falciparum* MDR1 polymorphisms among recurrent infections after artemether-lumefantrine treatment but not during initial parasite clearance. *Manuscript.*

Publications not included in this thesis:

Billy E Ngasala, **Maja Malmberg**, Anja M Carlsson, Pedro E Ferreira, Max G Petzold, Daniel Blessborn, Yngve Bergqvist, J.Pedro Gil, Zul Premji, Anders Björkman, Andreas Mårtensson. Effectiveness of artemether-lumefantrine provided by community health workers in under-five children with uncomplicated malaria in rural Tanzania: an open label prospective study. *Malaria Journal* 2011, Mar 16;10:64.

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Maria I Veiga, Pedro E Ferreira, **Maja Malmberg**, , Louise Jörnhagen, Anders Björkman, Francois Nosten, José P Gil. *pfmdr1* amplification is related to increased *Plasmodium falciparum in vitro* sensitivity to the bisquinoline piperaquine. *Antimicrob Agents Chemother*. 2012 Jul;56(7):3615-9. Epub 2012 Apr 16.

CONTENTS

LIST OF ABBREVIATIONS

ABC	ATP-Binding Cassette
ACPR	Adequate Clinical and Parasitological Response
ACT	Artemisinin-based Combination Therapy
AUC	Area Under Curve
CCC	Coartem to Children at Community level
CI	Confidence Interval
DEAQ	Desbutyl-Amodiaquine
DDT	Dichloro-Diphenyl-Trichloroethane
DHA	Dihydroartemisinin
G6PD	Glucose-6-Phosphatate Dehydrogenase
GSH	Reduced Glutathione
GSSG	Oxidized Glutathione
IC	Inhibitory Concentration
LC-MS-MS	Liquid Chromatography Mass Spectrometry Mass Spectrometry
µl	Microliter
MIM/TDR	Multilateral Initiative on Malaria / Research & Training in Tropical Diseases
ml	Millilitre
ng	Nanogram
nM	Nanomolar
OR	Odds Ratio
PCR	Polymerase Chain Reaction
pfcrt	*Plasmodium falciparum chloroquine resistance transporter* gene
*Pf*CRT	*Plasmodium falciparum* Chloroquine Resistance Transporter
pfdhfr	*Plasmodium falciparum dihydrofolate reductase* gene
pfglurp	*Plasmodium falciparum* Glutamate Rich Protein gene
pfmdr1	*Plasmodium falciparum* Multidrug Resistance Gene 1
*Pf*MDR1	*Plasmodium falciparum* Multidrug Resistance 1
pfmrp1	*Plasmodium falciparum* Multidrug Resistance Protein 1 gene
*Pf*MRP1	*Plasmodium falciparum* Multidrug Resistance Protein 1
pfmsp1	*Plasmodium falciparum* Merozoite Surface Protein 1 gene

pfmsp2	*Plasmodium falciparum* Merozoite Surface Protein 2 gene
RBC	Red Blood Cell
RDT	Rapid Diagnostic Test
RFLP	Restriction Fragment Length Polymorphism
RR	Relative Risk
SIDA	Swedish International Development Aid
SNP	Single Nucleotide Polymorphism
WWARN	World Wide Antimalarial Resistance Network
WHO	World Health Organization

PREFACE

My interest in malaria began when I had just turned 18. I had been looking forward to the day when I could start to donate blood and now I was finally 18 and Blodbussen was standing at Fyristorg in Uppsala. Before being allowed to donate blood I needed to fill out a form and one of the questions was if I had had malaria. I told the nurse that I had malaria when I was five years old and lived in Zambia. To my surprise she then told me that I was not accepted as a blood donor. I was disappointed but this also awakened my curiosity. Does this mean that I still have malaria parasites in my body? And if I do not, why have they decided upon a rule like this? To answer my questions I did my project work in upper secondary school (specialarbete) on this topic but I was not completely satisfied with my findings. When I again as a master student got the chance to choose my own project, I had just read about the success story of malaria control in Zanzibar, and was very happy that José Pedro Gil and Anders Björkman welcomed me back to the malaria world.

My interest for antimalarial drugs started already during my childhood. I had the fortune to live with my family in rural Zambia between the ages of four and six, when my father worked for SIDA as an agricultural advisor. To avoid getting malaria we took antimalarial prophylaxis, crushed in jam, every day. My grandparents Pelle and Gertrud Malmberg came to visit us and took antimalarial prophylaxis just as we did. However, my grandmother Gertrud was unlucky and got a very unusual side effect. She could no longer produce her own platelets and was therefore dependent on donated blood. She was sick for two years and then she died. My five year old I believed that my dear grandmother only came because it was my biggest wish. In my self-centred world I also then drew the conclusion that it was my fault that she had to take the prophylaxis which had made her sick and eventually killed her. It was quite a burden for me to carry. Today I know better, this book is for you Gertrud.

1 INTRODUCTION

1.1 GLOBAL BURDEN OF MALARIA

Malaria is a devastating disease killing approximately one million people every year (*1*). The vast majority (~90%) of these lives are lost in Sub-Saharan Africa, mainly among children under five years of age and pregnant women. In 2010, while World Health Organization (WHO) estimated there to be 655 000 cases, a systematic analysis of global malaria mortality estimated the figure for 2010 to be 1 238 000 (95% uncertainty intervals 929 000-1 685 000) (*1*). It is to note that this report by Murray *et al*. has been challenged by the malaria community, especially regarding the use of clinical malaria (i.e. not microscopy confirmed cases), which is known to overestimate malaria cases (*2, 3*). Nevertheless, it is estimated that 3.3 billion people are at risk of getting malaria and an estimated 216 million episodes of malaria to occurred in 2010 (*4*).

Malaria is an entirely preventable and treatable disease. This reinforces the strong link between malaria and poverty (*5*). Poverty can influence the risk of getting sick in and dying from malaria, for example by not being able to afford adequate treatment and insecticide treated bed nets. Malaria can also play a role in maintaining poverty by for example reducing the number of working days, increase health expenses etc.

The global burden of malaria increased between 1980 and 2010, with a peak at 2004. This increase was partly explained by increasing malaria death rates in the late 1980s and early 1990s, influenced by drug resistance, and an increased population at risk of malaria. Great achievements in reducing the number of deaths due to malaria have been seen, with a 32% reduction from 2004 to 2010 (*1*).

1.2 SPECIES OF MALARIA

Malaria is caused by a unicellular apicomplexan parasite of the genus *Plasmodium*. There are five species of *Plasmodium* that can infect humans, *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi*. *P. falciparum* will be the focus of this thesis as it is the by far the most common one in Africa, and the most lethal. *P. vivax* is the most geographically spread of the species infecting humans. It has the ability to enter a metabolically inactive state called hypnozoite and can relapse months or even years

after infection. Such a capacity has proven advantageous in settings with seasonal transmission. *P. knowlesi* is primarily a macaque parasite but was recently shown to be able to infect humans.

1.2.1 Origin of *Plasmodium falciparum*

The genus *Plasmodium* evolved about 130 million years ago during the period when flowering plants rapidly spread across the globe. *P. knowlesi* and *P. falciparum* belong to the linage *Laverania* subgenus and diverged most recently as compared to the other species able to infect humans (*6*). Recent evidence indicates that *P. falciparum* is actually not unique to humans as it has also been detected in gorillas and monkeys (*7, 8*). This raises concerns regarding the feasibility to eliminate malaria from areas where these reservoirs are maintained.

1.3 THE *PLASMODIUM FALCIPARUM* PARASITE

The *P. falciparum* parasite contains approximately 5 500 genes, on 14 chromosomes. The vast majority (>95%) of the genome was sequenced in 2002. By then, it was determined as the most A-T rich genome ever sequenced (81%) (*9*).

1.3.1 The *Plasmodium falciparum* life cycle

The *P. falciparum* parasite has a complex lifecycle (Fig. 1). The infection is initiated when a parasite infected *Anopheles* mosquito, while taking a blood meal, injects saliva containing *P. falciparum* sporozoites, into the human host. The sporozoites are injected to the skin where at least according to mice models quite a few will stay (*10*). The remaining enters either the lymph or the blood stream. Mice models have shown that those who enter the lymph will reach the closes lymph node and invade there, and then get cleared by the immune system (*11*). Those who enter the peripheral blood system will have the liver as their final destination. They rapidly reach the liver, invade the hepatocytes and start the process of maturation towards schizonts. After 5-16 days the hepatocytes burst and release up to 40 000 merozoites (per schizont) into the blood stream. These invade red blood cells (RBC) and start the erytherocytic part of the life cycle. This is initiated with the ring stage, followed by the trophozoite stage, and thereafter schizont stage. After the schizont stage the RBC are lysed and 8-24 new

merozoites are released from each schizont, ready to invade new RBC and reinitiate the erytherocytic cycle. This synchronized rupture of RBC causes periodical fever attacks, which is a main clinical characteristic of malaria.

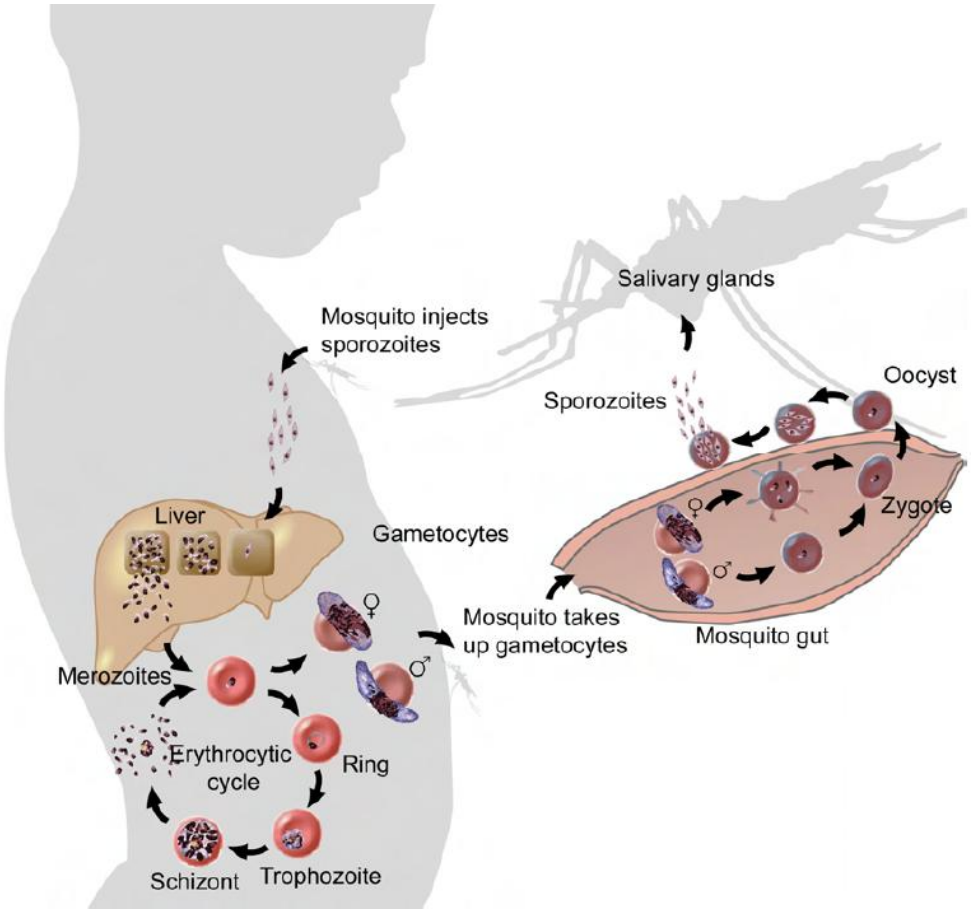

Figure 1. The *P. falciparum* life cycle. Printed with kind permission from Dr. Christin Sisowath (Karolinska Institutet). Illustration by Leopold Roos.

In a fraction of the intra-host parasite population, sexual forms, referred to as gametocytes, develop. These forms are essential for the second part of the malaria life cycle to take place. When an *Anopheles* mosquito takes a blood meal containing gametocytes, the parasite sexual phase is initiated within the gut of the mosquito. The male and female gametocytes fuse into a zygote and after sexual recombination an ookinete is formed. The ookinate traverse the lining of the midgut and until it gets between the two membranes facing the hemocoel. An oocyst is formed and sporozoites start to bud off (sporogony). When the oocyst ruptures, thousands of sporozoites are released into the hemocoel and migrate to the salivary glands of the vector. The *P. falciparum* life cycle is completed when these sporozoites are injected into a human.

1.3.1.1 The digestive vacuole

The cell biology of the parasite is complex, including regular organelle structures such as the endoplasmatic reticulum, the golgi system, the mitochondrion (even though not as involved in energy metabolism as in other organisms (*12, 13*), but also specific structures, like the apicoplast. In the context and the theme of this thesis the major compartment of interest is the digestive vacuole. This will be described in further detail. During the intra-erytherocytic cycle the parasite develops within the RBC, where it degrades ~80% of the haemoglobin within the RBC. Haemoglobin is the parasites main source of amino acids. The degradation of this complex molecule takes place within an acidic compartment called the digestive vacuole. The heamoglobin is ingested and transported to the digestive vacuole already during the early stages after invasion (12-16h) (*14*). The ingestion is mediated by endocytic structures (cytostomes) at the surface of the parasite (*15*). Within the acidic digestive vacuole haemoglobin is degraded by specific peptidases (i.e. plasmepsins, falcipains, falciysin, dipeptidylpeptidases and aminopeptidases) into di-peptides which are transported to the cytoplasm and utilized by the parasite as amino acid source for proteins (*16*). When haemoglobin is degraded, the toxic rest-product haem is released. Haem is neutralized through crystallization towards a polymeric complex, the haemozoin, which is also referred to as the "malaria pigment".

1.3.2 The mosquito

The origin of the name malaria comes from Latin's *mal aira* which means bad air. The name referred to the badly smelling swampy areas which were associated with the disease. In 1735, Carolus Linnaeus presented in his thesis entitled "*Hypothesis nova de febrium intermittentium causa*" his theory that the intermittent fever was caused by clay particles in the water (*17*). This was later proven to be wrong when it became known that the agent causing the malaria disease was transmitted by mosquitos.

All the *Plasmodium* species causing malaria in humans are transmitted by mosquitos of the genus *Anopheles*. The *Anopheles* is characterized by having its abdomen pointing upwards, as compared to parallel to the surface, when it is a resting position. There are many different species of *Anopheles* with different geographic locations. For example in south and coastal regions of Sweden the *A. messeae* is present. Different species differ in their capacity to transmit *Plasmodium* and *A. gambiae* and *A. funestus* which

dominate in sub-Saharan Africa are particularly efficient transmitters (*18*). One factor that influences transmission is the feeding behaviour of the mosquito, i.e. if they preferably feed on humans (anthropophily) or other animals (zoophily). The *A. gambiae* and *A. funestus* are strongly anthropophilic.

The *Anopheles* life cycle has four stages; egg, larva, pupa and imago. The first three stages are aquatic and last between 5-14 days, depending on temperature. Within a few days of becoming adult the anopheles mate, takes a blood meal, rest for 2-3 days and lay the eggs. This process is then repeated for the whole life span of in total 2-4 weeks. The female mosquito bites humans to get proteins from the blood, which are required for egg production. The time taken for *P. falciparum* to complete its life cycle within the mosquito depends on temperature. The parasites life cycle within the mosquito is expected to take 10-21 days. It has been shown in a mice model that development of *P. yoelii* increased with temperature, however maximum transmission prevalence was reached at 22°C (*19*).

The use of insecticide treated bed nets is a highly effective tool to prevent transmission of *Plasmodium* as it blocks the mosquito's ability to feed human blood. Indoor residual spraying of insecticides on indoor walls is used to kill mosquitos that prefer to rest indoors. The mosquito has a relative fast life cycle and thereby evolution. This favours resistance development, and its ability to develop resistance is a big threat to malaria control. The control measures insecticide treated net and indoor residual spraying were developed based on the assumption that the mosquitos feed and rest indoors (*20*). However, if/when the mosquitos change behaviour, the use of these control measures might lose its importance. A recent report of previously unidentified *P. falciparum* vectors with the ability to feed outdoors and early in the evening is of great concern. These vectors were found in the highlands of western Kenya but there is no reason to believe that they could not be present also in other parts of Africa (*21*).

1.3.3 The host

Malaria has been with human since the origin of our species and it is the strongest known force for evolutionary selection in the recent history of the human genome. Malaria has been the main driving force behind for example sickle-cell disease, thalassemia and glucose-6-phosphatase dehydrogenase (G6PD) deficiency (*22*).

Erythrocyte (i.e. RBCs) defects can influence the malaria parasites ability to invade and cause disease. It was recently shown in both Zanzibar and Burkina Faso that slow metabolizers were more likely to be infected with resistant parasites suggesting that human genetics is also associated with parasite drug resistance (*23, 24*)

1.4 DISEASE CHARACTERISTICS

Malaria symptoms are fever, general malaise, headache, body ache, vomiting, diarrhoea, coughing and stomach ache. Other symptoms such as hypoglycemia, hyperlactatemia, anaemia and altered consciousness can also be signs of malaria infection (*25*). The erytherocytic cycle of *P. falciparum* becomes synchronized a few days after hepatocyte burst, and then fever occurs with 48h time intervals. These are the general characteristics of the disease, defining what is generally referred as "uncomplicated malaria". This term is used to separate the more common, generally non-lethal forms of *P. falciparum* malaria, from "severe malaria". If the "uncomplicated malaria" infection is not treated it can quickly develop into severe malaria and result in a fatal outcome

Severe malaria is characterized by high parasite densities, above 200 000 parasites/µl. The symptoms of severe disease are unrousable coma, acute respiratory distress syndrome, severe anaemia, renal failure, cerebral malaria, metabolic acidosis, hypoglycemia splenomegaly and circulatory collapse.

