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Surveillance of artemisinin and partner drug efficacy in uncomplicated *Plasmodium falciparum* malaria in Coastal Kenya

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Village grown large

I return to the womb.

Smell of fish and sweat of ages

Stored in a bundle

Of palm leaves.

Where bark canoes used to dry

With salt on their spines

Fisherman patching nets

At edge of sea.

Shark spread on table

Under a mango tree–

Nearby heaps of cassava.

Maize and banana was market.

Sand and mosquitoes.

From: “Mombasa” by Amin Kassam

For Kenya, a country that became my second home

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List of abbreviations A – M

_rec	indicates the samples of isolates of **rec**urrent parasitaemia
µg	**M**ikro**g**ramm
µl	**M**ikro**l**itre
ACT	**A**rtemisnin-based **c**ombination **t**herapy
AL	**A**rtemether-**l**umefantrine
AS	**A**rte**s**unate
BSA	**B**ovine **a**lbumine **s**erum
CATMAP	**C**onfirmation of **A**rtemisinin **T**olerance in **M**alaria **P**arasites **T**rial
CCM	**C**omplete **c**ulture **m**edium
CI	**C**onfidence **i**nterval
CMM	**C**ell **m**edium **m**ixture
DDT	**D**ichlor**d**iphenyl**t**richlorethan
DHA	**Di**hydro**a**rtemisinin
dhfr	**Di**hydro**f**olate **r**eductase
dhps	**Di**hydro**p**tearate **s**ynthase
DNA	**D**esoxyribo**n**ucleic **a**cid
ELISA	**E**nzyme-**l**inked **i**mmuno**s**orbent **a**ssay
et al.	**et al**ii / **et al**iae
etc.	**et c**etera
glurp	**Glu**tatmate-**r**ich **p**rotein
HEPES	4-(2-**h**ydroxy**e**thyl)-1-**p**iperazineethane**s**ulfonic acid
HRP	**H**orse**r**adish **p**eroxidase
hr(s)	**H**our(s)
HRP2	**H**istidine-**r**ich **p**rotein **2**
IC50	**I**nhibitory **c**oncentration **50** %
KEMRI	**K**enya **M**edical **R**esearch **I**nstitute
log	**log**arithm
LUM	**Lum**efantrine
M	**M**olar
MFQ	**M**e**f**lo**q**uine
ml	**M**illi**l**iter
mM	**M**ikro**m**olar
***msp* 1/ 2**	**M**erozoite **s**urface **p**rotein **1** / **2**

List of abbreviations N – Z

ng	**N**ano**g**ramm
nM	**N**ano**m**olar
Nm	**N**ano**m**etre
OD value	**O**ptical **d**ensity value
PBS	**P**hosphate-**b**uffered **s**aline
PCE	**P**arasite **c**learance **e**stimator
PC50 / **PC90** / **PC99**	**P**arasite **c**learance **50** / **90** / **99**
PfCRT	***P**lasmodium **f**alciparum* **c**hloroquine **r**esistance **t**ransporter
pfmdr1	***P**lasmodium **f**alciparum* **m**ultidrug-**r**esistance gene **1**
pH	**P**otentia **h**ydrogenii
PfPI3K	***P**lasmodium **f**alciparum* **p**hosphatidyl**i**nositol-**3**-**k**inase
PI3P	**P**hosphatidyl**i**nositol-**3**-**p**hosphate
PIP	**P**hosphatidyl**i**nositol
pLDH	**P**arasite specific **l**actate **d**ehydrogenase
PCR	**P**olymerase **c**hain **r**eaction
PPQ	**P**i**p**era**q**uine
PRR	**P**arasite **r**eduction **r**atio
rpm	**R**ounds **p**er **M**inute
SOP	**S**tandard **o**perating **p**rocedure
RPMI medium	**R**oswell **P**ark **M**emorial **I**nstitute medium
TMB	**T**etra**m**ethyl**b**enzidine
WHO	**W**orld **H**ealth **O**rganization
WWARN	**W**orld**w**ide **A**ntimalarial **R**esistance **N**etwork

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1. Introduction

1.1 The burden of malaria and (the burden of) its treatment

Despite already made great achievements, malaria, and especially the infection with *Plasmodium falciparum,* still remains one of the major causes of morbidity and mortality worldwide (**W**orld **H**ealth **O**rganization (WHO), World Malaria Report 2016; Snow, R. W. et al. 2005). The World Health Organization estimated 200 million malaria episodes in the year of 2015, causing the death of 450.000 people. 90 % of all malaria deaths occur in Africa. The main age group affected are children under five years of age with 300.000 deaths. An estimated 3.3 billion (3.3×10^9) people worldwide are living at risk of malaria infections, which is about half the world´s population (WHO, World Malaria Report 2016).

Though a reduction in malaria transmission was achieved between 2000 and 2015 (**Fig. 1**), measures need to be taken in order to maintain and further improve the situation with regard to reports concerning drug resistant parasites.

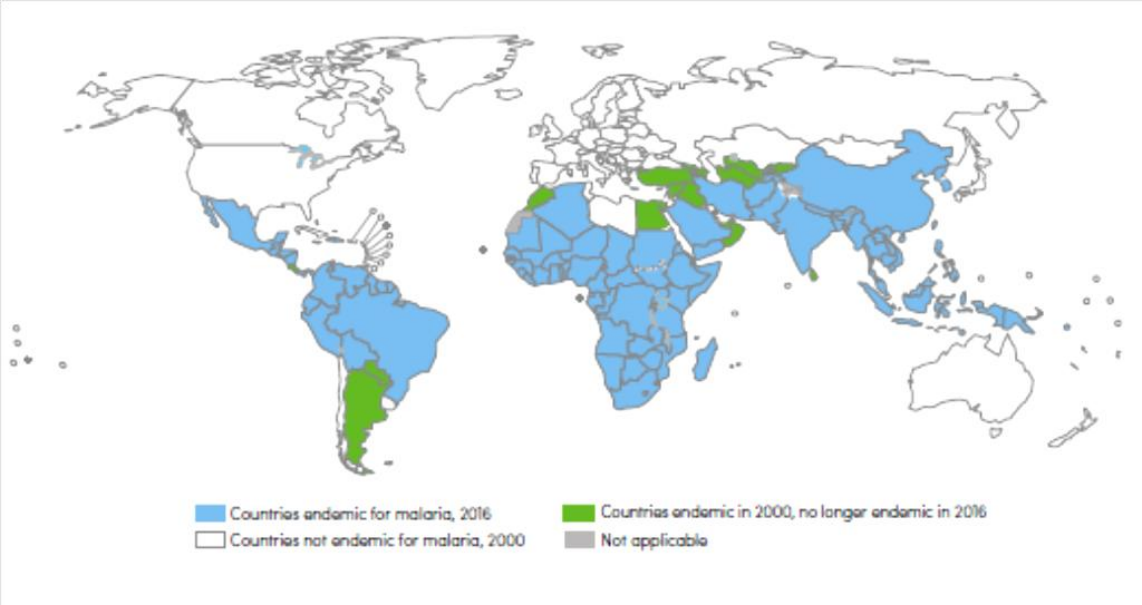

Fig. 1: Countries endemic for malaria in 2000 and 2015.[1]

[1] World Malaria Report 2016, World Health Organization

The economic costs of malaria are estimated to be US$ 12 billion per year for Africa alone, counting only direct losses such as illness, treatment and premature death.[2]
The funding for malaria control programs reached a sum of US$ 2.9 billion in 2015– still leaving a gap of US$ 2.2 billion to the yearly needed amount of US$ 5.1 billion (WHO, World Malaria Report 2016).

Up to now malaria vaccinations have proven to be only of modest protection (The RTS, S. Clinical Trial Partnership 2012) or are still undergoing trials (Seder, R. A. et al. 2013). Just recently the RTS, S / AS01 malaria vaccine phase III trial was completed showing a moderate statistically significant reduction in malaria cases after vaccination (RTS, S Clinical Trial Partnership 2015). Based on these modest results, RTS, S (Mosquirix©) has recently been approved by the European Medicine Agency. Up to now the highest impact still lies with control strategies consisting of

- prevention methods (e.g. insecticide treated nets) and vector control through (e.g. indoor spraying) and
- prompt and effective treatment.

WHO estimated that between 2001 and 2015 almost 7 million lives have been saved due to, inter alia, malaria control efforts (WHO, World Malaria Report 2016).
Out of Kenya's 34 million people population there are 25 million still living at risk of contracting malaria– with geographically varying predictions of the risks (Noor, A. M. et al. 2009).

To sustain and further improve the significant, if modest, achievements it is pivotal to evaluate the effectiveness of antimalarial drugs in order to prevent the emergence and spread of drug resistance in malaria parasites.

[2] http://www.rollbackmalaria.org/keyfacts.html (July 08th, 2015)

Presently artemisinin derivatives are the most efficient weapon against *P. falciparu*m. Treatment guidelines promote the combined use, ideally in co-formulation, with other drugs to sustain this effectiveness as long as possible. Alarmingly, studies from South-East Asia report about multiple foci of emergence of parasite tolerance against artemisinins. Drug surveillance has to provide timely information on which drug combination should be used in each endemic region to delay the emergence of artemisinin-tolerant parasites as long as possible.

The combination of **a**rtemether-**l**umefantrine (AL) is the first-line-treatment of uncomplicated *P. falciparum* malaria in Kenya since 2006. This decision, and the finding of the paediatric formula, was mainly based on data collected in Kilifi (Falade, C. et al. 2005, Abdulla, S. et al. 2008), where also the **C**onfirmation of **T**olerance in **Ma**laria **P**arasites trial (CATMAP) study was conducted.

1.2 Aims of the work presented

The aims of this work are, therefore,

- to present an overview and evaluation of the current artemisinin drug susceptibility in Kenyan children infected with *P. falciparum in vivo*, using parameters such as **p**arasite **c**learance **e**stimation (PCE), **p**arasite **c**learance **t**ime (PCT), **p**arasite **r**eduction **r**atio (PRR) and rate of recurrent parasitaemia by day 28 and

- to establish currrent *P. falciparum ex vivo* drug responses using the **h**istidine-**r**ich **p**rotein **2** (HRP2) **e**nzyme-**l**inked **i**mmunosorbent **a**ssay (ELISA) towards artemisinins and artemisinin partner drugs in order to discuss susceptibility phenotypes in Coastal Kenya.

2. Background

In order to understand the public health impact of malaria and the high importance of continuous drug surveillance first the evolution of the pathogen will be presented and its life cycle will be shown. Afterwards the clinical features of malaria as well as its prevention and treatment possibilities will be explained. That will lead to the main focus of this work, antimalarial drug resistance and surveillance of *P. falciparum*.

2.1 Evolution and development of *Plasmodium* into a human pathogen and the origin and spread of malaria

Human malaria is caused by the protozoon *Plasmodium*, a parasite that is transmitted by the female Anopheles mosquito. Known for being pathogenic in humans are *P. ovale, P. vivax, P. malariae* and *P. falciparum*. Recently there have been reports of a fifth species, *P. knowlesi*, which is mainly found in Old World monkeys and, therefore, considered a zoonotic pathogen (Knowles, R. M. and Das Gupta, B. 1932, Singh, B. et al. 2004; Cox-Singh, J. et al. 2008; Lee, K.-S. et al. 2009; Galinski, M. R. and Barnwell, J. W. 2009).

Plasmodium parasites are unicellular eukaryotes with a complex life cycle. According to Wilson and Williamson (1997) the ancestor was a free-living protozoon that developed into a plastid-containing organism through endosymbiosis of red algae. Before evolving into a vertebrae parasite, *Plasmodium* supposedly inhabited the gut of aquatic insect larvae, multiplying by sexual replication (Wilson, R. J. M. and Williamson, D. H. 1997). These aquatic insects were ancestors to the *Anopheles* mosquito. Since the first appearance of the fly species 150 to 200 million years ago, *Plasmodium* has broadened its host range and started to infest multiple different hosts such as reptiles and birds. Mosquitoes are still essential in the life cycle of *Plasmodium:* The Anopheles mosquito functions as the site for sexual replication and as the

vector, while humans are infected only by haploid asexual stages, including the newly formed gametocytes (refer to **2.2**).

Numerous studies looked into the evolution of some *Plasmodium* species into human pathogens. Waters et al. (Waters, A. P. et al. 1991) performed molecular experiments with small subunit RNA (ssRNA), finding only remote relatedness between the different species of human *Plasmodium.* They concluded that, rather than one incidence giving way to all four human parasites, multiple different transfers must have taken place.

Following this assumption, *P. falciparum* could also have evolved by lateral transfer from birds. The next hypothesis was in accordance with the formerly proposed distant relationships between the human pathogens, while also suggesting a co-evolution of the chimpanzee parasite *P. reichenowi* and *P. falciparum* 6 – 10 million years ago (Escalante, A. A. and Ayala, F. J 1994). The reliability of these early works was questioned due to limitations in their experimental methods such as the length in analysed gen loci or the under-representation of certain taxa (Prugnolle, F. et al. 2011).

Many groups have focused on overcoming these limitations since then. In 2008 Martinsen, E. S. et al. published a full phylogram of the five genera of haemosporidian parasites. One year later Rich, S. M. et al. (2009) proposed a fairly recent transfer of *P. falciparum* from chimpanzees instead of a co-evolution like Waters et al. had suggested. Then Krief, S. et al. (2010) published data pointing to the origin of *P. falciparum* being Bonobos and work by Liu, W. et al. (2010) claimed gorillas were the source of *P. falciparum* malaria.