The mature stages (late trophozoites and schizonts) transform the RBC to a rigid and sticky cell which easily attach to the walls of the capillaries, also referred to as sequestration. The infected RBCs can also attach to other RBCs by a phenomenon called rosetting. Sequestered RBCs cannot be cleared by the spleen and can thereby avoid the immune system. This property of sequestration, unique to *P. falciparum*, can result in clogging of fine capillaries in for example the brain and lungs, giving rise to lethal conditions.

1.5 IMMUNITY

Malaria is not a chronic disease. Individuals who live in stable transmission settings develop non-sterile immunity against malaria. During a child's first months it is protected by antibodies from the mother (*26*). Then immunity towards severe disease develops during the first years in life, followed by immunity towards clinical symptoms of malaria. Older children and adults are less likely to develop severe disease but remain vulnerable to infection. The non-sterile immunity against malaria is only maintained if the individual continues to be exposed.

It is still not known why immunity towards malaria develops so slowly. Recent evidence indicates that it is the interplay between different T cells, and the regulation of their response, throughout infection that influences immunity and pathogenesis of malaria. The immune response is context dependent, influenced by *Plasmodium* species, stage of the infection, and host factors (*27*). It was recently found that dendritic cells, highly specialized antigen-presenting cells, are reduced during malaria infection. Suggested that this might in part explain the slow and inefficient development of immunity to malaria infection (*28*). Due to the clear link between previous exposure to malaria and immunity development, there has been high hopes on vaccine development. The biggest challenge for vaccine development is the diversity of the *P. falciparum*. Both in terms of variation within the population and in terms of variation among the proteins displayed on the surface at any given time point.

One peculiar and unfortunate feature of the malaria parasites is its ability to cause severe disease in pregnant women. Despite the woman being immune for many years, as soon as she becomes pregnant she is highly susceptible to get sick from malaria again. The risk is greatest during the first pregnancy and then gradually decreases for consecutive pregnancies (*29*). The increased susceptibly of pregnant women to malaria infection is thought to be caused be pregnancy associated immunological and hormonal changes as well as the presence of the placenta, a new niche for the parasite (*30, 31*).

1.6 CONTROL OF MALARIA

Malaria has been eliminated (i.e. local transmission is completely stopped) in many parts of the world, like for example in Europe and US. In Sweden the last locally acquired malaria case was reported in 1930. These malaria elimination achievements were possible due to several factors such as; adequate treatment of sick patients, improved living conditions, removal of swampy areas. The mosquito that transmits malaria feeds only at night, therefore protecting humans from mosquito bites during night is an important tool for malaria control. Although these aspects are important also for elimination of malaria in still endemic countries there are several factors that make this task more difficult e.g. the climate and behaviour of the mosquito. Before elimination can be considered there has be sustainable malaria control.

The tools currently used for malaria control are:

- Prompt diagnosis and early effective chemotherapeutic treatment
- Insecticide treated bed nets
- Indoor residual spraying
- Intermittent preventive treatment of risk groups
- Larvicides

The main focus of this thesis is chemotherapeutic treatment and this will be discussed further in the next section. However, in the following paragraph the other tools will be described in brief.

Historically, the **diagnosis** for malaria was based only on symptoms with fever being the main symptom. Since fever is not exclusively a symptom of malaria, many patients have been given the wrong treatment and antimalarials have been grossly overused. Parasitological confirmation of the diagnosis of malaria before malaria treatment is started is recommended by WHO. Diagnosing malaria by using microscopy is still the gold standard but it is time consuming and requires well-trained staff. A major improvement therefore came when rapid diagnostic tests (RDTs) were introduced (e.g. in Tanzania in 2007). RDTs are fast and can be performed with very basic training. In stable high transmission settings WHO recommend the use of RDTs where high-quality microscopy is not available.

The use of **insecticide treated bed-nets** is very important for malaria control and major achievements have been seen. From the year 2000 to 2011 the percentages of households, in sub-Saharan Africa, owning at least one insecticide treated net has increased from 3% to 50%.

Indoor residual spraying is a method to kill mosquitos by spraying the inside walls of houses with insecticides. In 2009 the most used insecticide was pyrethroids accounting for approximately 77% of area sprayed, followed by dichlorodiphenyltrichloroethane (DDT) (~20% of areas sprayed). Due to resistance development towards pyrethroids, there is currently an on-going shift towards non- pyrethroids such as carbamates and organophosphates.

Intermittent preventive treatment is prophylactic treatment recommended for risk groups such as pregnant women. **Larvicides** is an insecticide used to kill the mosquito larva stage.

1.7 IMPORTANCE OF CHEMOTHERAPY FOR THE CONTROL OF MALARIA

Chemotherapy is fundamental for malaria control. In Zanzibar, the number of malaria cases and deaths decreased dramatically after 2003 following wide scale deployment of antimalarial interventions (artemisinin combination therapies (ACTs), insecticide treated bed-nets and indoor residual spraying) (*32*). The main obstacles for malaria control today are the financial crisis, loss of immunity and resistance development towards antimalarial drugs and insecticides.

1.8 DRUGS FOR MALARIA TREATMENT

The first malaria treatments came from natural products that had proven to be effective against treatment of fever. For example, in 1735, Carolus Linnaeus described the antimalarial properties of bark from ash tree (ask in Swedish) (*33*). The major driving force for development of antimalarial drugs has been to protect military forces from malaria. This section will give an overview of the main drugs used for malaria treatment.

1.8.1 Quinolines

All quinolines contain the quinoline ring structure (Fig. 2). There are two sub-classes of quinolines; the aminoquinolines and the aryl-amino alcohols.

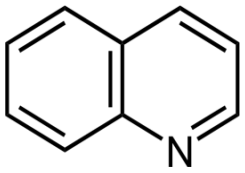

Figure 2. The quinoline ring structure

1.8.1.1 The aminoquinolines

The aminoquinolines are composed of 4-aminoquinolines (chloroquine, amodiaquine) and 8-aminoquinolines (primaquine). They are weak bases that are deprotonated and hydrophilic at neutral pH.

C**hloroquine** (molecular weight: 319.872 g/mol) (RS)-N'-(7-chloroquinolin-4-yl)-N,N-diethyl-pentane-1,4-diamine (Fig. 3), was developed in 1934 but came in clinical use first in 1945. Chloroquine is currently only used in areas where resistance has not developed e.g. Honduras. It is also used for treatment of *P. vivax* malaria in areas where chloroquine remains effective.

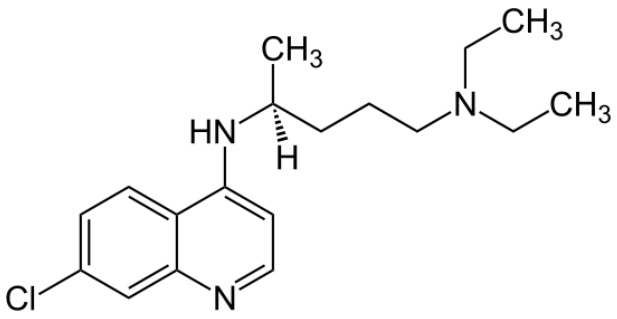

Figure 3. The chemical structure of chloroquine

Amodiaquine (molecular weight: 355.861 g/mol) g/mol4-[(7-chloroquinolin-4-yl)amino]-2-[(diethylamino)methyl]phenol (Fig. 4), is structurally related to chloroquine and used as a partner drug in the artesunate-amodiaquine combination.

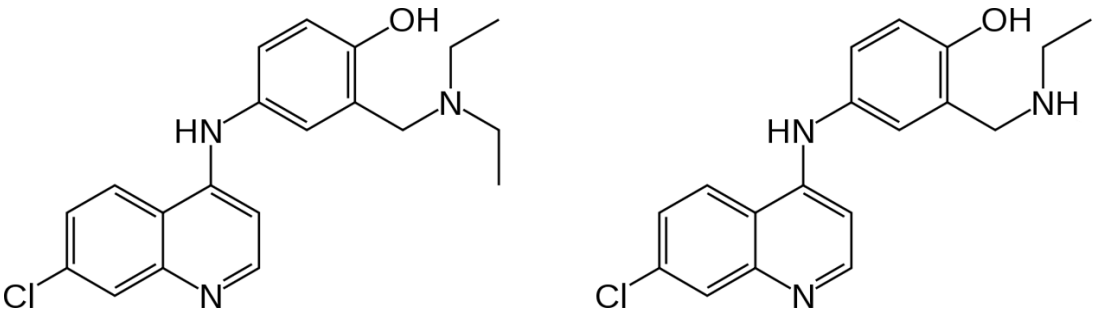

Figure 4. The chemical structure of amodiaquine (left) and its metabolite desethylamodiaquine (right)

Primaquine (molecular weight: 259.347 g/mol) (RS)-N-(6-methoxyquinolin-8-yl)pentane-1,4-diamine (Fig. 5), is an 8-aminoquinoline. It is recommended by WHO as anti-gametocyte treatment, especially in areas where pre-elimination or elimination is the target. It has also recently been recommended to be given in addition to ACT in areas threatened by artemisinin resistance (*34*). Primaquine is however limited because of its associated risk of haemolysis in patients with G6PD deficiency. To prevent relapses of *P. vivax*, a 14-day treatment course of primaquine should always be used if the G6PD status allows it.

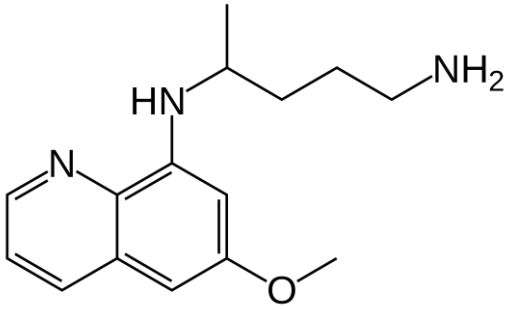

Figure 5. The chemical structure of primaquine

1.8.1.2 Arylamino alcohols

Aryl amino alcohols are weak bases that are lipid soluble at neutral pH.

Mefloquine (molecular weight: 378.312 g/mol) [(R*,S*)-2,8-bis(trifluoromethyl)quinolin-4-yl]-(2-piperidyl)methanol (Fig. 6), is an 4-methanolquinoline developed to save the lives of American solider during the Vietnam War. It is currently used as prophylaxis (Lariam®, F.Hoffmann-La Roche Ltd, Basel, Switzerland) and in combination with artesunate for treatment of uncomplicated malaria in South East Asia.

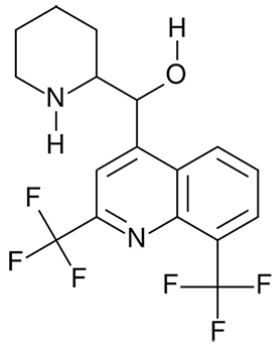

Figure 6. The chemical structure of mefloquine

Quinine (molecular weight: 324.417 g/mol) (R)-(6-methoxyquinolin-4-yl)((2S,4S,8R)-8-vinylquinuclidin-2-yl)methanol (Fig. 7), was the first quinolone drug used for malaria treatment. Quinine came originally from the bark of the Cinchona tree (named by Carolus Linnaeus in 1742). It was used as a medicinal plant by tribes in Peru and Ecuador to treat fevers, its antimalarial effect was recognized in the XVII Century by the Spanish settlers and subsequently taken to Europe. Despite its long history as antimalarial, the therapeutic mechanism of quinine has not been fully resolved. It is recommended as treatment of severe malaria.

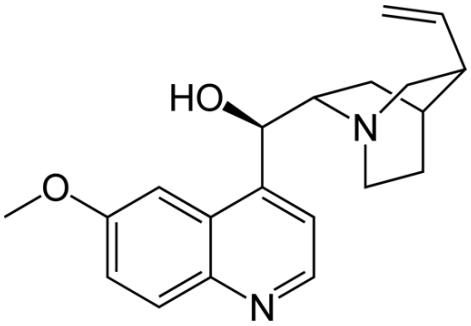

Figure 7. The chemical structure of quinine

Lumefantrine (molecular weight: 528.939 g/mol) 2-(dibutylamino)-1-[(9Z)-2,7-dichloro-9-(4-chlorobenzylidene)-9H-fluoren-4-yl]ethanol (Fig. 8), is also an aryl amino alcohol and it is only available in combination with artemether. As lumefantrine is one of the main focuses of this thesis it will be descried more extensively in a separate section.

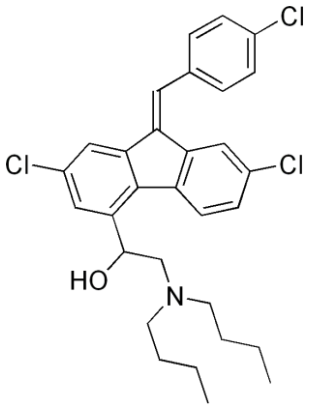

Figure 8. The chemical structure of lumefantrine

1.8.2 Other drugs

Piperaquine (molecular weight: 535.5 g/mol), 1,3-bis-[4-(7-chloroquinolyl-4)-piperazinyl-1]-propane, 7-chloro-4-[4-[3-[4-(7-chloroquinolin-4-yl)piperazin-1-yl]propyl] (Fig. 9), is a bisquinoline. It has been used in South East Asia as prophylaxis and is now used in combination with dihydroartemisinin (DHA) for treatment of uncomplicated malaria in both South East Asia and Africa.

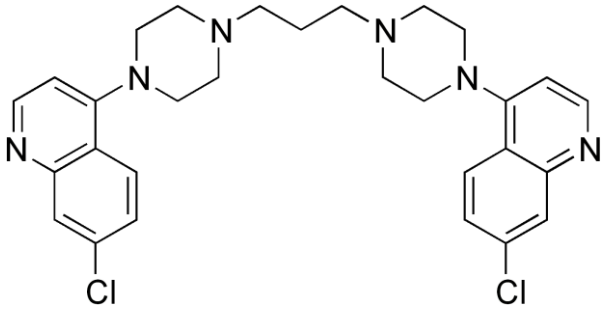

Figure 9. The chemical structure of piperaquine

Pyronaridine (molecular weight: 518.05 g/mol) 4-[(7-chloro-2-methoxy-pyrido[3,2-b]quinolin-10-yl)amino]-2,6-bis(pyrrolidin-1-ylmethyl)phenol (Fig. 10), is a benzonaphthyridine derivative first synthesized in China in 1970 and has been used there as monotherapy for treatment of malaria during 30 years (*35*). It has recently been combined with artesunate as a new combination therapy.

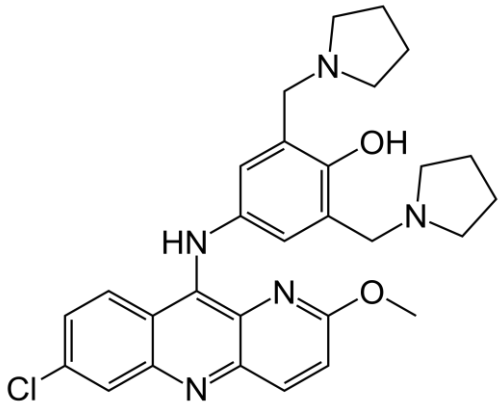

Figure 10. The chemical structure of pyronaridine

1.8.3 Sesquiterpene lactones

Artemisinin (molecular weight: 282.332 g/mol) (3R,5aS,6R,8aS,9R,12S,12aR)-octahydro-3,6,9-trimethyl-3,12-epoxy-12H-pyrano[4,3-j]-1,2-benzodioxepin-10(3H)-one (Fig. 11), is a class of drugs completely different from all other antimalarials.

The artemisinin (qinghaosu) comes from the plan *Artemisia annua* L. (qinghao in Chinese, sweet wormwood in English, malört in Swedish) that has been used for at least the last 2000 years in China for treatment of fever. The project leading to the discovery of artemisinin was initiated in response to a request from North Vietnamese leaders, suffering heavily losses of soldiers' lives due to malaria during the Vietnamese war (*36*). The Chinese leaders engaged to find solutions and in 1967 Chinese scientist started to screen Chinese herbs for antimalarial activity, and made extensive literature review. In Ge Hong's "A Handbook of Prescriptions for Emergencies" published in 341 AD, they found the key notes that made them changes the recipe and thereby increased the effect of their best compound (*37*). In 1972, artemisinin was isolated and purified (*38*) and in 1979 the finding was announced (*39*)

Artemisinin derivatives are used in all ACT combination therapies and for treatment of severe malaria. Due to their important role in this thesis they will be further described in a separate section.

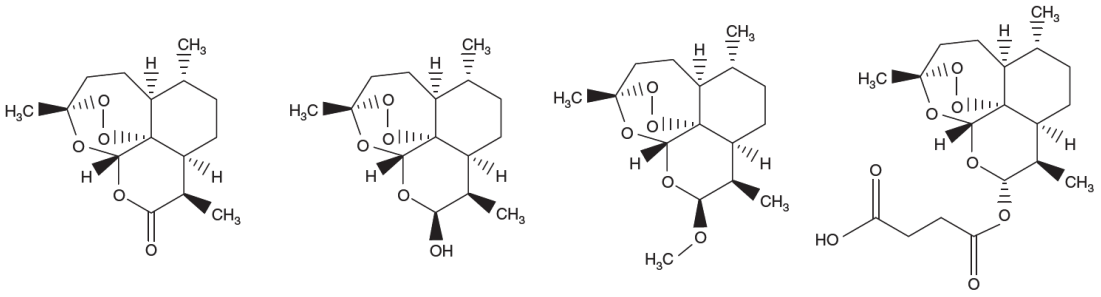

Figure 11. The chemical structure of artemisinin, dihydroartemisinin, artemether, artesunate.

1.8.4 Antifolates

Antifolates is a class of drugs that interfere with the synthesis of folic acid. The two most common drugs within this class are **sulfadoxine** (molecular weight: 310.33 g/mol) 4-Amino-N-(5,6-dimethoxy-4-pyrimidinyl)benzenesulfonamide and **pyrimethamine** (molecular weight: 248.71 g/mol) 5-(4-chlorophenyl)-6-ethyl- 2,4-pyrimidinediamine (Fig. 12) which are used in combination. Sulfadoxine-pyrimethamine is currently used for intermittent preventive treatment of pregnant women and as malaria treatment for pregnant women in the first trimester. There is also a combination therapy with sulfadoxine-pyrimethamine and artesunate.