Focusing on *P. falciparum*, work on mitochondrial **d**esoxyribo**n**ucleic **a**cid (DNA) proposed an origin of human malaria within Africa from where it was able to spread (Conway, D. J. et al. 2000). The authors place the first “big wave” of human malaria between 4000 and 5000 years ago, the time of agricultural revolution in Africa. The shift from a nomadic lifestyle towards the formation of villages with plenty resources of water to function as mosquito breeding sites as well as humans providing a constant blood supply for nourishment provided optimal conditions for the reproduction of the mosquitoes.

Further development in agriculture is proposed to have had an immense influence on the distribution of *Plasmodium* (Hume, J. C. C. et al. 2003). With trade thriving and a developing travel culture the parasite was able to spread easily – now affecting billions of people around the world.

2.2 *Plasmodium* and its life cycle

In 1880 the French military physician Alphonse Laveran discovered *Plasmodium* as the parasite causing malaria. *Plasmodium* is a unicellular parasite that belongs to the phylum *Apicomplexa*, which is characterized by an apical organelle (or, "apical complex", hence the name) essential for host cell invasion (Werk, R. 1985; Mitchell, G. H. and Bannister, L. H. 1988; Joiner, K. A. and Dubremetz, J. F. 1993; Sam-Yellowe, T. Y. 1996).

There are more than one hundred *Plasmodium* species[3], but only four are considered pathogenic to humans (refer to **2.1**). *Plasmodium falciparum* is by far the most malignant and will be the focus for this work.

The parasite undergoes a complex life cycle which partly takes place in the mosquito and partly in the human host.

2.2.1 The life cycle of *Plasmodium falciparum*

Through the bite of an infected female Anopheles mosquito *P. falciparum* sporozoites are transferred into the tissues of a human host.[4]

These sporozoites move actively towards nearby blood vessels and enter the blood stream (Boyd, M. F. and Kitchen, S. F. 1939; Matsuoka, H. et al. 2002; Aly, A. S. I. et al. 2009). Within minutes sporozoites that have penetrated into the blood stream are transported to the liver, where they invade hepatocytes

[3] http://malaria.wellcome.ac.uk/doc_WTD023864.html (August 10th, 2015)

[4] A visualisation of the lifecycle can for example be found in Portugal, S. et al. 2011.

and develop into liver schizonts[5]. After a single round of asexual replication liver schizonts then release the invasive blood stage forms, termed merozoites, into the blood stream (Frederich, M. et al 2002; Schlitzer, M. 2007). It has been shown that when leaving the hepatocyte, merozoites remain wrapped in a remnant of the hepatocyte host membrane (this vesicle is called merosome), possibly to escape the immune system until the infection of red blood cells (Sturm, A. et al. 2006). The apical organelle is considered essential for both quick and specific invasion of erythrocytes (Cowman, A. F. and Crabb, B. S 2006). Inside the red blood cell, the parasite enlarges and increases its metabolism. Due to its characteristic morphology seen by light microscopy in Giemsa stained blood smears, this early trophozoite form is also referred to as “ring stage”. The parasite now undergoes DNA replication and develops into a schizont. After completing this intraerythrocytic stage (duration approximately 48 hours), 16 – 32 new merozoites are released into the bloodstream for invasion of further red blood cells (Schlitzer, M. 2007). This asexual part of the life cycle causes the typical clinical symptoms of malaria.

Instead of forming merozoites, a varying proportion of asexual blood stage parasites develop into gametocytes, the sexual haploid form of the organism. When a mosquito takes in gametocytes with its next blood meal, certain factors in the gut of the fly (drop in temperature, pH changes and mosquito metabolites) in addition to a mosquito-derived molecule, xanthurenic acid, induce what is called gametogenesis (Billker, O. et al. 1997; Billker, O. et al. 1998; Aly, A. S. I. et al. 2009): the gametocyte escapes the red blood cell and becomes either a macrogamete (female gamete) or microgamete (male gamete). Upon fertilisation of the female gamete by a male gamete, a diploid zygote is formed. Then, after meiotic cell division motile ookinetes emerge. Ookinetes penetrate the epithelial wall of the gut, where they differentiate and replicate as so called oozysts.

[5] Schizgony is a form of asaexual reproduction typical for apicomplexa wherby DNA, organelles etc. are multiplied for up to 10.000 daughter cells within the mother cell. Cell fission occurs only at the end of schizogony.

When an oozyst ruptures it releases sporozoites into the body of the mosquito. These sporozoites now will find their way into the salivary glands of the mosquito, from where they will be injected into the new host when the mosquito takes its next blood meal.

2.3 Clinical features of malaria

An infection with *Plasmodium falciparum* can result in severe or uncomplicated malaria or even in an asymptomatic infection. This reflects increasing levels of acquired immunity to the parasite in patients in high transmission areas; therefore, severe malaria mainly occurs in young children. The symptoms become milder and eventually disappear as a function of repeated re-infections during childhood. Most adults in such areas are asymptomatically infected. The onset of clinical signs after sporozoite inoculation usually takes between 12 to 15 days (Verhage, D. F. et al. 2005; Roestenberg, M. et al. 2009). Symptoms result exclusively from the asexual erythrocytic stages of *Plasmodium.* Early symptoms before the typical fever episodes are unspecific and can be described as flu-like with headaches, nausea and fatigue. Other symptoms can include coughing, slight jaundice, myalgia, abdominal pain and / or diarrhoea and loss of appetite as well as prostration. With increasing parasitaemia the patient develops irregularly recurring high fever attacks (White, N. J. 2003, pp. 1226 – 1229).

Uncomplicated malaria can develop into severe malaria within hours presenting with altered consciousness, respiratory distress, convulsions, pulmonary oedema, disseminated intravascular coagulopathy, severe anaemia and hypoglycemia. Despite optimal treatment with intravenous artesunate, the mortality rate of patients with severe malaria remains between 8.5 – 15%. If parenteral artesunate is not available and treatment is conducted with quinine, this mortality rate ranges from 10.9 – 22 % (Dondorp, A. M. 2010; Dondorp, A. et al. 2005).

2.4 Malaria control strategies

In May 1955 the World Health Assembly initiated a malaria eradication campaign to eliminate the disease worldwide. By 1967 the use of **D**ichlor**d**iphenyl**t**richlorethan (DDT) against mosquitoes and the treatment of patients with antimalarial drugs had lead to the eradication of malaria in all developed countries and in some areas of Asia as well as Latin America. Unfortunately, due to limited practicability in some countries, emergence of resistance against the insecticide and the development of drug-resistant parasites imposed problems that prevented worldwide eradication.

A close to 100 % effective vaccine has been a long-term goal of malaria research. Recently, a recombinant vaccine, RTS, S, has been approved as the first malaria vaccine. However, it has shown only modest protection for a limited period of time (The RTS, S Clinical Trials Partnership 2012 and 2015). Therefore, current control strategies remain focused on prevention and fast and effective treatment.

Insecticide treated nets and indoor spraying have been implemented for many years now. Free distribution has led to a wide coverage of nets. For the most vulnerable groups the WHO recommends chemoprophylaxis, called the intermittent preventive treatment. This reaches out to infants and pregnant women. It is carried out with sulfadoxine-pyrimethamine given in the absence of the disease to suppress infections and prevent malaria.

Efficient treatment of *P. falciparum* malaria relies on **a**rtemisinin-based-**c**ombination **t**herapies (ACT) in all endemic countries (WHO, Guidelines for the treatment of malaria, 2015).

2.5 Antimalarial drugs

The complex life cycle (refer to **2.2**) offers different targets for chemotherapeutics. Antimalarials can be used for treatment and prevention of infection. Most drugs inhibit asexual blood stage replication (**blood schizontocides**); the preerythrocytic (liver stage) effect of medication remains mainly uncharacterized. The first known (herbal) medicine against malaria was powdered bark of the cinchona tree, containing the quinoline alkaloids **quinine** and **quinidine**. Sparking off synthetic drug manufacturing in 1891, **methylene blue** went through only a short period of usage as antimalarial drug but gave way to numerous synthetic drugs. Regiments such as **plasmochin**, **primaquine** and **mepacrine** were replaced by **chloroquine (CQ)**, which became the major antimalarial drug after the Second World War and sustained its importance for almost two decades. CQ is thought to interfere with the ability of the parasite to form haemozoin and, thus, to lead to accumulation of toxic haeme, a product from the parasite's haemoglobin metabolism (Chou, A. A. and Fitch, C. D. 1992; Sullivan, D. J. et al. 1996 b).

In the 1960s the first CQ-resistant strains were reported and by 2005 it was estimated that around 80 % of the wild *Plasmodium falciparum* strains are resistant to this highly sufficient drug regimen (Ginsburg, H. 2005). Much effort has been put into finding other drugs and synthesising CQ derivatives by modifying its chemical structure, leading to substances such as **amodiaquine, ferroquine**, **pyronaridine, and piperaquine** (**PPQ)** to overcome CQ resistance (Edgcomb, J. H. et al. 1950; Grewal, R. S. 1981; Geary, T. G. and Jensen, J. B. 1983; Ridley, R. G. et al. 1996; Foley, M. and Tilley, L. 1998). Other antimalarial drugs developed, like **mefloquine** (**MFQ**), **lumefantrine** (**LUM**) and **halofantrine** are analogues of the active antimalarial metabolite quinine and quinidine from the above mentioned cinchona tree. The artemisinins like **artemether** and **artesunate** (**AS**) derive from the herb known as sweet wormwood.

Available compounds nowadays are grouped into the following classes:

- **aryl-aminoalcohols** (quinoline-related) such as quinine, quinidine, chloroquine, amodiaquine, mefloquine, halofantrine, lumefantrine, piperaquine, primaquine, tafenoquine, ferroquine, pyronaridine
- **antifolates** such as pyrimethamine, proguanil, chlorproguanil, trimethoprim
- **artemisinins** such as artemisinin, **dih**ydro**a**rtemisinin (DHA), artemether, **a**rte**s**unate (AS)

Since *P. falciparum* treatment guidelines in Kenya are mainly based on artemisinins combined with either LUM or PPQ, more detailed information on these compounds will be provided in the following subchapters.

2.5.1 Artemisinins

Over the past years Artemisinin and its derivatives have proven to be the most efficient antimalarial drug since CQ. In traditional Chinese medicine “qinghao” was known for its ability to treat fever. In 1971 the antimalarial effects of the extract “qinghaosu” from the herb “qinghao” were demonstrated for the first time (Qinghaosu Antimalaria CoordinatiTeg Research Group 1979; Editorial The Lancet 1992) and its discovery resulted in a Nobel Prize in 2015 (Nobel Media AB 2016).

Artemisinin is extracted from the leaves of sweet wormwood, *Artemisia annua*. To increase its poor bioavailability, artemisinin is structurally modified and semi-synthetic derivatives in clinical use are **artesunate**, artemether, arteether, and **dihydroartemisinin** (**Fig. 2**) (Shen, J. X. 1989; Hien, T. T. and White, N. J. 1993; Woodrow, C. J. et al. 2005).

Artemisinins rapidly clear the patients' blood from malaria parasites and have the broadest time window of antimalarial effect (from ring forms to early schizonts) (White, N. J. 2004). *In vivo* the derivatives are hydrolyzed into the main active metabolite DHA. This metabolite has a very short elimination half time of about 45 minutes (Teja-Isavadharm, P. et al. 1996; Batty, K. T. et al. 1998).