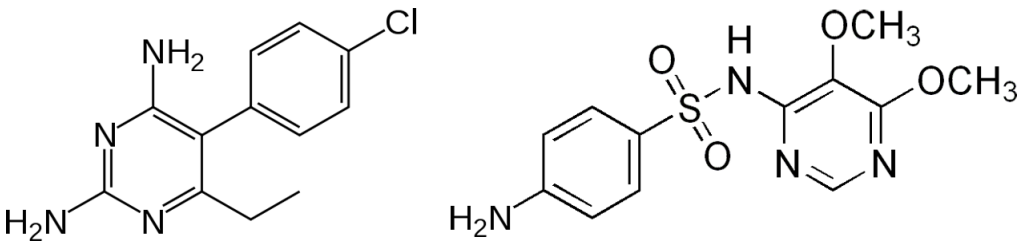

Figure 12. The chemical structure of pyrimethamine (left) and sulfadoxine (right)

1.8.5 Artemisinin combination therapy

According to the 2nd edition of the WHO Guidelines for the treatment of malaria published in March 2010, ACT is globally the recommended treatment for uncomplicated *P. falciparum* malaria (*40*).

The basic principle behind ACT is to improve drug efficacy of the artemisinin derivatives by combining it with an effective partner drug that can kill off the remaining parasites (*41*). This scenario markedly reduces the amount of parasites that remains for the partner drug as compared to if the partner drug were to be used as

monotherapy (Fig. 13). The two drugs combined should have independent modes of action and thereby prevent or at least delay the emergence of resistance. If a mutation associated with resistance to one of the drugs arises *de novo* in one parasite during the treatment, this resistant parasite will be killed by the other drug. The pharmacokinetic profiles of the two drugs should ideally be matched, so that both drugs protect each other during the full treatment. Due to the very short half-life of the artemisinin derivatives, the pharmacokinetic profiles of all currently used ACTs are not matched.

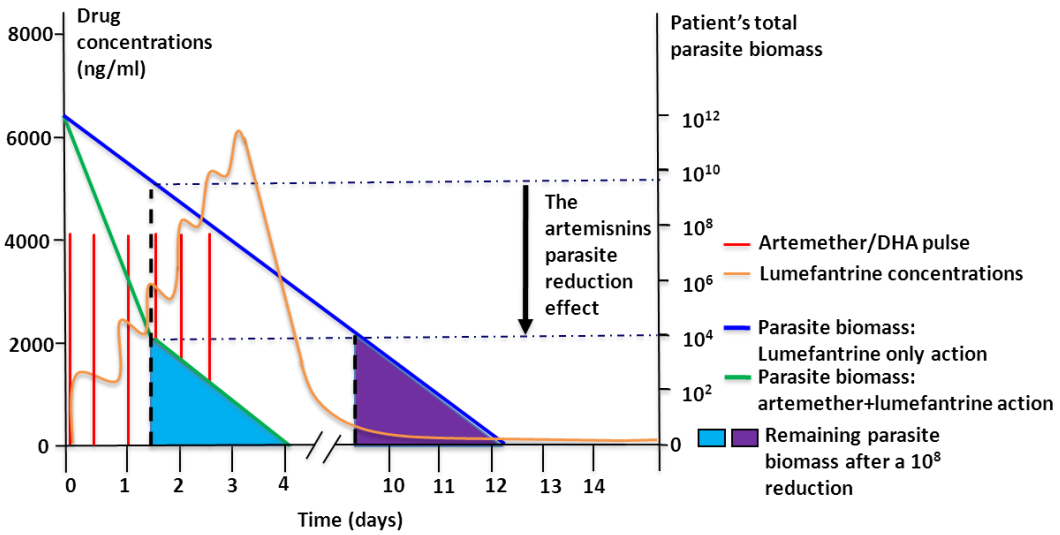

Figure 13. The principle behind ACT treatment with artemether-lumefantrine as the example. The large triangle under the blue line represents the total parasite biomass when exposed to a lumefantrine in monotherapy. In ACT, the artemisinin derivative rapidly reduces the parasite biomass (green line) and only a small number of residual parasites (turquoise triangle) will be exposed to lumefantrine. These parasites meet a much higher concentration of lumefantrine (area under orange curve) than the same parasite biomass exposed to lumefantrine in monotherapy (purple triangle). Adapted from (White *et al*. 1997)(*42*)

There are presently five ACTs approved by WHO; **artemether-lumefantrine**, **artesunate-amodiaquine**, **artesunate-mefloquine**, **artesunate-sulfadoxine-pyrimethamine** and **dihydroartemisinin-piperaquine.** The most recent ACT, **pyronaridine-artesunate** is yet only approved by the European Medicines Agency.

1.8.6 Counterfeit and substandard drugs

The use of counterfeit and substandard drugs is a major problem and it has been estimated that up to 35% of the antimalarials in sub-Saharan Africa are substandard (*43*). Counterfeit drugs can contain too low quantities of the active substance, potentially leading to resistance selection. Considering the artemisinins importance in

the first hours of therapy, the use of such substandard medicines can have serious consequences. In sub-Saharan Africa in 2002, WHO estimated there to be a traditional medicine practitioner per 500 people, and only one regular medicine practitioner per 40 000 people (*44*). There is a risk that counterfeit and substandard drugs would reduce the public's confidence in medicines and modern health care and make people turn to traditional medicine. This could result in reduced intake of potentially life-saving medicines (*45*).

1.9 MALARIA IN TANZANIA

United Republic of Tanzania constitutes of mainland Tanzania and Zanzibar. The focus of this thesis will be on mainland Tanzania, situated in East Africa. In 2009, the population of United Republic of Tanzania was 43.7 million, out of which 45% were 0-14 years old. The average birth rate was 5.3 children per woman. The life expectancy at birth was 59 years for women and 57 years for men (*46*).

In Tanzania, 73% of the population lives in areas with high malaria transmission, defined as ≥1 case per 1000 population. More than 40% of all outpatient visits are attributed to malaria and the number of annual malaria deaths is estimated to be 60 000 (*47*). Indoor residual spraying is recommended since 2006, intermittent preventive treatment for pregnant women was adopted in 2001. In 2004 artemether-lumefantrine (Coartem®, Novartis Pharma AG, Basel, Switzerland) (Fig. 14) was adopted as first line treatment of uncomplicated malaria, having been implemented throughout the country in the subsequent years. In Bagamoyo district, coastal Tanzania, it was implemented late 2006 (*48*). Quinine was adopted 2004 as treatment of severe malaria (*4*).

1.10 ARTEMETHER-LUMEFANTRINE

Artemether-lumefantrine is highly effective ACT with PCR adjusted cure rates exceeding 95% in 16 out of 22 studies in the latest Cochrane review (*49*). In 2001, Novartis agreed to make artemether-lumefantrine available without profit for distribution through the WHO to malaria-endemic developing countries. Since 2001, over 500 million treatments of artemether-lumefantrine have been delivered to more than 60 endemic countries. Artemether-lumefantrine is currently the most widely used

antimalarial, approved in 86 countries. In Africa 30 out of 47 countries have adopted artemether-lumefantrine as first line treatment, and an additional eight as second line treatment (*50*).

Figure 14. Coartem® Dispersible (artemether-lumefantrine).
Printed with permission from Erik Larsson

1.11 ARTEMETHER AND DIHYDROARTEMISININ

From the firstly discovered compound artemisinin, several derivatives have been made such as; artemether, artesunate, arteeter, DHA etc. (Fig. 11). Artemether is the artemisinin derivative in artemether-lumefantrine and therefore this section will focus specifically on it and its active metabolite DHA.

1.11.1 Pharmacokinetics of artemether and dihydroartemisinin

Artemether is quickly absorbed, reaching peak plasma concentrations ~2h after tablet ingestion (*41*, *51*). The absorption of artemether is increased by food intake (*41*). When artemether reaches the liver it is rapidly and extensively metabolised by demethylation into dihydroartemisinin.

During the distribution phase, artemether and dihydroartemisinin binds readily to human serum proteins (95.4% and 47-76%, respectively) (*52*). In patients infected with malaria, 93% of DHA was found to be protein bound (*53*). Both artemether and DHA are rapidly cleared from plasma with an elimination half-life of about 1-3h (*41*, *51*).

Artemether is mainly metalized to DHA via first-pass metabolism by enzymes belong to the cytochrome P450 enzyme (CYP) superfamily. The main actor is the CYP3A4, with possible contribution of CYP1A2 and CYP2B6 (*54, 55*) and CYP3A5 (Rita Piedade, personal communication). Artemisinins have auto inducing properties resulting in reducing concentrations for each dose (*56*). Artemether and DHA have been reported to have an inducing effect on CYP3A4 and CYP2B6 activity which results in increased levels of artemether being metabolized after each consecutive dose (*57*). The induction is driven by nuclear receptors.

1.11.2 Pharmacodynamics of artemether and dihydroartemisinin

The artemisinin component has a parasite reduction rate of 1:10 000, i.e. within 48 hours after *in vivo* treatment the parasitaemia has decrease by 99.99% (*42*). It is the fastest acting antimalarial ever developed and it is active against a broad range of stages within the malaria parasite life cycle. It is believed to be able to kill also the sexual stage to of the malaria parasite, the gametocytes (*58*), which is generally not true for other antimalarials. Artemisinin compounds are also active on the sequestered stages, which make them particularly useful to treat severe malaria (*59*). Accordingly, since April 2011, WHO recommends artesunate for treatment of severe malaria (*60*). The rapid elimination of artemisinin derivatives is advantageous as it theoretically limits the parasites ability to develop resistance due to reduced time of exposure. However the fast elimination represents a disadvantage as it limits the capacity of these compounds to clear the total parasite burden. Accordingly, recrudescences (i.e. treatment failure) are seen in ~10% of the patients after seven days of artesunate monotherapy (*61*) . To make the treatment more efficient and to protect the artemisinins derivative from resistance development, WHO presently recommends for these compounds to be used only in combination with a partner drug for the treatment of uncomplicated malaria.

In general, artemisinin derivatives are well tolerated at the presently prescribed doses. There has been concerns with neurotoxicity, especially in the first trimester of pregnant women (*62, 63*) Studies where increased dosing of artesunate from the standard 4mg/kg to 6mg/kg reported neutropenia in 19% suggesting that the maximum dose limit of artesunate has already been reached (*64*).

1.11.3 Artemether and dihydroartemisinin – mechanism of action

For the artemisinin derivatives, the mechanism of action is not known. It is known that artemisinin compounds are concentrated in the parasite infected RBCs. *In vitro* experiments have shown that DHA concentrations in infected RBCs are 300 times higher than in the medium, whereas uninfected RBCs had a less than two-fold increase as compared to the medium (*65*). Most studies agree that the peroxide bridge of the artemisinins is essential for antimalarial activity. There are several hypotheses as to how artemisinins exert its action.

- Interferences with haem-detoxification
- Induction of alkylation of translationally controlled tumor proteins(*66*)
- Inhibition of sarco/endoplasmic reticulum membrane calcium transporting ATPase6 (*67*)
- Interference with mitochondrial function

One theory hypothesise that the activity of artemisinin and its derivatives results from reductive scission of the peroxide bridge by reduced haem iron inside the highly acidic digestive vacuole (*68*). This theory was supported by a recent study where fluorescent artemisinin trioxane derivatives provided evidence for their rapid accumulation in the digestive vacuole and their activation by neutral lipid-associated haem (*69*). It has recently been shown that artesunate can inhibit haemozoin formation (*70*).

1.12 LUMEFANTRINE

Lumefantrine, previously known as benflumetol is a highly lipophilic compound (Fig. 8). It is an aryl-amino-alcohol structurally related to mefloquine, halofantrine and quinine.

1.12.1 Pharmacokinetics of lumefantrine

Absorption of lumefantrine starts after a lag-phase of up to 2h and peak plasma concentrations are reached first 6-8h after tablet taken (*71*). To get the maximum lumefantrine absorption it is recommended that artemether-lumefantrine is taken together with food or drink (*41, 71, 72*). It has been suggested that the fat content in

standard African diet or breast milk is enough to ensure optimal absorption (*73*). Lumefantrine is highly bound to human serum proteins (99.7%).

Elimination of lumefantrine is much slower than that of artemether and DHA. Lumefantrine has a terminal elimination half-life of 3-5 days (*71, 74, 75*). This results in a gradual increase in lumefantrine plasma concentrations throughout the three day artemether- lumefantrine treatment course (*41, 51*).

Lumefantrine is N-butylated, mainly by CYP3A4 (*54*). It is however only a small fraction (~1%) of lumefantrine that becomes the metabolite desbutyl-lumefantrine.

1.12.2 Pharmacodynamics of lumefantrine

When lumefantrine was introduced on the market it was as a combination therapy with artemether, therefore not much data exist on the pharmacodynamics effects of lumefantrine alone.

1.12.3 Lumefantrine – mechanism of action

The mechanism of action of lumefantrine is not fully elucidated. It was recently shown that lumefantrine inhibits haemozoin formation in the parasite cell, suggesting that lumefantrine similarly to chloroquine, interfere with the haemoglobin detoxification process within the digestive vacuole (*70*). This might be one but most probably not the only mechanism of action of lumefantrine.

1.13 ANTIMALARIAL DRUG RESISTANCE

As with other infectious diseases drug resistance is a major obstacle in the treatment and control of malaria. In general, whenever an antimalarial drug has been used for longer periods, resistance develops. This severely limits our ability to control this disease, with consequences not only directly in public health, but also as substantial economic costs.

1.13.1 Drug resistance and tolerance

WHO defines antimalarial drug resistance as "the ability of a parasite species to survive and/or to multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within the limits of tolerance of the subject" (WHO, 1973). In 1986 the definition was clarified with the addition of "the form of the drug active against the parasite must be able to gain access to the parasite or the red blood cell for the duration of time needed for its normal action".

Concerning drugs with expected multiple targets and pleiotropic effects, development of resistance is most likely a process not involving an on/off event, but rather an ongoing progression of stepwise increased changes leading initially to tolerance. Tolerant parasites are killed by the high drug levels achieved during the initial phase of treatment but can withstand higher levels than fully sensitive parasites. In clinical, real world terms, it means that although the action of the drug on these parasites is still inside the therapeutic window and hence being still cleared by the usual therapeutic doses, this reduced sensitivity will position these parasites nearer the top of this window. The parasites are still "clinically invisible", but represent populations probably developing towards a fully resistant phenotype.

This has implications in the post-treatment prophylactic period after ACT treatment. The ACT partner drugs have long half-lives and remain in the individual for weeks or up to months, providing the reoccurring parasites with a gradient of decreasing concentrations. During this window of selection it is possible to study tolerance development acquired through accumulation of favourable mutation and/or other modifications (*76, 77*). Usually these mutations are associated with a fitness cost, deeming them advantageous only in the presence of drug (*78, 79*).

1.13.2 Mechanisms of drug resistance

There are different ways for the malaria parasite to develop drug resistance. Without going into specific details the overall mechanisms are:

- Avoid drug-target interaction
    - By alteration of intracellular drug levels (e.g. decreased uptake, increased export, inactivation by metabolism or sequestration)

- By alteration of the drugs ability to affect the target (e.g. decreased target affinity for the drug of complete loss of target)

- Dormancy
- Overexpression of systems to handle indirect drug effects.

1.13.3 History of antimalarial drug resistance

Chloroquine was introduced in 1945 and became the first global chemotherapy for the control of malaria. Although extremely effective for near one decade, the first cases of clinical resistance to the drug emerged in 1958-1959 in the Thailand/Burma border and in remote provinces in Colombia and Venezuela (*80*), and later on spread globally. New drugs like the antifolate drug combination sulfadoxine-pyrimethamine and the synthetic quinoline derivative mefloquine were introduced during the 1960s and 1970s as an attempt to control the disease. Unfortunately, resistance to both drugs developed within less than five years (*81-83*). These observations and their clinical consequences are clear indications of the strong capacity of the parasite to adapt to new drug challenges. In this context, a common measure to delay the development of drug resistance is the introduction of combinations of drugs. A measure used since long for treatment of HIV/AIDS and tuberculosis.

1.13.4 Methods to assess antimalarial drug resistance

Antimalarial drug resistance can be assessed using different methods, i.e. *in vivo* (treatment failure in clinical trials), *ex vivo* (drug assays directly on blood from the patients, also referred to as "micro tests"), *in vitro* (parasites susceptibility to drugs in laboratory culture) or by analysis of molecular markers associated with drug resistance. There are advantages and disadvantages with each of these methods.

The way to evaluate drug efficacy *in vivo* is based on a 28 or 42-day test (*84*), where the patient's clinical and parasitological response is classified into "early treatment failure", "late clinical failure", "late parasitological failure", or "adequate clinical and parasitological response" (ACPR). The major limitation with this test for evaluation of therapeutic efficacy is that resistance may not always be detected, due to for example pharmacokinetic variation, re-infections, multiple infections, non-compliance or interference with the acquired immune response. There have been suggestions to

improve the definition by include for example *in vitro* tests, and measured drug concentrations to assure that treatment failure is not due to inadequate levels if drug (*85*).

Ex vivo methods have the advantage that they are applied to the actual parasites from the patient, and are possible to standardize. The disadvantages are that there could be influences from the immune system of the patient, minority clones could be lost due to lack of fitness, and the method requires well-trained personnel.

In vitro methods have the advantage that they are independent of the patient's immunity, can be performed in a controlled environment, repeated and used to test different drugs. The limitation are that some aspects of the parasite might be lost during long term adaptation to ideal conditions, the methodology is very costly and time consuming and require very well-trained personnel and advance laboratory facilities.

1.13.4.1 Molecular surveillance

Surveillance of molecular markers associated with drug resistance is a way to estimate drug efficacy. Genetic markers from a sub-set of the population are expected to reflect the prevalences of these single nucleotide polymorphisms (SNPs) in the total parasite population. For example, if molecular makers that accurately predict treatment failure are available these can be used for molecular surveillance and further on guide authorities in decisions regarding drug policies. Unfortunately, it is difficult to define biomarker with clinical value. It demands in general detailed knowledge not only of the mechanisms of action of the drug and resistance against it, but also of the drugs pharmacokinetic and pharmacodynamics characteristics. Due to this multi-factorial aspect of the clinical definition of resistance, no molecular marker is presently available with levels of specificity and sensitivity compatible with the demands of replacing phenotype determinations of resistance. Further studies in the several above mentioned aspects are needed, as such tool is, no matter the challenge, a fundamental factor for the ongoing malaria elimination plans.

A large advantage with molecular marker based surveillance as compared with the much more resource consuming drug efficacy clinical trials is that it is possible to scale up and feasible also when the patient population is small and time is scattered.

1.13.5 Artemisinin resistance

Due to its characteristic very short half-life and the rapid "pulse"- like exposures, it was originally thought that the malaria parasite would not be able to develop resistance towards artemisinin derivatives. To detect artemisinin resistance it is recommended to perform artesunate monotherapy clinical trials to avoid the influence of partner drugs.

It has been proposed that a clinical case of artemisinin resistance would have to fulfil all of the following criteria (*86*): a) persistence of parasites at seven days after the start of monotherapy with artemisinin compounds, or re-emergence of parasites within 28 days after the start of treatment; b) adequate plasma concentrations of DHA; c) prolonged parasite clearance time; and d) reduced *in vitro* susceptibility of the parasite.

The first reports on artemisinin resistance, as defined by the above mentioned criteria's came from the Thai-Cambodian border (*87, 88*). Thereafter there have been several reports of patients with prolonged parasite clearance time from; Thai-Burma border (*89*), Pursat region in Cambodia (*90*) , Vietnam (*91*) and Pailin in Cambodia (*92*). None of these reports have however fulfilled all the criteria's of artemisinin resistance. There in an ongoing controversies regarding whether only prolonged parasite clearance can be called artemisinin resistance and what the consequences of these findings are (*93, 94*).

Formally, artemisinin resistance is currently assessed as either:

- Suspected resistance: Microscopically confirmed positivity day 3 after ACT treatment (if ≥10%, containment activities should begin immediately),

Or

- Confirmed resistance: Treatment failure after treatment with an oral artemisinin-based monotherapy , as evident by persisting parasites day 7, or the presence of parasites day 3 and recrudescence within 28/42 days (adequate antimalarial blood concentrations confirmed)(*91*)

Anyway, the finding of artemisinin resistant parasites and the prolonged parasite clearance times are worrying. Artemisinin derivatives are basis of all ACTs, therefore the consequences of spread of resistance to these compounds should not be underestimated. After the identification of the South East Asia *foci* of suspected artemisinin resistance, strategies have been implemented to contain the spread of these

parasites. Unfortunately, it is becoming clear that these actions have not been sufficiently effective to halt its expansion (*95*).

The delayed parasite clearance phenotype has been proposed to have a genetic component (*96*). Accordingly, a major region on chromosome 13 has been identified to explain 35% of the slow clearance phenotype (*97*), although no molecular markers are yet available. It has been proposed that the observed delayed parasite clearance time is explained by reduced sensitivity in particularly the ring stage (*98*, *99*). This view was anyway not supported by a clinical trial designed for the administration of a split dose of artesunate, which showed no improvement on parasite clearance rates (*100*).There have also been reports on dormancy, which could potentially explain the phenotype observed (*99, 101*).

1.14 RESISTANCE ASSOCIATED GENES

1.14.1 *Plasmodium falciparum multidrug resistance gene 1* (*pfmdr1*)

The *P. falciparum multidrug resistance gene 1* (*pfmdr1*) codes for a transmembrane protein (*Pf*MDR1) belonging to the ATP-Binding Cassette (ABC)-transporter superfamily. The protein is located in the digestive vacuole membrane and to a minor extent in the plasma membrane (*102-104*). In the digestive vacuole the *Pf*MDR1 is believed to be faced towards the digestive vacuole, able to import molecules, including some antimalarial drugs, into the lumen of the digestive vacuole (*105, 106*).

Chloroquine resistance was early proposed to be based on the efflux of the drug from the parasite (*107*). These reports were later supported by several groups, further adding the observation that this was reversible by the calcium blocker verapamil (*108, 109*) . The same verapamil sensitization effect had been previously documented in resistant cancer cells, associated with the action of the efflux pump P-glycoprotein. Driven by this knowledge the search for the *P. falciparum* P-glycoprotein homologue started. Two genes were identified and named *pfmdr1* and *pfmdr2* (*110, 111*). The *pfmdr1* proved the best candidate to be associated with antimalarial drug resistance. Upon its discovery, promising initial associations between *in vitro* chloroquine resistance in adapted parasite clones and the presence of specific *pfmdr1* SNPs (*112*) were reported. These were rapidly dismissed by the discovery that the main genetic factor was actually

located in a different chromosome (*113*), as well as the frequent lack of association between clinical failure and the presence of these mutations (*114, 115*). Presently, *pfmdr1* is believed to represent a secondary – but non negligible - factor in the mechanism of chloroquine susceptibility.

Nevertheless, the *pfmdr1* gene has been found to be pivotal in the parasite response to presently more relevant antimalarials. The main SNPs found in *pfmdr1* are N86Y (N = asparagine, Y = tyrosine), Y184F (F = phenylalanine), S1034C (S = serine, C = cysteine), N1042D (D = aspartic acid), and D1246Y (*110*). SNPs in *pfmdr1* have been associated *in vivo* and *in vitro* with reduced susceptibility to important ACT drugs such as mefloquine (*116-119*), lumefantrine (*77, 120*), amodiaquine (*121, 122*) and artemisinin (*117, 118, 123, 124*).

In vivo, the *pfmdr1* has been shown to select for N86, 184F, D1246 among re-infections upon artemether-lumefantrine treatment (*77, 120, 125, 126*). This was further supported by culture adapted Kenyan isolates where isolates with *pfmdr1* N86 had significantly higher lumefantrine IC_{50} values as compared to the 86Y (*127*). Recently, a newly identified polymorphism in the *pfmdr1*, i.e. 1226Y, was correlated with reduced artemisinin, lumefantrine and mefloquine response in culture adapted field isolates from Thailand (*128*).

Duplications of the *pfmdr1* gene have been associated with decreased *in vitro* susceptibility to mefloquine, quinine and halofantrine (*129, 130*). *In vitro* development of mefloquine resistance has been shown to be associated with increased *pfmdr1* copy number (*130-132*). Knock down experiment removing one of the two copies of *pfmdr1* in the clone FCB, resulted in increased sensitivity to quinine, lumefantrine, mefloquine, artemisinin and halofantrine (*133*). These *in vitro* findings have been confirmed *in vivo* in mefloquine, artesunate-mefloquine and four dose artemether-lumefantrine clinical trial where increased *pfmdr1* copy number have been associated with treatment failure (*116, 134, 135*). All these *in vivo* findings are from South East Asia, where duplications of *pfmdr1* are frequently found, however presently *pfmdr1* duplications seem to be essentially non-existent on the African continent (*120, 136, 137*).

1.14.2 *Plasmodium falciparum chloroquine transporter gene* (*pfcrt*)

The *pfcrt* (*P. falciparum chloroquine resistance transporter*) gene has been found to play a central role in chloroquine resistance. It codes for a transmembrane protein *Pf*CRT, located in the digestive vacuole membrane, the compartment where haemoglobin (the parasites main amino acid source) is degraded and detoxified. It is currently not known how di-peptides generated from haemoglobin degradation are transported from the digestive vacuole to the cytoplasm. However, recent work using parasites from a genetic cross between a chloroquine resistant and a chloroquine sensitive strain, combined with a metabolomic approach, showed an accumulation of di-peptides in chloroquine resistant parasites (Manuel Llinás, personal communication). These results suggest that *Pf*CRT, a member of the drug/metabolite transport superfamily, may be involved directly or indirectly in the transport of di-peptides (*138*).

Through accumulations of SNPs the *Pf*CRT is capable of transporting chloroquine out of the digestive vacuole (*139, 140*). The SNP that is most strongly associated with chloroquine resistance is located on codon 76, where a lysine (K) in replaced by a threonine (T) (*141, 142*). Currently, the most accepted hypothesis for chloroquine mode of action is by the drugs capacity to interfere with the process of detoxification of haem inside the digestive vacuole, namely its polymerization to haemozoin (*70*). The 76T harbouring *Pf*CRT is hence able to decrease chloroquine concentration in the digestive vacuole, where the drug acts. On the contrary, the parasites harboring *Pf*CRT carrying the K76 allele are unable (or at least, less able) to efflux the drug (*143*), rendering the parasite more susceptible.

Besides its role in chloroquine resistance, allele exchange experiments have clearly shown that *Pf*CRT is potentially involved in the parasite's response to other antimalarials (*142, 144, 145*). Specifically in the ACT context, this gene might also play a role in resistance towards artemether-lumefantrine. It has been shown that the *pfcrt* K76 is selected among re-infection after artemether-lumefantrine treatment (*146*), an observation supported by *in vitro* works pointing for the K76 to be associated with reduced susceptibility to lumefantrine (*127, 146*).

1.14.3 *Plasmodium falciparum multidrug resistance protein 1* (*pfmrp1*)

The *pfmrp1* gene codes for an ABC-transporter (*Pf*MRP1) located in the parasites plasma suggested to act as a GSH/GSSG pump (*147*) and drug effluxer (*148, 149*). This hypothesis has recently been supported by the *in vitro* finding that parasites with disrupted *Pf*MRP1 accumulate GSH (reduced glutathione), GSSG (oxidized glutathione) and GSH-conjugates. The disruption also resulted in an accumulation of chloroquine and quinine, and reduced susceptibility to piperaquine, primaquine, artemisinin as well as chloroquine and quinine (*150*).

A significant association of parasites harbouring *Pf*MRP1 carrying the 1390I (I = isoleucine) allele with reduced susceptibility to lumefantrine, artemisinin and mefloquine have been seen in culture adapter clones from the Thai-Burma border (*128*). In clinical trials a significant selection of parasites carrying the *Pf*MRP1 I876 allele has been seen among recurrent infections upon artemether-lumefantrine treatment (*151*) and the *Pf*MRP1 K1466 allele has been shown to be selected within recrudescences after sulfadoxine-pyrimethamine treatment (*152*).

2 SCOPE OF THE THESIS

The overall aim of this thesis was to contribute to the understanding the molecular basis of emerging artemether-lumefantrine resistant *P. falciparum*.

The specific aims of the different studies were:

Paper I

- To investigate how well artemether-lumefantrine is working in real life and under ideal conditions and how this relates to whole blood day 7 lumefantrine concentrations.
- To assess if treatment with artemether-lumefantrine selects for genetically distinct parasite populations among recurrent infections, with focus on the major drug resistance associated genes *pfmdr1* and *pfcrt*.

Paper II

- To examine if selection of *pfmdr1* SNPs among re-infections after artemether-lumefantrine treatment is associated with lumefantrine drug concentrations.
- To assess the relative importance of different SNPs for the parasites capacity to withstand lumefantrine *in vivo*.

Paper III

- To analyse temporal trends of the prevalence of *pfmdr1* and *pfcrt* SNPs after the implementation of artemether-lumefantrine as first line treatment for uncomplicated malaria in Tanzania.

Paper IV

- To assess if artemether/dihydroartemisinin *in vivo* select for the same molecular markers as lumefantrine.
- To examine the characteristics of the lumefantrine window of *pfmdr1* and *pfcrt* SNP selection after artemether-lumefantrine treatment.

3 MATERIAL AND METHODS

3.1 STUDY LOCATION AND POPULATION

All studies included in this thesis were conducted in coastal region of mainland Tanzania from the year 2004 to 2011. The primary study site was Fukayosi dispensary in Bagamoyo District (Fig. 15). Studies were also conducted in Yombo, Bagamoyo District and in Ngeta and Mwanabwito villages, Kibaha district.

Figure 15. The Fukayosi dispensary, Bagamoyo District, Tanzania.
Printed with permission from Erik Larsson.

The area were the studies were conducted is primarily rural. Malaria transmission is high and occurs throughout the year with some accentuation during the rainy seasons in May–July and to a lesser extent between December-January. *P. falciparum* is the predominant malaria species and *Anopheles gambiae* complex the main vector.

Study participants were recruited among patients presenting at the study sites for routine care. All patients included in the studies were children ≤10 years with uncomplicated microscopy confirmed *P. falciparum* infection.

3.2 CLINICAL STUDIES

The clinical trials analysed in this thesis where conducted in accordance with WHO recommendations. Blood samples were collected on filter paper at day of inclusion and for studies with follow up at the pre-determined days of re-visit as well as at any day when the patient felt sick.

The MIM/TDR (Multilateral Initiative on Malaria / Research & Training in Tropical Diseases) study (n=359): was a two arm (efficacy versus effectiveness) randomized, open-label, parallel-group, controlled artemether-lumefantrine clinical trial conducted in 2007-2008 in the villages Fukayosi and Yombo, Bagamoyo district (*136*). The drugs intake was provided under supervision in the efficacy arm whereas patients and parents/guardians in the effectiveness arm were informed to take the drug at home. The complete analysis of this study is presented in Paper I. In Paper II, the first follow up of 56 days from this study is included. In Paper III the part of this study that was conducted in Fukayosi (n=258) is referred to as Study 3 (Fig. 16).

The CCC (Coartem to Children at Community level) study (n=244): was a single arm effectiveness artemether-lumefantrine clinical trial conducted in 2007 in the Ngeta and Mwanabwito villages, Kibaha district (*153*). This study was included in Paper II.

The Fukayosi I study (n=106): was a two arm (artemether-lumefantrine versus sulfadoxine-pyrimethamine) efficacy trial conducted in 2004 at Fukayosi dispensary, Bagamoyo district (*146, 154*). It is referred to as Study 1 in Paper III.

The Fukayosi II study (n=50): was an artemether-lumefantrine pharmacokinetic and pharmacodynamics study performed in 2006 at Fukayosi dispensary, Bagamoyo district (*75, 155*) . It is referred to as Study 2 in Paper III.

The Fukayosi IV study (n=200): was a pre-treatment blood sampling done in 2008 at Fukayosi dispensary, Bagamoyo district. It is referred to as Study 4 in Paper III.

The Fukayosi V study (n=33): was a pre-treatment blood sampling done in 2010 at Fukayosi dispensary, Bagamoyo district. It is referred to as Study 5 in Paper III.

The Fukayosi VI study (n=130): was a pre-treatment blood sampling done in 2011 at Fukayosi dispensary, Bagamoyo district. It is referred to as Study 6 in Paper III.

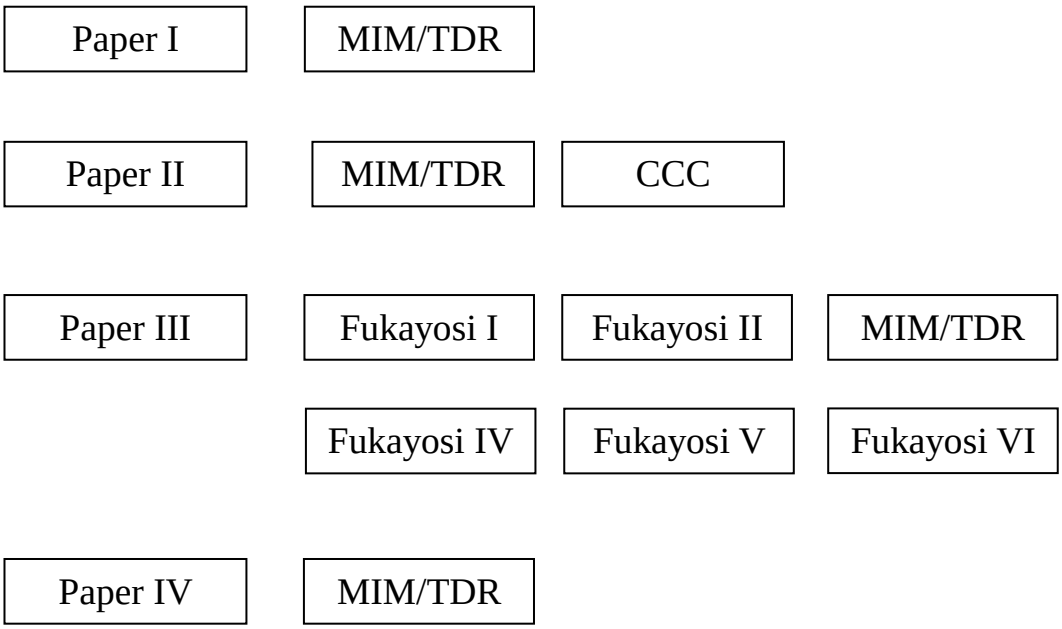

Figure 16. An overview of the clinical studies and the papers that they appear in.

3.3 TREATMENT

All patients included in the studies received a three day treatment with artemether-lumefantrine. The six doses were taken at 0h, 8h, 24h, 36h, 48h and 60h. The tablets contained 20 mg artemether and 120 mg lumefantrine and were administered based on weight; 1 tablet (5–14 kg), 2 tablets (15–24 kg), 3 tables (25-34 kg).

3.4 DNA EXTRACTION

Blood samples from patients were collected on filter paper (3MM; Whatman) and transferred to Karolinska Institutet, Stockholm, Sweden. Genomic DNA was extracted from the blood spots using the BloodPrep™ Chemistry on an ABI PRISM® 6100 Nucleic Acid PrepStation (Applied Biosystems, Fresno, CA, USA) according to the manufacturer's instructions. Day 3 samples included in Paper IV were extracted using a modified Chelex extraction protocol (*156*).