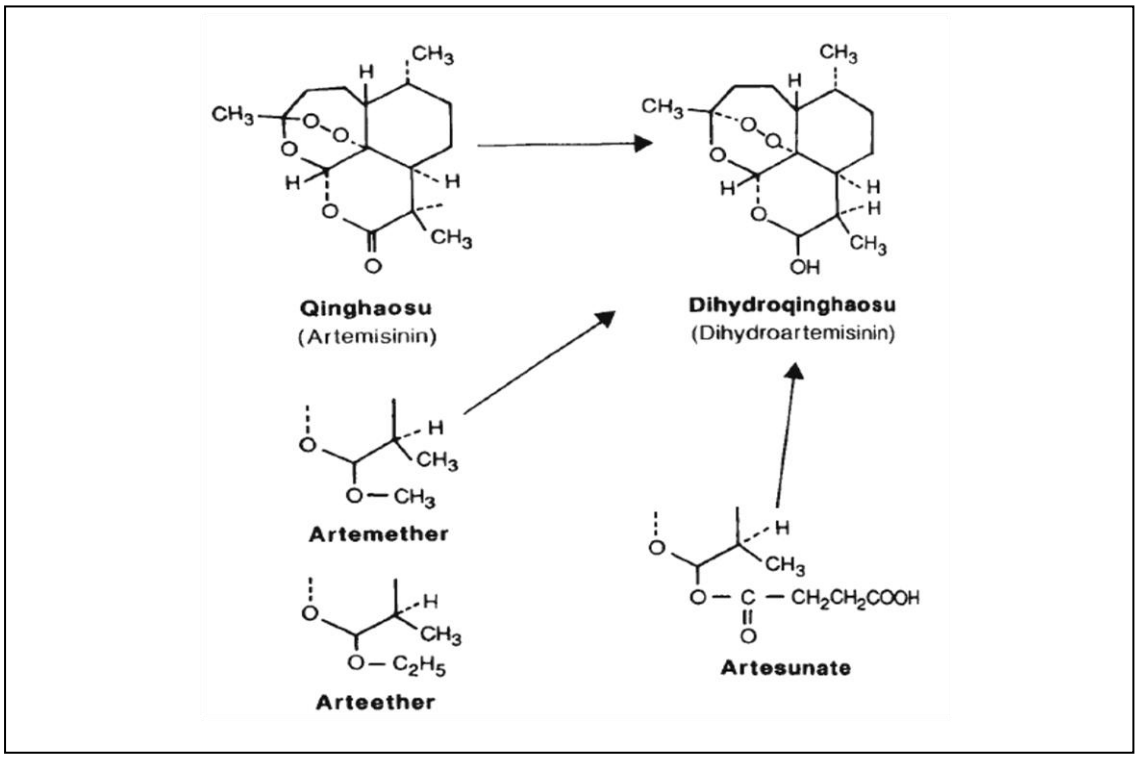

Fig. 2: Artemisinin and derivatives chemical structures.[6]

The exact mechanism of action of artemisinins remains unknown. The key structural feature of all artemisinins is an endoperoxide. Artemisinins also act against developing, but not mature, sexual blood stages (Kumar, N. and Zheng, H. 1990; Meshnick, S. R. 2002; Schlitzer, M. 2007). A reduction in gametocytaemia can additionally reduce transmission by decreasing the chances of another mosquito taking up gametocytes through a blood meal on a malaria patient (Price, R. N. et al. 1996; Okell, L. et al. 2008).

[6] Hien, T. T. and White, N. J. 1993

Since 2004 the WHO promotes artemisinin derivatives for the treatment of uncomplicated malaria as the main compound of **a**rtemisinin-based **c**ombination **t**herapies (ACT). In ACTs artemisinins account for a rapid onset of parasite clearance, the combination with a partner drug with a longer half time and different mode of action, e.g. lumefantrine, is thought to delay the emergence of resistance (White, N. J. 1999). After the artemisinin is cleared from the body, the partner drug eliminates the remaining (few) parasites, including those that possibly are still in or are leaving the dormant stage. Additionally, the partner drug plays a role in prophylaxis of a new infection by maintaining blood levels over a longer period of time.

2.5.2 Lumefantrine

LUM was developed by Chinese scientists. Today it is only available in fixed combination with artemether (Coartem®), which has been the first-line treatment in Kenya since 2004 (Ministry of Public Health and Sanitation, National Guidelines for the Diagnosis, Treatment and Prevention of Malaria in Kenya, 2010). Several studies have been conducted on this combination-therapy on the study site in Kilifi, including a first safety and efficacy study (Falade, C. et al. 2005), trials for paediatric formulations (Abdulla, S. et al. 2008) as well as drug surveillance studies (Borrmann, S. et al. 2011). The last mentioned study lead to the CATMAP trial in which this work was embedded. LUM is lipophilic and, thus, absorption increases with the intake of fatty food (Ezzet, F. et al. 1998). The elimination half-life is estimated to be 3 – 4 days (Ezzet, F. et al. 2000).

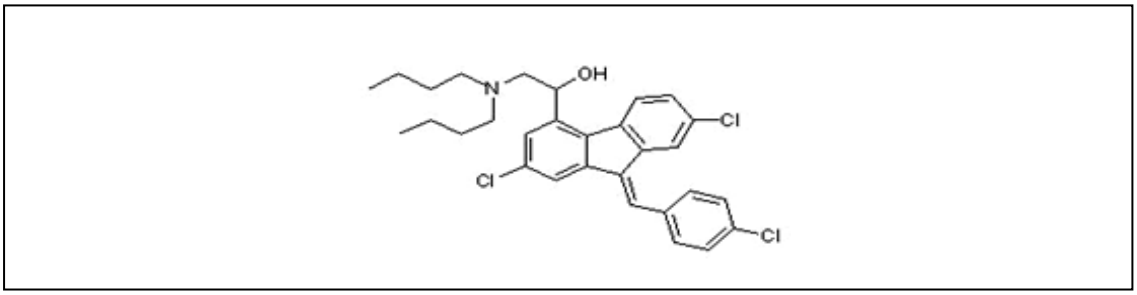

Fig. 3: LUM chemical structure.[7]

[7] http:\\www.sigmaaldrich.com (August 10th, 2015)

2.5.3 Piperaquine

PPQ is a bisquinoline and one of the drugs synthesised to overcome CQ resistance. Hypotheses suggest that its activity against CQ-resistant strains derives from its steric bulk, causing it to be not a suitable substrate for the ***P****lasmodium* ***f****alciparum* **c**hloroquine **r**esistance **t**ransporter (PfCRT), a transporter involved in chloroquine resistance (Fidock, D. A. et al. 2000). Another suggestion is that its four positive charges are trapping it inside the acidic digestive vacuole of *Plasmodium* (Warhurst, D. C. et al. 2007; Kaur, K. et al. 2010).

PPQ has mainly been used in China, leading to resistant strains in the country (Chen, L. et al. 1982; Lan, C. X. et al. 1989; Fan, B. et al. 1998). In Africa it is still considered highly effective and applied as a combination therapy with DHA. This combination has been reported to be safe and effective in numerous studies (Myint, H. Y. et al. 2007; Grande, T. et al. 2007; Abdulla, S. et al. 2008; Adam, I. et al. 2010), including a multicentre Phase III trial where the Pingilikani clinic (the study site of the CATMAP trial) was one of the study sites (Bassat, Q. et al. 2009).

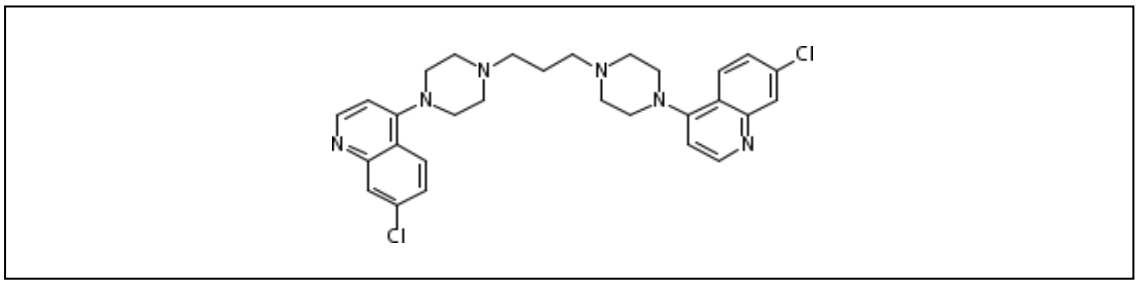

Fig. 4: PPQ chemical structure.[8]

[8] http:\\www.sigmaaldrich.com (August 10th, 2015)

3. Antimalarial drug tolerance and resistance

According to the WHO, antimalarial drug resistance is defined as “the ability of a parasite strain to survive and / or multiply despite the administration and absorption of a medicine in doses equal to or higher than those usually recommended but within tolerance of the subject, provided drug exposure at the site of infection is adequate” (WHO, Guidelines for the treatment of malaria, 2015, p. 113). This leads to a shift to the right in the dose-response curve of the drug (White, N. J. 2004).