3.5 MOLECULAR ANALYSIS

3.5.1 PCR-Restriction Fragment Length Polymorphism (RFLP)

PCR-RFLP is an old method based on the use of restriction enzymes to differentiate between SNPs. Restriction enzymes has the capacity to cut DNA at a very specific

sequence pattern. The presence of a SNP will be detected trough the different sizes of fragments as result of the restriction. In this thesis the *pfmdr1* N86Y, *pfmdr1* Y184F *pfmdr1* D1246Y and *pfcrt* K76T SNPs were analysed by PCR-RFLP.

3.5.2 Pyrosequencing

Pyrosequencing was developed by Pål Nyrén and Mostafa Ronaghi at the Royal Institute of Technology, Stockholm in 1996 (*157*). The technology relies on detection of pyrophosphate release upon nucleotide incorporation and is used to sequence short DNA fragments. This technology has been suggested to be particularly useful in malaria research as it can reliable detect mixed infections. In high transmission settings it is very common that patients are infected by more than one genetically distinct population of *P. falciparum* parasites, also referred to as multiplicity of infections. As the malaria parasite is haploid during its stay in the human host all possible proportions of allele distribution can be found within mixed infections (Zhou et al 2006).

In this thesis the *pfmdr1* Y184F, *pfmdr1* D1246Y, *pfmdr1* F1226Y, *pfmrp1* F1390I SNPs were analysed by pyrosequencing PyroMark™ ID instrument (Qiagen, Germany). Serial dilutions of different proportions of mutant and wild type alleles originating from laboratory clones were used to determine the cut-off for mixed infections. The definition of mixed infections was all samples with more than 10% of each allele at a particular locus.

3.5.3 Sequencing

DNA sequencing can provide data on the full DNA sequence for bigger fragments (i.e. up to ~700bp) and is therefore preferable when your interest is on a more than one SNP. The DNA sequencing was outsourced (Macrogen Inc, Seoul, South Korea).

In this thesis the *pfmdr1* N86Y, *pfmdr1* Y184F, *pfmdr1* D1246Y SNPs and the regions around them were analysed by sequencing.

3.5.4 Real Time PCR

Real Time PCR has the advantage of being faster than conventional PCR and is particularly useful for detection of copy number variation. The *pfmdr1* gene copy number was assessed through TaqMan® probe based quantitative real time PCR (ABI Prism® 7000 Sequence Detection System) (*116*). (*158*). To be able to make relative quantification two probes were used, one targeted the *β-tubulin* gene, known to be present in only one copy, the other probe targeted our gene of interest *pfmdr1*. In each experiment three *P. falciparum* clones with only one copy of *pfmdr1* were used as calibrators and two clones with more than one copy of *pfmdr1* were used as positive controls.

Using the so called "ΔΔCt method" (*159*) we calculated the copy number in accordance with modifications made in previous work (*116*) and a copy number > 1.5 was defined as an amplification of *pfmdr1*.

In this thesis *pfmdr1* copy number was assessed in Paper I and in Paper III.

3.5.5 Distinguish between re-infection and recrudescence

When efficacy of antimalarial drugs is evaluated using clinical trials, it is quite common, in particular in high transmission settings that patients get a recurrent *P. falciparum* infection. As these infections can be due to a new infection (i.e. re-infection) or a recrudescence (i.e. treatment failure), it is very important to be able to distinguish between these two types. This is done by comparing the infection at baseline and with the recurrent infection, using nested PCR. For this three highly polymorphic genes *P. falciparum merozoite surface protein 2* (*msp2*) and *1* (*msp1*), and *glutamate-rich protein* (*glurp*) are used (*160*).

By using nested PCR, distinction between recrudescence and re-infection was performed by stepwise genotyping of the *msp2* and *msp1*, and *glurp* according to previously described protocols (*161*). Recrudescences were defined as samples containing at least one matching allelic band in all markers between baseline and day of recurrent parasitaemia, whereas re-infections were defined as no matching allelic band for one or more of the loci tested. Patients with recurrent parasitaemia and no available blood sample or negative PCR results were considered to have uncertain PCR corrected outcome.

3.5.6 Gel electrophoresis

Restriction fragments were analysed by an agarose gel electrophoresis containing 0.1μg/ml ethidium bromide to visualize DNA fragments by UV transillumination (GelDoc System, Bio-Rad, Hercules, CA, USA).

3.6 DRUG CONCETRATION ANALYSIS

Liquid chromatography mass spectrometry mass spectrometry (LC-MS MS) is an analytical method that can measure concentrations with high accuracy. Seven days after treatment initiation, capillary blood was collected on filter paper pre-treated with 0.75 M tartaric acid, and stored at -20°C. Lumefantrine whole-blood concentrations were measured by solid-phase extraction and liquid chromatography at Bioanalytics and Pharmacokinetics laboratory of Dalarna University, Sweden (*162*).

This analysis was performed for all patients, both after initial and re-treatment for Paper I. All infections included in Paper II had lumefantrine concentrations day 7 measured.

3.6.1 Estimation of lumefantrine concentrations

Lumefantrine elimination is considered to be in its log-linear phase after day 7. Therefore individual drug concentrations can be extrapolated to the point of interest according to the individual pharmacokinetic characteristics. Population pharmacokinetic estimates were derived from a detailed population pharmacokinetic study previously performed in the same setting (*75*). The expected drug concentrations on the day of hepatocyte burst were calculated for all patients with re-infections. The estimated day of hepatocyte burst was assumed to occur 7 days before microscopy-based detection of recurrent parasitaemia during follow up after artemether-lumefantrine treatment (Fig. 17). This method permitted an *in vivo* estimate of the re-infecting parasite's ability to multiply under drug pressure.

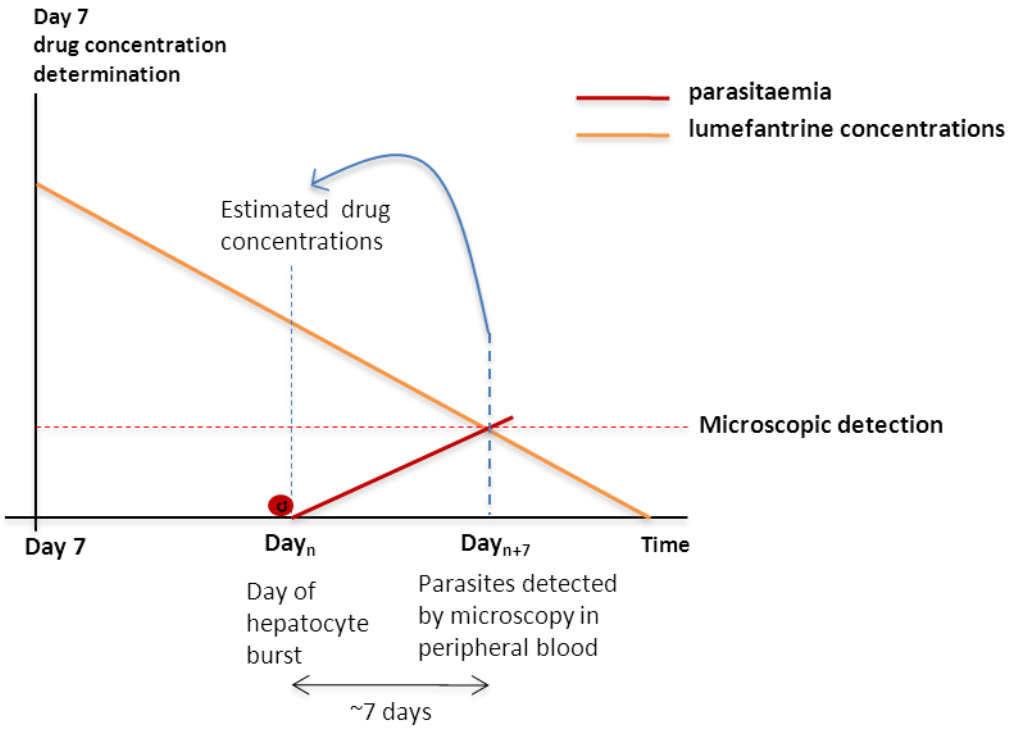

Figure 17. Schematic picture of parameters involved in estimating the lumefantrine concentrations at day of hepatocyte burst.

3.7 BIOINFORMATICS

Sequencher™ software version 4.6 (Gene Codes Corporation, Ann Arbor, MI, USA) was used to analyse all DNA sequences, assuming the 3D7 clone as the *pfmdr1* reference sequence (PFE1150w, Gene ID 813045 at NCBI RefSeq, National Center for Biotechnology Information Reference Sequence).

3.8 TRANSMISSION

Rainfall data was downloaded from the "Earth System Research Laboratory – Physical Science Division's" web page www.esrl.noaa.gov/psd/, using the coordinates for Fukayosi, Tanzania (latitude: 6º 24´ S and longitude: 38º 40´ E). The data was provided in mean mm rain/day for each month.

3.9 STATISTICS

The different statistic methods used are described in brief in the result section and more in detail in the papers and manuscripts. Statistical significance was defined as $p < 0.05$. The statistical softwares used were STATA v.12, SigmaPlot® and SPSS v.19.

3.9.1 Mixed infections

It is common that patients living in moderate/high transmission settings are infected with more than one parasite strain, i.e. another *P. falciparum* strain. There is currently no gold standard on how to analyse these mixed infections (both alleles present at a particular locus). In the result section of each paper, there is an explanation on how the mixed infections were treated. The mixes limits analysis of haplotypes as the bigger the haplotype of interest, the greater the risk of mixes. If two or more loci are mixed no precise haplotype can be determined.

3.10 ETHICS

All studies included in the thesis were approved by ethics committees at the National Institute for Medical Research in Tanzania and at the Regional Ethics Committee, Stockholm, Sweden. Before patients were enrolled in a trial, the study and its objectives were thoroughly explained to the patients and parents/guardians, in the local language (Swahili), thereafter written informed consent was obtained from the parents/guardians of the children.

The MIM/TDR study was registered at isrctn.org with identifier ISRCTN69189899. The CCC and Fukayosi II studies were registered at clinical.trials.gov with identifier NCT00454961 and NCT00336375, respectively.

4 RESULTS

4.1 PAPER I

Billy E Ngasala, Maja Malmberg, Anja M Carlsson, Pedro E Ferreira, Max G Petzold, Daniel Blessborn, Yngve Bergqvist, José P Gil, Zul Premji, Anders Björkman, Andreas Mårtensson

"Artemether-lumefantrine is a well-tolerated and highly efficacious treatment for uncomplicated *P. falciparum* malaria in children in Tanzania"

The aim of the study was to assess efficacy and effectiveness of artemether-lumefantrine, and how this relates to whole blood day 7 lumefantrine concentrations. As well as to assess if treatment with artemether-lumefantrine selects for genetically distinct parasite populations among recurrent infections, with focus on the major drug resistance associated genes *pfmdr1* and *pfcrt*.

An open label randomized trial was performed comparing supervised artemether-lumefantrine intake supervised (n=180) with unsupervised intake (n=179) in under five year old children with uncomplicated *P. falciparum* malaria, in rural Tanzania. Recurrent infections between day 14-56 were retreated within the same study arm. Main endpoints were PCR corrected cure by day 56 and 42 after initial and repeated treatment, respectively, as estimated by survival analysis. Mixed infections (i.e. both alleles present at a particular locus) were analysed together with the *pfmdr1* N86 and *pfcrt* K76, respectively. Efficacy and effectiveness end points were analysed by survival analysis. Continuous data were analysed with Student's *t* test, and comparison of categorical variables between groups was performed using the χ^2 test or Fisher's exact test, as appropriate.

PCR corrected cure after initial treatment was 98.1% (95% confidence interval (CI) [94.2% - 99.4%]) after supervised intake and 95.1% (95% CI [90.7% - 98.1%]) after unsupervised; p=0.29. Overall, 77 supervised and 84 unsupervised patients were retreated with PCR corrected cure of 92.9% (95% CI [81.8% - 97.3%]) and 97.6% (95% CI [89.3% - 98.8%]), respectively, p=0.58.

Early parasitological responses to treatment could only be assessed in the supervised group. After initial treatment, 28.7% (51 of 178), 93.3% (166 of 178) and 98.9% (176 of 178) of the patients had cleared parasitaemia on days 1, 2 and 3, respectively. During re-treatment, 49.4% (38 of 77), 98.7% (75 of 76) and 100% (75 of 75) of the patient achieved parasite clearance on days 1, 2 and 3, respectively.

Re-infections occurred in 46.9% (82 of 175) versus 50.9 % (86 of 169) (relative risk [RR] 0.92 [95% CI 0.74-1.14], p=0.46) after initial therapy; and 32.4% (24 of 74) versus 39.0% (32 of 82) (RR 0.83 [95% CI 0.54-1.27], p=0.39) after re-treatment.

Parasite genotyping showed that nearly all recurrent infections were due to re-infections. However, in total 16 recrudescent infections (treatment failures before day 7 and PCR confirmed recrudescent infections) occurred, of which 37.5% (6 of 16) after day 28. PCR corrected ACPRs were high and similar in both treatment groups after both initial and repeated treatment.

The proportion of PCR confirmed re-infections were similar between the supervised and unsupervised groups after initial treatment (46.9% [82 of 175] versus 50.9 % [86 of 169], relative risk 0.92 [95% CI 0.74-1.14], p=0.46) and after repeated treatment (32.4% [24 of 74] versus 39.0% [32 of 82], relative risk 0.83 [95% CI 0.54-1.27], p=0.39).

The frequency of parasites carrying the *pfmdr1* N86 allele (in pure form or mixed with 86Y) increased from 66.9% at baseline to 80.0% and 90.3% in recurrent infections after initial and repeated treatment, respectively (p<0.002). No significant change in prevalence of *pfcrt* K76 allele was observed. It is to note that the baseline prevalence of *pfcrt* K76 in pure form or mixed with 76T was high, (81.5%). No child carried infections with *pfmdr1* gene duplications at baseline or in recurrent infections after initial treatment. One recurrent infection carrying two copies of *pfmdr1* was registered after the re-treatment of a previous breakthrough infection.

The median lumefantrine blood concentrations on day 7 among the patients in this study were significantly higher in the supervised compared to the unsupervised group: after initial treatment (575 nM versus 367 nM; p<0.001) and after re-treatment (478 nM versus 310 nM; p<0.001). A significantly higher proportion of patients with

lumefantrine concentrations of <280 ng/ml (i.e. 529 nM), a cut-off value previously associated with risk of recrudescence (*163*), were observed in the unsupervised than in the supervised group ($p<0.02$ for all comparison). However, the lumefantrine concentrations did not correlate with different treatment outcomes (recrudescent infection, re-infection or no parasite reappearance).

4.2 PAPER II

Maja Malmberg*, Pedro E. Ferreira*, Joel Tarning, Johan Ursing, Billy Ngasala, Anders Björkman, Andreas Mårtensson, José P. Gil. (*shared first authorship)

"Using day 7 drug concentrations to estimate the drug levels that parasites withstand *in vivo* is a powerful tool to assess molecular markers relative importance"

The aim of this study was to examine if selection of *pfmdr1* SNPs among re-infections after artemether-lumefantrine treatment is associated with lumefantrine drug concentrations, and to assess the relative importance of different SNPs for the parasites capacity to withstand lumefantrine *in vivo*.

We analysed two clinical trials that assessed the efficacy and effectiveness of artemether-lumefantrine. As a proof of concept we used measured day 7 lumefantrine concentrations to estimate the concentrations at which re-infections multiplied. *Pfmdr1* genotypes of these parasites were then correlated to drug susceptibility. Only pure re-infections were included in the analysis. Mann-Whitney Rank Sum Test was used to compare estimated lumefantrine blood concentrations for different genotypes.

Re-infecting parasites carrying *pfmdr1* N86, 184F or D1246 pure alleles were able to survive at significantly higher median estimated lumefantrine blood concentrations compared with parasites harbouring their alternative alleles. The largest difference was observed for the N86Y SNP with 25.4 nM versus 2.08 nM (12.2 fold) between the N and the Y carrying parasites. For *pfmdr1* Y184F and D1246Y the corresponding differences were 4.09 versus 34.5 nM (8.4 fold) and 15.9 versus 3.23 nM (4.9 fold), respectively.

P. falciparum with the N86/184F/D1246 haplotype was statistically significantly less sensitive than the alternative haplotypes 86Y/Y184/1246Y, 31.4 versus 2.16 nM (14.5 fold, $p<0.001$), and 86Y/Y184/D1246, 31.4 versus 0.678 nM (46.3 fold, $p<0.001$), respectively (Fig. 18).

The highest estimated lumefantrine concentration that re-infecting parasites carrying N86 versus 86Y could withstand differed by a factor of 35 (1184.3 nM versus 34.3 nM).

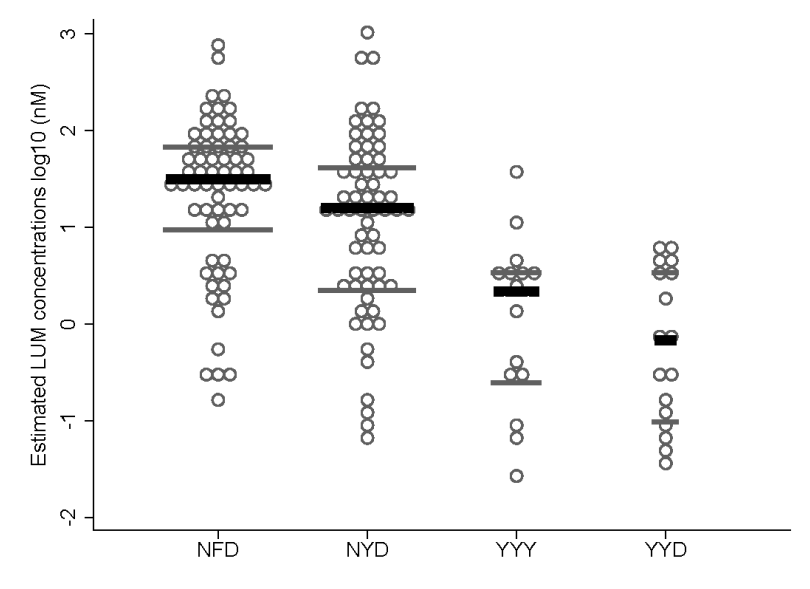

Figure 18. Estimated lumefantrine concentrations for re-infections carrying different *pfmdr1* haplotypes at codon 86, 184 and 1246. Each open circle represents a re-infection. The black lines denote the median values, the grey the inter-quartile range.

There was a distinct sub-set of parasites (n=8) able to grow at estimated lumefantrine concentrations above 550 nM, whereas no other parasites grew at concentrations higher than 300 nM. The subset represented 4.57% (8/175: CI 1.99-8.81) of the re-infections occurring up to 35 days after treatment initiation. These least susceptible parasites all carried the *pfmdr1* N86 allele and had lumefantrine day 7 levels 1.8-10.3 fold higher than 328 nM, confirming adequate treatment and bioavailability.

In Paper II the mixed infection (i.e. both alleles present at one locus) were excluded. It is to note that, when mixed infection were included as a separate group the median estimated lumefantrine concentrations for the mixed group was lower than the median of the resistant group and higher than the median of the sensitive group, for all the *pfmdr1* SNPs.

A separate analysis, also not included in the Paper II, was performed on *pfcrt* K76T. Re-infecting parasites with *pfcrt* K76 survived at higher median estimated lumefantrine concentration as compared to those with the 76T allele, 14.3 nM versus 4.1 nM (3.4 fold difference), this did not reach statistical significance (p=0.083).

4.3 PAPER III

Maja Malmberg, Billy Ngasala, Pedro E. Ferreira, Erik Larsson, Irina Jovel, Angelica Hjalmarsson, Max Petzold, Zul Premji, José P. Gil, Anders Björkman, Andreas Mårtensson.

"Molecular surveillance of *pfmdr1* and *pfcrt* SNPs unveiled temporal trends of markers associated with artemether-lumefantrine tolerance/resistance"

The aim of this study was to analyse temporal trends of the prevalence of *pfmdr1* and *pfcrt* SNP alleles after the implementation of artemether-lumefantrine as first-line treatment for uncomplicated malaria in Tanzania 2006.

SNPs in the *pfmdr1* N86Y, Y184F, D1246Y and *pfcrt* K76T were analysed from dried blood-spots collected during six consecutive studies from children with uncomplicated *P. falciparum* malaria in Fukayosi village, Bagamoyo District, Tanzania, between 2004-2011. For prevalence analysis of individual SNPs, mixed infections (i.e. both alleles present at one locus) were analysed together with the *pfmdr1* 86Y, Y184, 1246Y and *pfcrt* 76T. For haplotype analysis, minority haplotypes (≤ 5%) and infections that were mixed at two or more loci were excluded. Infections that were mixed at only one locus were analysed as having both haplotypes. Logistic regression with year included as a continuous covariate was used to estimate the yearly changes in prevalence from 2006 to 2011.

There was a statistically significant yearly increase of *pfmdr1* N86, 184F, D1246 and *pfcrt* K76 between 2006-2011 from 14% to 61% (yearly OR=1.38 [95% CI 1.25-1.52] p<0.0001), 14% to 35% (OR=1.17 [95% CI 1.07-1.30] p=0.001), 54% to 85% (OR=1.21 [95% CI 1.03-1.42] p=0.016) and 49% to 85% (OR=1.33 [95% CI 1.17-1.51] p<0.0001), respectively.

During the same period the *pfmdr1* NFD haplotype increased from 10% to 37% (OR=1.25 [95% CI 1.12-1.39] p<0.0001), whereas the YYY haplotype decreased from 31% to 6% (OR=0.73 [95% CI 0.56-0.98] p=0.018) (Fig. 19).

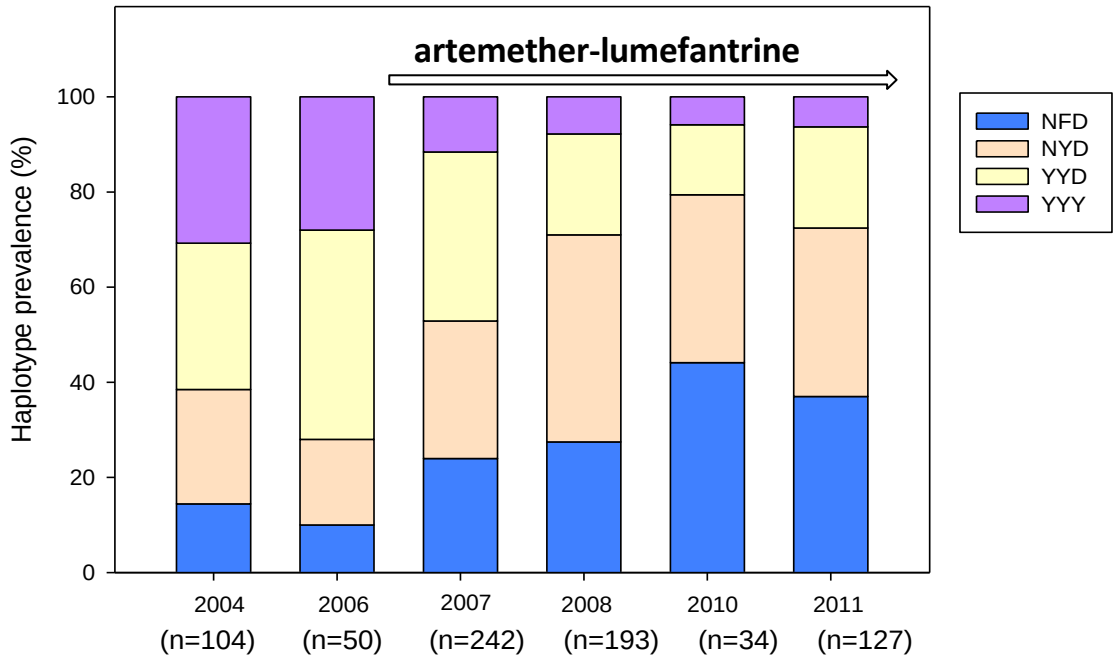

Figure 19. Prevalence of *pfmdr1* haplotypes (N86Y, Y184F, D1246Y) in the parasite population in the village of Fukayosi, Bagamoyo District, Tanzania from 2004 to 2011. The arrow indicates when artemether-lumefantrine was introduced as first line treatment of uncomplicated malaria.

When *pfmdr1* haplotypes were combined with *pfcrt* K76T there was a statistically significant increase of the NFD+K haplotype from 12% to 36% (OR 1.27 [95% CI 1.12-1.45] p<0.0001) between 2006-2011. During the same period there was a significant decrease of the YYD+K and YYD+T from 29% to 20% (OR 0.86 [95% CI 0.74-1.00] p=0.041) and 17% to 0% (OR 0.59 [95% CI 0.41-0.83] p=0.003), respectively. There was also a decrease in prevalence of YYY+T from 19% to 2% (OR 0.63 [95% CI 0.39-1.00] p=0.05), confirmed statistically significant by non-parametric trend test (p=0.003).

All 390 successfully analysed samples had one copy of the *pfmdr1* gene.

4.4 PAPER IV

Maja Malmberg, Pedro E. Ferreira, Aminatou Kone, Berit Aydin-Schmidt, Billy Ngasala, Anders Björkman, Andreas Mårtensson, José P. Gil.

"PfMDR1 SNPs are not selected during initial parasite clearance but play a role in the post-treatment prophylactic effect of artemether-lumefantrine"

The aim of this study was to assess if artemether/dihydroartemisinin *in vivo* select for the same *pfmdr1* and *pfcrt* polymorphisms as lumefantrine. As well as examine the characteristics of the lumefantrine window of selection after artemether-lumefantrine treatment.

A total of 359 children were enrolled and followed-up weekly to 56 days after initiation of artemether-lumefantrine treatment (Paper I). A sub-population of 178 children also underwent daily blood sapling during the early treatment phase, i.e. day 0 to day 3. Genotyping of *pfmdr1* and *pfcrt* main SNPs was compared between day 0 and day 3 (initial parasite clearance), and day 0 and day of recurrent parasitaemia during follow-up. For the day 0 versus recurrent infection SNP prevalence, mixed infections (i.e. both alleles present at one locus) were combined with the *pfmdr1* 86Y, Y184, 1246Y and *pfcrt* 76T. For haplotype analysis mixed infections and minority haplotypes (<3%) were excluded from further analysis. To compare prevalence of SNPs at baseline and at recurrent infection, χ^2 test or Fisher's exact test was used, as appropriate. Time to recurrent infection was analysed using survival analysis and comparisons between the different times were done using Log-Rank test and an all pairwise method.

Out of 178 patients sampled at day 3, only two (1.1%) were positive by microscopy whereas 117 (65.7%) were positive using PCR. The analysis of these infections did not reveal evidence of *pfmdr1* and/or *pfcrt* SNP selection between day 0 and day 3, during the artemether-lumefantrine treatment course. Genotype changes between day 0 and day 3 were seen in 29% (23/80), 24% (17/71), 22% (14/63) and 20% (18/91) of patients, for *pfmdr1* N86Y, Y184F, D1246Y and *pfcrt* K76T, respectively. Nevertheless, this fluctuation did not follow any specific trends.

A total of 186 patients (52%) experienced microscopically confirmed recurrent infection within 56 days after initiation of artemether-lumefantrine treatment. Only 7 of

these were defined as recrudescences according to PCR analysis, precluding any meaningful analysis between re-infections and recrudescences.

When comparing prevalences of SNPs day 0 and among recurrent infections a statistically significant selection was seen for *pfmdr1* N86, 184F and the *pfmdr1* NFD haplotype. The prevalence of the *pfmdr1* haplotype N86, 184F and D1246, increased from 28.1% (77/274) at day 0 to 100% (2/2) D7-D14 (p=0.15), 64.7% (11/17) D15-D21 (p=0.004) and 50.0% (17/34) D22-D28 (p=0.016) among recurrent infections (Fig. 20).

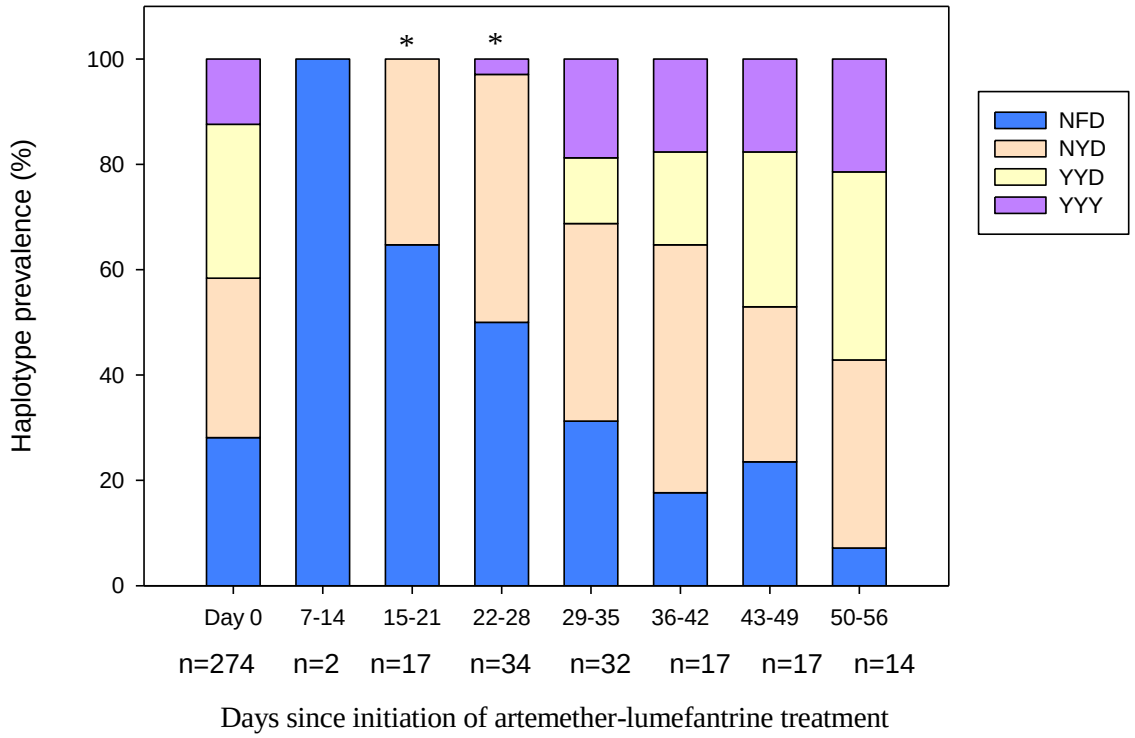

Figure 20. Window of selection for *pfmdr1* haplotypes (codon 86, 184, 1246) after artemether-lumefantrine treatment. The stars indicated significant difference in prevalence of NFD as compared to day 0.

There was a 3 week difference in median day to recurrent infection between the NFD and the YYD haplotypes, 28 days versus 49 days respectively (p=0.0034). There was also a significant difference in median time to recurrent infection between NFD and YYY (28 days versus 38 days; p=0.0037) as well as between NYD and YYD (35 days versus 49 days; p=0.0039).

4.5 OTHER RESULTS

4.5.1 Seasonal fluctuations

It is to note that the results presented here are not included in any paper. As selection of *pfmdr1* SNPs were seen within four weeks after treatment, we carried out an analysis to identify during which time of the year this was most likely to happen, i.e. how the risk of getting a recurrent infection during the first four weeks post-treatment varied by month.

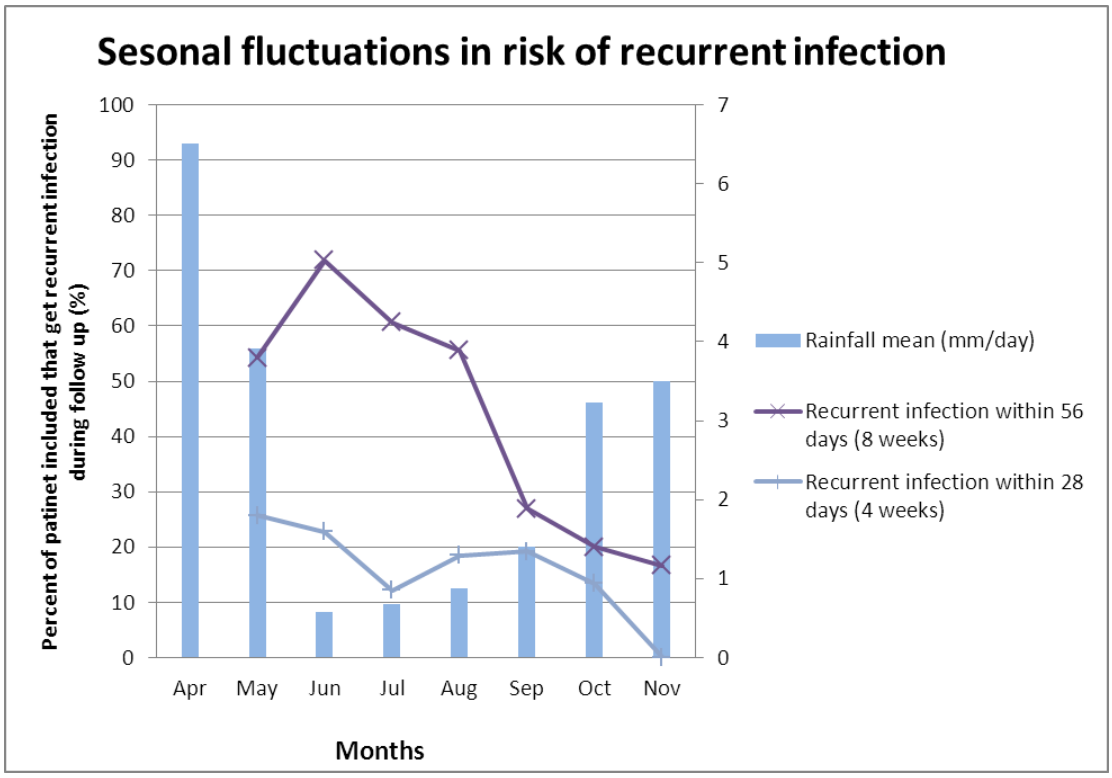

Figure 21. Seasonal fluctuations in risk of recurrent infection after treatment with artemether-lumefantrine and rainfall in mean mm/day.

Seasonal variation in the risk of recurrent infection was observed during the MIM/TDR study used in Paper IV. For example, patients enrolled during the month of June experienced the highest risk of getting a recurrent infection, 64 out of 89 (72%) experienced a recurrent infection within 56 days after treatment initiation, compared with an average of 47% during the other months (Fig. 21). The risk of recurrent infection within four weeks was highest during May and June (26% and 22%, respectively), but did not fluctuate as much as for the 56 day follow up. This peak in risk of recurrent infection followed ~1-2 months after the peak in rainfall.

To find out if the difference in risk of recurrent infection was reflected among the baseline prevalence of our SNPs of interest (*pfmdr1* N86, 184F, D1246 and *pfcrt* K76), a logistic regression with month as a covariate was performed.

A significant increased prevalence of the *pfcrt* K76 was observed among pre-treatment infections (day 0) from May to August (Monthly OR 1.46 [95% CI 1.04-2.06] p=0.028). There was also a trend for increased prevalence of the *pfmdr1* N86 among pre-treatment infections during the full period May to October; i.e. 37% (13/35) in May to 67% (10/15) in October (Fig. 22). No major changes in *pfmdr1* 184F and D1246 was observed.