Increased tolerance to antimalarial drugs has been reported for all classes of antimalarials, which imposes a great threat to the control of the disease.

Often, the onset of parasite resistance is very easily overlooked by medical professionals, as most patients seem to improve clinically while undergoing treatment. In most cases, even parasitaemia will eventually disappear below the microscopic detection threshold. Three to six weeks after completion of treatment symptoms may then reoccur. This will commonly be interpreted as a newly acquired infection with *Plasmodium* rather than a recrudescence of the same (resistant) strain. The intervals between fever clearance and reoccurrence of the clinical symptoms will then become increasingly shorter until the drug regimen will fail to clear the patient from symptoms completely (WHO, Guidelines for the treatment of malaria, 2015). This clearly indicates the importance of constant drug surveillance studies to monitor drug sensitivity continuously be able to react fast to declining responsiveness.

In 2006 the national Kenyan guidelines adapted the WHO recommendations and announced AL as the first-line treatment for uncomplicated *P. falciparum* malaria. This combination has been proven effective even in areas of multi-drug-resistant parasites (van Vugt, M. et al. 2000; Hutagalung, R. et al. 2005). Despite its fairly short time in use, there have already been reports about reduced artemisinin sensitivity in *P. falciparum*. In 2009 Dondorp, A. M. et al. described delayed *in vivo* parasite clearance times after treatment in Western

Cambodia. However, no evidence for reduced *in vitro* drug sensitivity was found. This was followed by a study which also showed declined responsiveness to ACTs *in vivo* in Kenya (Borrmann, S. et al. 2011) with no indication of drug resistance *in vitro*.

In the past years much progress has been achieved in understanding artemisinin resistance better. As said before, by the time of the first description of delayed response after artemisinin treatment there was no correlate *in vitro* when testing drug sensitivity. Cheeseman, I. H. (2012) aimed to identify genome regions under selective pressure by artemisinin treatment in Western Thailand and found areas of interest on chromosome 13. Also Borrmann, S. et al. (2013) studied genes associated with naturally occurring phenotypic variation of artemisinin susceptibility in Kenya and reported about associations with chromosome 13.

In 2013 an altered *in vitro* phenotype of parasites with reduced artemisinin susceptibility was reported. Ring stages showed a higher survival rate and better ability to recover compared to mature stages when exposing parasites to DHA (Witkowski, B. et al. 2013b). Witkowski et al. then performed a so called **r**ing stage **s**urvival **a**ssay (RSA, refer to **3.1.2**) on ring stage parasites at different ages during the life cycle (refer to **2.2.1**), explicitly at hours 0 – 3 (RSA^{0-3}) and 9 – 12 ($RSA^{9\text{-}12}$) post invasion. Additionally, the survival assay was done on trophozoites 18 – 21 hours post invasion (then called **t**rophozoite **s**urvival **a**ssay, $TSA^{18\text{-}21}$) and on *ex vivo* samples (Witkowski, B. et al. 2013a). The survival rate of parasites in the *in vitro* RSA^{0-3} proved to be much higher in slow clearing parasites. They were also able to show a correlation between the RSA performed on *ex vivo* samples and the *in vivo* parasite clearance half life. Thus, the RSA^{0-3} provides a tool for further characterization of artemisinin resistance, e.g. as the authors mention association between genome studies and RSA^{0-3} results. The *ex vivo* RSA can be performed in drug surveillance studies in areas where artemisinin resistance has not yet been established.

Using the established RSA^{0-3} as reference, Ariey, F. et al. (2014) were then able to identify a molecular marker on chromosome 13, namely K13 propeller[9] polymorphism, for artemisinin resistance in Cambodia. Straimer, J. et al. (2015) genetically modified parasites by inserting resistance-associated mutations in the K13-propeller domain and were able to demonstrate higher survival rates under DHA drug pressure after the insertion compared to the wild type. Transcriptome analysis of clinical isolates then revealed an up-regulation of proteins involved in reparation and degradation of proteins damaged by artemisinins in drug resistant parasites. Additionally, a slowed progression through the ring stage was reported (Mok, S. et al. 2015). Hence, artemisinin resistance seems to be a result of a decelerated ring stage development together with protection against protein damage caused by artemisinin.

Mbengue, A. et al. (2015) reported inhibition of *P. falciparum* **p**hosphatidyl**i**nositol-**3**-**k**inase (PfPI3K) by DHA. This kinase is involved in phosphorylation of **p**hosphatidyl**i**nositol (PI) leading to **p**hosphatidyl**i**nositol-**3**-**p**hosphate (PI3P) which promotes cell survival. Ergo, the inhibition of PfPI3K by DHA leads to parasite death. In artemisinin resistant parasites it is proposed that the mutation in the K13-propeller domain may lead to increased levels of PfPI3K and therefore PI3P, promoting the survival of the parasite. Interestingly, and supportive in favour of above mentioned hypothesis regarding artemisinin resistance, Mita, T. et al. (2016) reported that prior to the introduction of ACTs there was little polymorphism at the K13-propeller locus worldwide.

In summary, regarding South East Asia, where artemisinin resistance is established, the K13-propeller polymorphism seems to be an appropriate tool to

[9] K13 [PF3D7_1343700 (PF13_0238)] is a one exon gene encoding for a putative kelch protein. "K13 has a predicted 3-domain structure, with an approx. 225 residue long, Plasmodium-specific and well conserved N-terminal domain, followed by a BTB / POZ [broad-complex, tramtrack and bric-abrac / poxvirus and zinc-finger] domain and a 6-blade C-terminal propeller domain formed of canonical kelch motifs 43,48. Little is known about the protein in malaria parasites. Proteomics data indicate that it is produced by asexual (trophozoites, schizonts, merozoites and rings) and sexual blood stages (gametocytes) of *P. falciparum*, and that it possesses phosphorylated residues in the N-terminal Plasmodium-specific domain (www.plasmodb.org). The M476I mutation is located between the first and second blade of the propeller domain. "(Ariey, F.et al. 2014)

track the spread of the resistance. The importance for the African region remains the subject of further studies. K13-polymorphisms have been reported in Mali, where parasite clearance under ACT remains fast, but were also present before the introduction of ACTs (Outtara, A. et al. 2015). Kamau, E. et al. (2015) detected different K13-propeller polymorphisms than the ones found in Asia in Sub-Saharan Africa, but so far no study investigated the RSA^{0-3} in association with these findings. Thus, it is crucial to further study molecular markers and carry out *in vivo* as well as *ex vivo* and *in vitro* drug sensitivity testing in Africa in various regions to clarify significance of already found markers and detect others.

3.1 Monitoring drug efficacy

Drug monitoring consists of two main aspects: the efficacy of the drug in an infected patient (*in vivo*) and the efficacy outside the patient (*ex vivo or in vitro*). Whereas *in vitro* and *ex vivo* data are important tools in research and can give additional information, it is always the result of an artificial set-up. *In vivo* data on the other hand provides the actual clinical outcome of treatment that also depends on various host-related factors and, therefore, cannot be replaced by *in vitro* tests. At first sight inter-individual differences of patients in clinical trials such as e.g. drug metabolism, immunity etc. might seem like an obstacle to get comparable results. This data is taken under “real life” conditions though and absolutely necessary for the development of clinical guidelines, the development of new drugs and much more. This shows a necessity in drug surveillance for both clinicians and researchers to be aware of and responsive to changes.

3.1.1 Drug susceptibility *in vivo*

Besides measuring clinical outcomes such as body temperature and haematological variables (e.g. haemoglobin), the level of parasitaemia is documented over time and used as a parameter for the efficacy of the drug(s) applied. According to WHO standards the duration of required follow-up-time

depends on the half-life of the drug(s) applied (at least 28 days, for combinations with MFQ or PPQ at least 42 days) (WHO, Guidelines for the treatment of malaria, 2015, pp.112 – 113).

During an infection with *Plasmodium*, the parasite multiplication rate is used to describe the rate of multiplication of the parasite. In the absence of any antimalarial drug activity in non-immune adults this multiplication rate is estimated to exceed the rate of 10 per asexual cycle (Simpson, J. A. et al. 2002).
The ratio between parasitaemia at onset and at a point of interest after initiation of treatment (commonly hour 6, 12, 24 and 48) is called parasite reduction ratio (White, N. J. 1997). The PRR varies depending on the drug applied. Artemisinins are currently known as the fastest acting antimalarials *in vivo* (White, N. J. 2004).

Until recently the PCT, i.e. the time from first drug exposure to the recording of multiple consecutive negative slides, was the most commonly used parameter to describe drug efficacy *in vivo*. The PCT was found to be dependent on numerous factors such as baseline parasitaemia and the frequency of sampling. Thus, it proved to be an inaccurate parameter that does not allow comparisons between individuals or across cohorts of patients (White, N. J. 2011).
A recently proposed method is the PCE. This method requires frequent sampling and uses a logarithmic transformation of the parasite density data over time. It starts by identifying different segments of the parasite clearance curve in the patient (Flegg, J. A. et al. 2011). Clearance times are then estimated using only the robust, linear segment of the clearance curve over time. The segments excluded from this estimation are the lag-phase, the tail and outliers. The lag-phase is caused by effects on mainly sequestered parasites instead of parasites in the peripheral blood. The tail is the part in the clearance curve where parasitaemia approaches the detection limit and counting errors are likely to occur. The slope in the fall of parasitaemia in the clearance curve after exclusion of lag and tail is proposed to be much less vulnerable to influences and, consequently, better for comparison of real drug

efficacy than the parasite clearance time. The slope half life, thus, indicates the time needed for the parasitaemia to be reduced by half after excluding lag and tail. Parasite clearance 50 and 99 are also adapted to the linear part of the curve and refer to the time it takes for the parasitaemia to be reduced to 50 and 99 % of the admission parasitaemia. The clearance rate constant shows the fractional reduction of parasites per unit in time. To be able to compare obtained results to numerous other studies which used either PCT or PCE, including data from the same study area (Borrmann, S. et al. 2011), both parameters will be shown and discussed here.

The rate of occurrence of recurrent parasitaemia is another parameter used for the determination of drug efficiency. Recurrent parasitaemia can occur due to reinfection with a new parasite or recrudescence of the same parasite. The second scenario indicates treatment failure. To distinguish between reinfection and recrudescence, genotyping of highly diverse parasite loci, such as **m**erozoite **s**urface **p**rotein 1(*msp1),* **m**erozoite **s**urface **p**rotein 2 (*msp2*) and / or **glu**tatmate-**r**ich **p**rotein *(glurp)*, is done.

Blood concentrations of the drug of interest can be measured in order to distinguish between real treatment failure and failure to achieve effective blood concentrations, e.g. due to non-adherence of the patient or variable bioavailability of the drug.

3.1.2 Drug susceptibility *ex vivo* / *in vitro*

When measuring drug sensitivity outside the patient, it is crucial to distinguish between *ex vivo* and *in vitro* methods. Also genetic methods can be performed for specific forms of drug resistance that are confirmed to be associated with molecular markers (e.g., certain mutations in the *pfcrt* or **D**i**h**ydro**f**olate **r**eductase *(dhfr)* and **D**i**h**ydro**p**tearate **s**ynthase *(dhps)* genes in case of chloroquine or antifolate resistance, respectively). The drug sensitivity testing is in general performed by exposing parasites to different drug concentration levels for a specific of time. Afterwards inhibition will be determined by the effect of the drug on growth and multiplication.

In vitro applies to methods that are carried out on parasites that have been adapted to parasite culture. *Ex vivo* methods on the other hand are performed directly on the patients´ sample. Various different methods have been developed and established, each with its advantages and disadvantages (Noedl, H. et al. 2003). Below, a few examples of the many methods are mentioned.

The simplest method of drug sensitivity testing is the **WHO schizont maturation test** that is based on microscopic assessment of schizonts development following drug exposure in a 24-hour culture. This method can easily be used in the field but is very work-intensive and is susceptible to inter-individual variability of assessment.

Isotopic methods, such as the widely used **radio-labelled [3H]-hypoxanthine assay** (Desjardins, R. E. et al. 1979), are based on the uptake of the labelled hypoxanthine by the parasite and the measurement of radioactivity in a liquid scintillation counter. Reproducible results for high quantities of isolates are the advantages of this assay, but costly equipment (e.g. liquid scintillation counter) and the exposure of laboratory staff to and the disposal of radioactive materials are limiting the use of this assay. The main problem though is the fairly high parasite density of 0.5 % needed in order to obtain reliable results.

Another method is the detection of **parasite specific lactate dehydrogenase** (pLDH) (Piper, R. et al. 1999), which measures the activity of pLDH (an enzyme actively produced by the parasite during growth and differing from the human LDH) and allows for quantification of parasites.