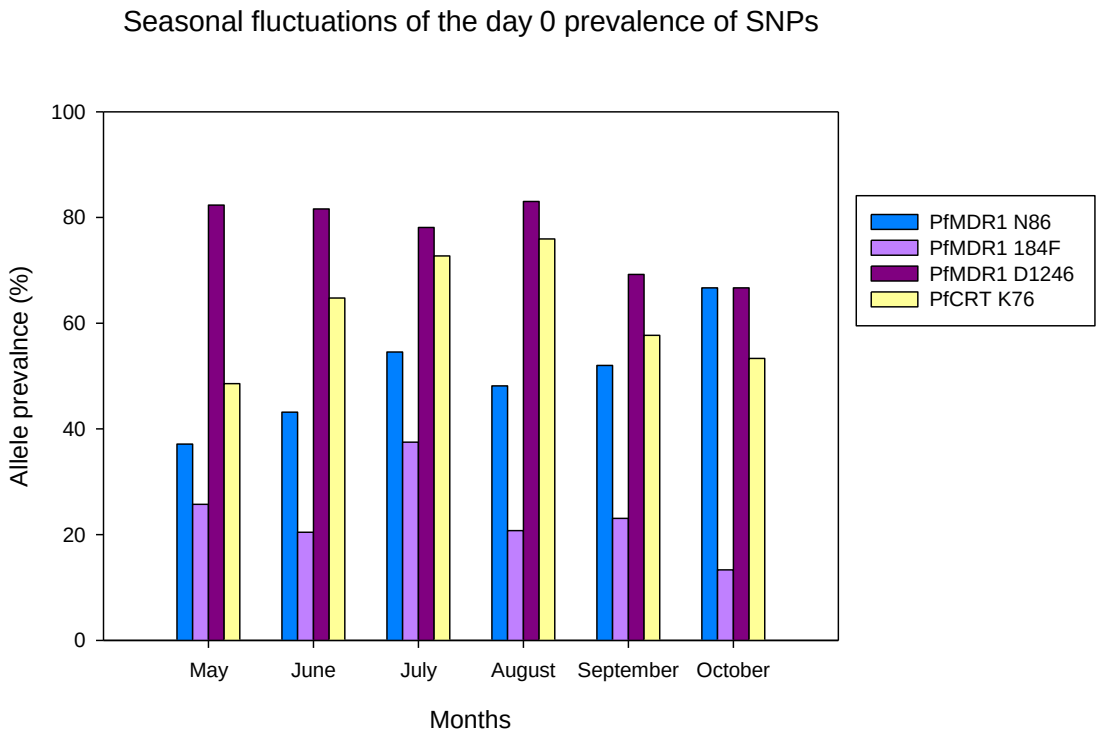

Figure 22. Prevalences of *Pf*MDR1 N86, 184F, D1246 and *Pf*CRT K76 SNPs among pre-treatment infections in Fukayosi village, Bagamoyo District, Tanzania, May to October 2007. Mixed infections (i.e. both alleles present at one locus) were combined with the *Pf*MDR1 86Y, Y184, 1246Y and *Pf*CRT 76T, respectively.

5 DISCUSSION

5.1 ARTEMETHER-LUMEFANTRINE

5.1.1 Efficacy and effectiveness of artemether-lumefantrine

In Paper I it was concluded that artemether-lumefantrine is a highly effective and well-tolerated treatment for children with uncomplicated malaria. This has previously been seen in several other studies (*50*). Both supervised and unsupervised intake resulted in high cure rates. This is supported by other effectiveness studies from East Africa (*153, 164-166*) and other parts of Africa (*167, 168*) where artemether-lumefantrine has been proven to be highly effective even when the treatment was administered at home.

It was shown in Paper I that re-treatment with artemether-lumefantrine within 56 days after initial treatment was highly efficacious. This has previously been seen in Uganda, Ghana, Mali and Senegal (*169-174*). Importantly, artemether-lumefantrine was also safe and well tolerated after repeated treatment, which is also supported by several other studies (*169, 170, 172, 175*).

5.2 LUMEFANTRINE

5.2.1 The use of drug concentrations day 7 to assess tolerance

Paper II introduced a new method to assess the development of tolerance/resistance *in vivo*. By using day 7 lumefantrine concentrations and the pharmacokinetic information of the terminal elimination rate for our study population, the drug levels present when the recurrent parasites initiate its intra-erytherocytic life cycle could be estimated. This defines a new *in vivo* phenotype theoretically more similar to the *ex vivo* assays (i.e. sensitivity assessment at controlled drug exposure) than the currently used standard PCR-adjusted *in vivo* testing phenotype. By genotyping these reappearing parasites, we correlated the exposed drug concentrations with the presence of different SNPs alleles, defining the importance of these polymorphisms on the parasites ability to withstand lumefantrine. The proposed method has the potential of evaluating old and defining new potential molecular markers of *in vivo* tolerance/resistance.

The described method has limitations. One is the fact that our model of lumefantrine clearance was based on a population average terminal elimination half-life, derived from a previous population pharmacokinetic study. Although still valuable and sufficient for the proof of concept objectives in Paper II, it is evident that measured individual lumefantrine concentrations from two time points during the drug elimination phase would provide a more precise determination of the concentrations the parasite is exposed to. For future work using this method I propose to measure concentrations e.g. at day 7 and day 14, to get individual terminal elimination half-lives.

Another issue that must be taken into account is the fact that lumefantrine is highly protein bound (*52*). Due to this it is not known with absolute precision the actual intra-erytherocytic lumefantrine concentrations. This makes it difficult to compare results from our method with *in vitro* methods, in terms of actual concentrations. However when comparing within a study, like what was done in Paper II, it is not expected that the high protein binding would influence the correlation between drug concentrations and SNPs. I assume that protein binding does not differ too much between individuals and that comparisons *ex vivo* would be more accurate in terms of actual concentration but this need to be further explored.

The use of drug concentrations to assess tolerance/resistance is not specific for lumefantrine as it is a concept applicable to any long half-life antimalarial drug, where concentration determination methods are available. The method might be useful to study drug resistance in *P. vivax,* where the possibility to perform *in vitro* work is yet very limited. Finally, other infectious diseases, where recurrent infections and the use of long half-life drugs are common, could also apply this approach, e.g. leishmaniasis.

5.2.2 The role of *pfmdr1* in lumefantrine resistance

Throughout this thesis significant contribution of specific *pfmdr1* polymorphisms have been shown to reduce lumefantrine susceptibility and shorten post-treatment prophylactic effect after artemether-lumefantrine treatment (Paper I, II, and IV).

From our work in Paper II it was concluded that among the SNPs studied, the *pfmdr1* N86 was most important for reduced lumefantrine susceptibility. These results are consistent with early *in vitro* work using parasite clones resulting from a genetic cross (*124*), as well as from a more recent study on laboratory adapted field isolates from Kenya showing an association between *pfmdr1* N86 and reduced IC_{50} values for lumefantrine (*127*). In Paper II *pfmdr1* 184F and D1246 were associated with reduced lumefantrine susceptibility. Paper IV showed that the *pfmdr1* N86 and 184F are selected among recurrent infections upon artemether-lumefantrine treatment. These results are in accordance with previous studies (*77, 120, 125, 126, 176*). In Paper IV selection of *pfmdr1* N86 was shown already 14 days after treatment initiation (n=3), which could be interpreted as selection starting almost immediately after ended treatment course. This points towards the importance of the *pfmdr1* gene in the parasite response to this drug, supposedly at even relatively high lumefantrine concentrations. This observation is consistent with the finding in Paper II, where the *pfmdr1* N86 was shown to be associated with a marked difference in the capacity of the parasite to grow *in vivo* under drug pressure.

Reinforcing the epidemiological importance of this SNP, the World Wide Antimalarial Resistance Network (WWARN) is currently performing a meta-analysis of artemether-lumefantrine clinical trials, and the preliminary results point towards *pfmdr1* N86 as a possible risk factor for artemether-lumefantrine recrudescence (*177*).

The *pfmdr1* gene codes for the PfMDR1 transmembrane transporter, an ABC protein mainly located in the digestive vacuole membrane. It transports molecules from the cytoplasm into the lumen of the digestive vacuole (*105*). *In silico* models of PfMDR1 have shown that amino acid 86 is located adjacent to the drug binding pocket suggesting a role in the kinetics of the transporter (*106*).

The amino acid 184 is located on the outer surface of the transporter and is expected to also be involved in transport kinetics rather than direct interactions with drugs (*106*). This less direct interaction with the transport mechanism might explain why in our (Paper II) and several other studies, the Y184F has proven to be less consistently associated with drug susceptibility than other SNPs in *pfmdr1* (*116, 120, 123, 129, 178-181*).

The amino acid 1246 is located in the nuclear binding domain, and as such it is important for the ATPase activity of the protein. Mutations in the 1246 have been suggested to interfere with ATPase activity by influencing the communication between the nuclear binding domain and the trans-membrane domain (*106*). This is expected to affect the protein's transport capabilities in a general manner, not drug specific. Accordingly, this SNP has been related *in vitro* with more or less intensity in the response to a broad range of quinoline drugs (*117, 118*). In our works (Paper IV), the D1246Y SNP clearly modulates the lumefantrine IC_{50}, when comparing parasite clones with engineered exchanges in only the D1246Y position.

Increased copy number of *pfmdr1* is relatively common in South East Asia, whereas on the African continent *pfmdr1* duplication has very rarely been found (*182-184*), e.g. none in Guinea Bissau despite ~600 samples analysed (*137*). In Sudan, despite low numbers (3/100), amplified *pfmdr1* at baseline was associated with recurrent infections upon artemether-lumefantrine treatment (*185*). Interestingly, this reminds of the previous report by Price *et al*., 2006 where *pfmdr1* copy number was related with less effective four dose artemether-lumefantrine treatment in the Thai-Burma boarder (*134*). Curiously, in South East Asia parasites analysed with amplified *pfmdr1* are N86 carriers (*116, 128, 134*), whereas in the African context, the few cases found have been 86Y carriers. Accordingly, in Paper I one patient infected with a parasite carrying two copies of 86Y *pfmdr1*, was reported. This finding was confirmed by Gadalla *et al*., 2011, where all three *pfmdr1* amplifications detected were 86Y (*185*).

Increased copy number of *pfmdr1* is associated with an increased fitness cost (*186*). *Pfmdr1* amplifications have been more frequently found among travellers returning from Africa (*187*), which might be because these non-immune subjects provide a niche for parasites with a reduced fitness. This raises a concern; as malaria incidence, in part due to improved malaria control, is decreasing in several African regions (*32, 188-190*), there is a risk that this might increase the possibility of parasites carrying *pfmdr1* duplications to become more established in the parasite population.

This thesis provide additional evidence that *pfmdr1* is associated with decreased parasite response to lumefantrine. The mechanism behind this phenotype remains to be understood. The topology of the *pfmdr1* suggests this protein to be oriented towards the lumen of the digestive vacuole, with both the N- and C-terminals located in the

cytoplasm (*110*). Its transport activity is hence expected to concentrate substrates in the digestive vacuole (*105*). Assuming that the main target(s) for lumefantrine is/are located in the cytoplasm, hence by pumping the drug into the digestive vacuole the concentration of lumefantrine decreases in the cytoplasm. The digestive vacuole is discarded during the formation of the upcoming merozoites by the end of the intra-erytherocytic cycle, therefore using it as a waste bin could represent a temporary but effective solution for evading the drug action (Fig. 23).

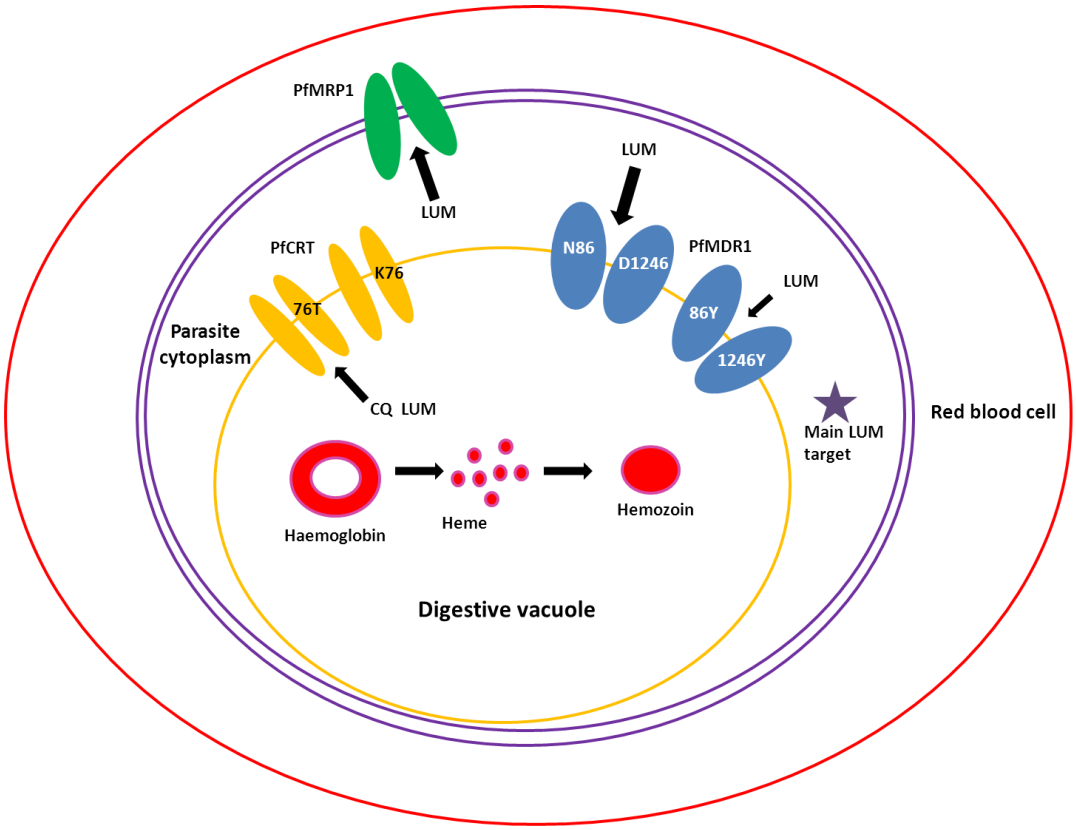

Figure 23. Possible model of PfMDR1, PfCRT and PfMRP1 mediated lumefantrine resistance. The main target of lumefantrine (LUM) is located in the parasites cytoplasm (purple star); therefore the parasite can become resistant by transporting the drug into the digestive vacuole. This can be done more efficiently via the PfMDR1 N86/D1246 (big arrow), than the PfMDR1 86Y/1246Y (small arrow). Increased copies of PfMDR1 will also increase the transport capacity into the digestive vacuole. The PfCRT K76 cannot transport lumefantrine out from the digestive vacuole (same is true for chloroquine (CQ)), this results in lumefantrine accumulation in the digestive vacuole of parasites with PfCRT K76. The PfMRP1 can transport lumefantrine out of the cytoplasm and thereby remove it from its target and render the parasite resistant.

5.2.3 The role of *pfcrt* in lumefantrine resistance

In Paper I and IV no significant selection of *pfcrt* K76 among recurrent infections was seen. In Paper I, the *pfcrt* K76 baseline prevalence was high (82%), and therefore although a trend was seen no significant selection was observed. Nevertheless, it has been shown to be selected among re-infections after artemether-lumefantrine treatment (*146*), and confirmed to play a role in the response to lumefantrine *in vitro* both through the analysis of culture adapted parasites (*127*), as well as through the study of genetically engineered clones that only differ at the K76T position (*146*). This is to a certain extent consistent with the influence of the *pfcrt* K76T polymorphism on mefloquine IC_{50}s in allelic exchanged clones (*142, 191*). In Paper II it was observed that *pfcrt* K76 were able to re-infect at 3.4 fold higher estimated lumefantrine concentrations than the 76T, however this did not reach statistical significance (p=0.083). These somehow inconclusive results could be due to the relatively high baseline prevalence of *pfcrt* K76 or the greater importance of other genes (e.g. *pfmdr1*) for lumefantrine susceptibility. In any case, the precise importance of *pfcrt* K76T polymorphisms in lumefantrine resistance deserves a more detailed examination.

5.2.4 The window of lumefantrine-driven *pfmdr1* SNP selection and the short post-treatment prophylactic effect of artemether-lumefantrine

The window of selection is mainly determined by the terminal half-life of the drug, which in the case of lumefantrine is circa 3-5 days (*71, 74, 75*). Assuming that the log-linear phase starts at day 5, it is expected that no major concentrations of lumefantrine remain in the patient after approximately 20-30 days. Supposing that it takes one week for parasites from hepatocyte burst until they reach levels detectable by microscopy one could assume that selection seen within approximately five weeks after treatment initiation is in part driven by lumefantrine. By using modelling the lumefantrine window of selection has been estimated to end at approximately 38 days (*192*). The window of selection for lumefantrine observed in Paper IV (i.e. 28 days), was in accordance with previous reports from Zanzibar (*77, 120*). In Paper IV an earlier opening of the window of selection (i.e. day 14) was observed, indicating that some parasites can actually start growing almost immediately after completed treatment course.

Lumefantrine has a limited post-treatment prophylactic effect, which is partly explained by the relatively short terminal elimination half-life compared to other ACT partner drugs, i.e. 3-5 days (*71, 74, 75*) as compared with 10-14 days for DEAQ (desbutyl-amodiaquine) - the active metabolite of amodiaquine (*193, 194*), and ~23 days for piperaquine (*195*) (*196*). The disadvantage with a short post-treatment prophylactic effect is that it provides a shorter recovery period for patients and thereby limits the time for haematological recovery. From a resistance development point of view it could be an advantage with a short post-treatment prophylactic effect as it shortens the period of sub-therapeutic drug concentrations that can select for resistant infections (*197*).

In Paper IV, it was shown that the prophylactic effect varied depending on the *pfmdr1* haplotype carried by the recurrent parasite. Hence, the presence of specific *pfmdr1* SNP alleles in the parasite populations might directly reflect the artemether-lumefantrine post treatment prophylactic period. That is, as the prevalence of for example *pfmdr1* NFD in the parasite population is increasing, the post-treatment prophylactic effect could decrease. It has been shown that chloroquine and amodiaquine both select for *pfcrt* 76T and *pfmdr1* 86Y (*198*). Due to this there is reason to believe that parasites that are less susceptible to lumefantrine have increased susceptibility to chloroquine and amodiaquine. As an additional evidence to show that these molecular markers can have a role in the post-treatment prophylactic effect a comparison can be made between the different ACTs ability to protect against recurrent infections in relation to the baseline prevalence of *pfcrt* K76. Table 1 shows the percentage of recurrent infections up to 28 days after ACT treatment, in order by percentage of chloroquine sensitive parasites (i.e. *pfcrt* K76 carriers).

Table 1. Percentage of recurrent infections up to 28 days after ACT treatment, in order by percentage of chloroquine sensitive parasites (i.e. *pfcrt* K76 carriers).

	chloroquine sensitivity (*pfcrt* K76)	artemether-lumefantrine arm	artesunate-amodiaquine arm	DHA-piperaquine arm	Site	
4ABC Study Group (*199*)	Low	16% (12/75)	8.7% (6/69)	0% (0/67)	Ndola	Zambia
4ABC Study Group	0%	7.1% (5/70)	11.8% (9/76)	1.6% (1/63)	Fougamou, Lambaréné	Gabon
Martensson *et al* 2005 (*200*)	3%	7% (14/197)	28% (57/206)		2002-2003	Zanzibar
4ABC Study Group	19%		17.4% (26/149)	6.9% (10/145)	Mbarara	Uganda
4ABC Study Group	22%		19.1% (35/183)	5.5% (10/182)	Manhica	Mozambique
4ABC Study Group	50%	1.3% (1/75)		5.3% (4/76)	Mashesha	Rwanda
Bukirwa *et al* 2006 (*201*)	55%	51% (102/202)	66% (133/201)		Tororo, 2004-2005 dec-jul	Uganda
4ABC Study Group	55%	43.7% (94/215)		24.5% (53/216)	Tororo	Uganda
4ABC Study Group	55%	8.8% (7/80)	7.6% (6/79)	7.7% (5/65)	Pamol	Nigeria
4ABC Study Group	55%	3.4% (3/87)	6.0% (5/83)	6.9% (5/72)	Afokang	Nigeria
4ABC Study Group	60%	9.9% (7/71)		2.9% (2/70)	Rukara	Rwanda
4ABC Study Group	72%	6.8% (14/206)		4.4% (9/206)	Jinja	Uganda
Sagara *et al* 2012 (*172*)	63%	36.4% (231/634)	19.5% (117/601)		2005-2007	Mali
4ABC Study Group	76%	60.6% (177/292)	31.4% (91/290)	14.6% (32/219)	Nanoro	Burkina Faso

In Paper III it was shown that the prevalence of *pfmdr1* NFD haplotype and all the individual SNPs have increased significantly since the introduction of artemether-lumefantrine. The *pfmdr1* SNPs have been shown to increase also in other settings where artemether-lumefantrine has been implemented (*202-204*). As the *pfmdr1* NFD haplotype is associated with a decreased post-treatment prophylactic effect there is a risk that this will lead to an increased malaria incidence.

5.3 ARTEMETHER/DIHYDROARTEMISININ

In Paper IV we report that ~25% of the patients had different *pfmdr1* and/or *pfcrt* genotype, day 3 and day 0. Similar parasite population dynamics have been previously described upon the analysis of very polymorphic genes such as *pfmsp1/2* and *glurp* (*155*), and concerning *pfcrt* and *Plasmodium falciparum dihydrofolate reductase* (*pfdhfr*) SNPs in travellers (*205*). Such differences could partly be because of the sequestration phenomenon, where only early and late ring stages are visible in the peripheral blood. They could also reflect a context of high baseline multiplicity of infection. The parasite dynamics indicate a possible within patient selection process among the drug exposed parasite populations. Accordingly, Paper IV investigated if there were trends of *pfmdr1* selection between day 0 (pre-treatment) and day 3, a period in the treatment where the pulse "area under curves" (AUCs) of artemether/DHA are expected to be the pharmacodynamic driving force of the treatment. Such hypothesis was put forward, as *pfmdr1* has been shown *in vitro* to modulate *P. falciparum* response to artemisinin derivatives (*116, 118, 123, 124, 133*).

The absence of significant selection of any of the SNPs does not support the involvement of *pfmdr1* in the parasite early response to the treatment. This is in contrast with a recent study from Kenya where the selection of the *pfmdr1* N86/184F/D1246 haplotype has been documented during the day 0 to day 3 period (*206*). Presently, no definitive explanation for these contradicting results exists. This may simply be due to differences between the malaria settings (a high baseline prevalence of the N86+D1246 haplotype (55%) in our location), or trial design (differences in drug intake). Another possibility could be that the parasites populations are genetically different in other – yet to be identified – *loci* associated with artemisinin susceptibility.

Anyway, our negative results are in line with the present lack of evidence of significantly slow parasite clearance in the African continent (*155, 207*). Nevertheless,

one study from Africa has reported increased prevalence of day 1 positivity (microscopy based) after ACT treatment (*208*). Although this observation could be interpreted as a first sign of parasites with declining responsiveness to artemisinin, it might just reflect a decreased immunity in the population upon the reduction in malaria incidence experienced in this region.

As no selection was seen day 0 to day 3, we conclude that in our setting and within the number of patients and SNPs analysed the artemisinin component does not select for the same molecular markers of tolerance/resistance as does lumefantrine. This suggested lack of resistance mechanism overlap is expected to prolong the useful life span of artemether-lumefantrine as an effective combination therapy in Africa.

5.4 DO WE SEE RESISTANCE TOWARDS ARTEMETHER-LUMEFANTRINE?

ACT efficacy is based not only on the powerful anti-parasitic capacity of the artemisinin component, but also on a highly efficacious long half-life partner drug. The collapse of one of the drugs is likely to be enough to severely compromise the combination. In other words, resistance to artemether-lumefantrine – defined as treatment failure upon the administration of appropriate doses leading to adequate exposure – will probably happen upon a significant decrease in lumefantrine efficacy. Using the same argument, it is possible that a three day artemether-chloroquine treatment would fail in a chloroquine resistant area, as indirectly suggested in a study testing this (*209*).

Treatment failure may however be due to inadequate drug bioavailability, i.e. not only truly drug resistant parasites. For slowly eliminated antimalarial drugs, day 7 concentrations have been proposed to be a surrogate marker of the AUC and risk of clinical failure. Day 7 lumefantrine concentrations above 280ng/ml (529 nM) have been associated with low risk of treatment failure in a small pharmacokinetic pharmacodynamic study among adult Thai malaria patients (*163*). In a later clinical efficacy trial, at the Thai-Burma border it was shown that day 7 concentrations below 175ng/ml (331 nM) predict recrudescence with 75% sensitivity and 84% specificity (*134*).

In Paper II parasites able to proliferate in conditions of estimated lumefantrine concentrations above the mentioned thresholds were identified. Although one should be cautious as to state that we have identified artemether-lumefantrine resistant parasites in our studies, it seems likely that the observed parasites with decreased lumefantrine susceptibility are the precursors of future artemether-lumefantrine resistance.
Overall, this work supports the view that the development of resistance against artemether-lumefantrine is possible, probably through a process starting with an expected decreased of post-treatment prophylactic effect progressively reducing the combination treatment to an artemether monotherapy (*210*), and only three days of artemether is sufficient for therapeutic cure.

In the study described in Paper I no clear correlation between low day 7 lumefantrine concentrations and recrudescence was seen. This finding was also reported in previous trials (*167*). However in the effectiveness study included in Paper II there is a significant association between low day 7 lumefantrine concentrations and recrudescence (i.e. recrudescences had median lumefantrine concentrations of 183 nM versus re-infection 388 nM and no recurrent infection 410 nM) (*153*). In a multicentre trial comparing a dispersible tablet formulation with a tablet formulation administered crushed, it was noted that there was a tendency for lower lumefantrine concentrations among treatment failures (*211*).

5.5 CONTRIBUTING TO THE UNDERSTANDING OF A COMPLEX SCENARIO: *IN VIVO* PARASITE DRUG SUSCEPTIBILITY

The current PCR adjusted *in vivo* testing used to evaluate antimalarial drug efficacy has some concerns. By only using one time point, not all parasite populations are visible in the peripheral blood, and important information is thereby lost (*154*). Also the method used to extract and amplify DNA will influence the sensitivity and hence the detection of minor parasite populations (*212*). This leads to different laboratories having different outcomes for the same markers, with the same biological material (*213*).

There is also the risk that - in particular in a setting with low genetic diversity in the parasite population - a patient might be re-infected with a parasite that looks identical to the one that was there pre-treatment, and thereby wrongly classified as a recrudescence.

Conversely, in setting with diverse parasite populations, recurrent infections occurring up to day 14 are often classified as re-infections (*214*). Such "re-infections" are unlikely, considering that it takes approximately 7 days from the moment of the infective bite until the hepatocyte burst, and approximately 7 days from hepatocyte burst to microscopically detectable levels of parasites in the peripheral blood

The WHO guidelines were designed to evaluate how well the drug cleared parasites and cured the patient. However it was not designed to study resistance development. Another limitation with the WHO guidelines currently used to evaluate antimalarial drug efficacy is that it does not take into consideration the post-treatment prophylactic effect. In Paper II we propose that by using drug levels day 7 and estimating the concentrations that emerging parasites can withstand, will provide a more accurate definition of individual infections as carrying tolerant/resistant parasites. For improved estimates we suggest to determine drug levels also at day 14, to get individual estimates of the terminal elimination rate. This method is expected to be highly specific, i.e. a highly tolerant/resistant parasite will be detected and a false classification is unlikely. Our proposed method will only be able to detect tolerant/resistant parasites that appear during the early post-treatment period, as such it is prone to low sensitivity, i.e. parasites that have the ability to withstand high drug concentrations may just happen to emerge late during the follow up and thus not to be considered as tolerant/resistant.

Recent findings from work done in rodents suggest that superinfections (infection with several *Plasmodium* strains at the same time) are regulated trough the host's iron regulatory hormone hepcidin (*215*). It is likely that this is also the case in humans. If that is so, then when several sporozoites with genetically different parasite strains are injected and migrate to the liver, it could be so that not all strains then immediately start multiplying, and burst from the hepatocyte and infect RBCs. Some strains could become growth arrested in the liver cells, a process probably mediated by the host's hepcidin-levels. The synthesis of hepcidin has been shown to follow blood stage parasite in a density dependent manner. Therefore, when one infection has been cleared, hepcidin levels decrease and the parasite strain previously growth arrested in the liver can start growing again (*215*). This new understanding of the complex interactions between the parasite and the host challenges our view of a re-infection as this concept proposes that a newly appearing parasite strain does not necessarily need to come from a new infective mosquito bite.

5.5.1 Seasonal fluctuations

Malaria incidence is highly dependent on mosquito prevalence. Access to water needed for the development of mosquitos increases dramatically during rainy seasons, leading to strong natural correlation between rainfall and malaria transmission, resulting in an increased incidence of the disease, usually with a few weeks lag time. The re-infection rate is therefore observed to vary throughout the year, following the seasons (*172, 201*).

In the MIM/TDR study, a seasonal variation in the rate of recurrent infections was observed (Fig. 21). During the period when the risk of recurrent infection four weeks after treatment is high, i.e. during the high transmission season, the drug selective pressure will also be higher. This could give rise to an increased selection of tolerant/resistant parasites.

To assess if these differences in risk of recurrent infection was reflected among the baseline prevalence of our SNPs of interest (*pfmdr1* N86, 184F, D1246 and *pfcrt* K76), a logistic regression with month as a covariate was performed. During the time period when the risk of getting a recurrent infection within four weeks was greatest (i.e. patients who started treatment in May and June), no major increase of our SNPs of interest were observed at baseline infections. A significant monthly increase in the *pfcrt* K76 among pre-treatment infections May to August was seen, which could be due to the increased fitness of the K76 as compared to 76T (*216*). A trend for increased *pfmdr1* N86 was also observed from May to October. It is to note that the sample size for this analysis was relatively small, wherefore the results should be interpreted with caution.

In Paper IV, it was concluded that the days of post-treatment prophylactic effect is highly dependent on the *pfmdr1* status of recurrent parasites. Therefore, the risk of getting a recurrent infection varies with rainfall and *pfmdr1* SNPs (*217*).

6 CONCLUSIONS

These are the overall conclusions from this thesis:

Paper I

- Artemether-lumefantrine is highly effective both in a real life like scenario and under ideal conditions.
- Patients with unsupervised drug intake have lower lumefantrine blood concentrations day 7 than the patients with supervised intake, but the cure rates between the two groups are not different.
- Recurrent infections that appear after artemether-lumefantrine have specific genetic characteristics. There is a significant selection of *pfmdr1* N86 and a trend for increased prevalence of *pfcrt* K76.
- One patient was infected with a parasite carrying two copies of the *pfmdr1* gene.

Paper II

- Selection of *pfmdr1* SNPs among re-infections after artemether-lumefantrine treatment is associated with lumefantrine drug concentrations.
- Patients' lumefantrine concentrations in combination with pharmacokinetic parameters and genotyping of recurrent parasites post-treatment can be used to assess the relative importance of different SNPs for the parasites capacity to withstand lumefantrine *in vivo*.
- *In vivo*, the *pfmdr1* N86/184F/D1246 is able to withstand 15 fold higher lumefantrine concentrations than the *pfmdr1* 86Y/Y184/1246Y.

Paper III

- From 2004 to 2011, the prevalences of parasites with *pfmdr1* N86/184F/D1246 and *pfcrt* K76 have increased significantly after the implementation of artemether-lumefantrine as first line treatment for uncomplicated malaria in Tanzania.

Paper IV

- Artemether/dihydroartemisinin does not appear to select for the same *pfmdr1* and *pfcrt* molecular markers as lumefantrine *in vivo*.
- The lumefantrine window of selection appears to start almost immediately after completed artemether-lumefantrine treatment course and lasts for four weeks.
- Within the lumefantrine window of selection *pfmdr1* N86 and 184F were significantly selected.
- The post-treatment prophylactic effect after artemether-lumefantrine treatment can vary up to three weeks depending on the *pfmdr1* polymorphisms of parasites causing the recurrent infection.

7 PERSONAL REFLECTIONS AND FUTURE PERSPECTIVES

I would like in this section to share some of my personal reflections and future perspectives in relation to the main findings of this thesis.

To me the most important contribution of this work is the development of a new method to study tolerance/resistance development *in vivo*, based on the use of drug concentrations (Paper II). This method has the potential of accelerating the discovery and establishment of molecular markers of resistance, and is applicable to other drugs than lumefantrine and to other diseases.

This thesis has shown that *pfmdr1* SNPs contribute to the parasites lumefantrine susceptibility. In order to fully understand the importance of these SNPs and the mechanism behind lumefantrine resistance more work is needed and it would be very interesting to follow up these findings with controlled transfection experiments.

Trend analysis is a crude but important tool to understand resistance development and spread. The trend of increased *pfmdr1* NFD shown in Paper III could be an early warning sign of decreased lumefantrine efficacy and should be followed up with close monitoring of artemether-lumefantrine efficacy. I believe that molecular markers play an important role in the evaluation and surveillance of emerging drug resistance especially in settings where malaria incidence is decreasing, as this makes clinical trials even more costly and time consuming to conduct. Molecular markers may also be valuable in resource poor settings, where RDTs can be collected and later on used as a DNA source for molecular genotyping/surveillance (*218*).

When artemether-lumefantrine was introduced it was in the era of chloroquine resistance. During many years chloroquine had been selecting for a parasite population with mainly *pfcrt* 76T and *pfmdr1* 86Y. From what we know now about lumefantrine, at least in regards to these two SNPs, highly chloroquine resistant settings were the optimal environment for artemether-lumefantrine to be efficient.

In Paper III it was shown that the vast majority of the parasite population in Fukayosi, just like in other places in Africa, have now become *pfcrt* K76 (and therefore supposedly chloroquine sensitive). If this is the case maybe reintroducing chloroquine would be an option. The advantage with chloroquine is that it is a very cheap, safe and well-tolerated drug. The disadvantage is the risk for resistance to develop rapidly again. This could potentially be overcome by giving higher total dose of chloroquine, but divided into smaller and more frequent doses as was previously done successfully in Guinea Bissau (*219*).

Artemether-lumefantrine has been shown to be highly effective, however considering the previously mentioned limitations with evaluation of antimalarial drug efficacy and with the results from this thesis, I believe we need to raise the question: how long will artemether-lumefantrine remain effective? I think that we in a proactive manner should start thinking about and implementing ways to prolong the life-span of artemether-lumefantrine. One option could be the use of multiple first line treatments (*220*), e.g. introducing DHA-piperaquine as an additional first line treatment to be used in parallel with artemether-lumefantrine. An alternative to parallel use could be to use DHA-piperaquine during the period when the risk of recurrent infection within four weeks after treatment is highest, and to use artemether-lumefantrine during the remaining year.

The differences in post-treatment prophylactic effect observed in Paper IV makes me wonder; what is the reason behind it? Can it get even shorter? And how short post-treatment prophylactic effect can be considered acceptable from a clinical point of view?

One way that might help to prolong the post-treatment prophylactic effect of artemether-lumefantrine is by increasing the exposure to lumefantrine. It was recently shown among children in Uganda on antiretroviral therapy, that those receiving a lopinavir-ritonavir based antiretroviral therapy had a 41% reduced incidence of malaria as compared to those on Non-nucleoside Reverse Transcriptase Inhibitor (NNRTI)-based antiretroviral therapy. This was primarily due to a major reduction in the risk of recurrent malaria after treatment with artemether-lumefantrine. Children in the lopinavir-ritonavir arm had significantly higher lumefantrine concentrations day 7 which was thought to be the result of inhibition of P450 3A4 metabolism by ritonavir

(*221*). This way of increasing exposure to lumefantrine is likely to be more efficient than increasing the dosing as it appears that absorption of lumefantrine is a saturable process (*74*). In line with this work, future studies should investigate the use of pharmacological enhancement to prolong the post-treatment prophylactic effect of artemether-lumefantrine.

I'm hesitant to state that artemether-lumefantrine resistant parasites are seen in the studies included in this thesis. However, I do believe we need to improve the current tools used to assess antimalarial efficacy and I'm concerned about the observed evolution of less lumefantrine susceptible parasites.

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