Widely used is the **SYBR-Green assay**. A fluorescent dye, the SYBR-Green, with affinity to double-stranded DNA, is added to the drug exposed isolate and fluorescence is measured. This assay produces reliable results from a fairly high parasitaemia of 0.75 % on. Also samples need to be purified from white blood cells in order to avoid unspecific DNA staining. This makes it fairly cost-intensive and unreliable for usage in the field (Vossen, M. G. et al. 2010).

One method which can be performed *ex vivo* as well as *in vitro* and which was performed in this study is the **H**istidine **R**ich **P**rotein **2** (HRP2) **E**nzyme-**l**inked **i**mmuno**s**orbent **a**ssay (ELISA) (Noedl, H. et al. 2002a). *P. falciparum* HRP2 has been identified as a protein associated with *Plasmodium falciparum* infected erythrocytes. HRP2 is localized in several cell compartments, including the parasite cytoplasm. It is known to be histidine-and alanine-rich and synthesised in each *P. falciparum* parasite, regardless of its knob-phenotype[10] (Rock, E. P. et al. 1987). HRP2 is found in concentrated packages in the host-erythrocytes as well as in the host plasma (or culture supernatant) due to secretion by the parasite (Howard, R. J. et al. 1986). The exact role of HRP2 remains unknown but it has been suggested to function in the haemoglobin metabolism of *P. falciparum* (Sullivan, D. J. et al. 1996a). There is only a slight inhibition in the production of HRP2 under drug pressure; therefore, it remains a good tool for drug sensitivity testing (Noedl, H. et al. 2002b). Besides sensitivity testing, rapid diagnostic tests are also based on the detection of HRP2.

The HRP2 ELISA as an assay for drug sensitivity testing in *P. falciparum* was first described in 2002 (Noedl, H. et al. 2002a). Compared to other assays it does not only require little equipment but also produces reliable results in low parasitaemia samples, which occur frequently. The assay can be performed *ex vivo*, directly on the isolates taken from the patient without prior parasite culture, leukocyte depletion or usage of radioactive markers. Compared to the WHO schizont maturation assay it is not subject to individual variability, is far less labour intensive and provides more accurate results concerning inhibitory concentrations.

After drug exposure an enzyme-linked immunosorbent assay is performed, measuring the concentration of HRP2, a protein that is produced by the parasite throughout its life cycle. This assay needs only fairly low parasitaemia starting from 0.01 % on and only little equipment. It is easy to reproduce and can quickly be performed on a great number of isolates at the same time.

[10] Erythrocytes infected with P*. falciparum* show an alteration of the cytoskeleton in the form of protrusions called knobs. These knobs are induced by the parasite (Deitsch, K. W. and Wellems, T. E. 1996) and play a role in cytoadherence (Crabb, B. S. et al. 1997).

On *ex vivo* samples (especially without prior depletion of leukocytes and samples with low parasitaemia) it has been shown to be superior to the SYBR-Green method (Chaorattanakawee, S. et al. 2013) and the radio-labelled-hypoxanthine-reuptake assay (Tritten, L. et al. 2009).

Very recently, as described above, a novel sensitivity assessment test gained importance with regards to artemisinin drug testing (Witkowski, B. 2013a). The **Survival Assay** is performed on ring stage parasites (ring survival assay) and mature stage parasites (mature survival assay, MSA). Parasites are synchronized to the stage of interest and at a parasitaemia 1 – 2 % exposed to six hours of high DHA levels (700 nM). The short exposure to high doses of the drug is assumed to imitate the drug exposure *in vivo* better than continuous exposure to lower doses as it is being done in other sensitivity tests. Depending of the parasite age post invasion the assay is called RSA^{0-3}, referring to parasites 0 – 3 hours post invasion etc. Then viable second generation rings or trophozoites will be determined by microscopy after e.g. another 66 hours (RSA) or 42 hours (MSA) (specific time depends on the age of parasites when performing drug exposure) of incubation.

4. Material and Methods

4.1 Material

Sample processing

Albumax II	Gibco Invitrogen, New Zealand
Gentamicin (stock 10 mg / ml)	Sigma Aldrich, USA
Giemsa	Sigma Aldrich, USA
Glucose 10 %	Sigma Aldrich, USA
4-(2-**h**ydroxy**e**thyl)-1-**p**iperazine**e**thane**s**ulfonic acid (HEPES)	Gibco, USA
Human European AB serum	Universitätsklinikum Heidelberg, Germany
Hypoxanthine 10 mM	ccpro, Germany
L-Glutamine 200 mM	Sigma Aldrich, USA
Methanol	Scharlab SL, Spain
NaOH 12 %	Sigma Aldrich, USA
Roswell **P**ark **M**emorial **I**nstitute medium (RPMI) 1640 without L-Glutamine	Sigma Aldrich, USA

Solutions

Complete culture medium (CCM): RPMI 1640 without glutamine
plus 5 ml L- glutamine
plus 3 ml $NaHCO_3$
plus 5 ml hypoxanthine
plus 18.5 ml HEPES
plus 1.25 ml gentamicin
plus 50 ml 5 % albumin II
plus 10 ml glucose
plus 10 ml human AB serum

The CCM was stored at 4 °C and used for a maximum of one week.

Blocking solution: BSA diluted at 2 % in PBS

Washing solution: Tween 20 diluted at 0.05 % in **p**hosphate **b**uffered **s**aline (PBS)

Stop solution: 1 M sulphuric acid (10 ml sulphuric acid added to 90 ml water)
Diluent for second antibody: 2 % BSA + 1 % Tween 20 in 97 ml PBS (10 ml per plate)
Antibody dilutions: Following the **s**tandard **o**perating **p**rocedure (SOP) for the HRP2 ELISA[11] the first antibody was diluted to 1.0 µg / ml in PBS. The second antibody was transferred into the diluent for the second antibody (see above) to 0.2 µg / ml. Depending on the activity of the conjugate this was adjusted, if required.

Drug sensitivity assay

AS	Sigma Aldrich, USA
Bovine **S**erum **A**lbumin (BSA)	Sigma Aldrich, USA
CQ	Sigma Aldrich, USA
DHA	Sigma Aldrich, USA
LUM	Novartis, Switzerland
MFQ	Sigma Aldrich, USA
MPFG-55G (second antibody)	Immunology Consultants Inc, USA
MPFM-55A (first antibody)	Immunology Consultants Inc, USA
PBS tablets (Dulbecco A)	Thermo Scientific, Oxoid limited, UK
PPQ	SigmaTau, Italy
Tetra**m**ethyl**b**enzidine (TMB) substrate	Sigma Aldrich, Germany
Tween 20	Sigma Aldrich, USA

Software

HN-NonLin V1.1	Available at www.malaria.farch.net
Microsoft Office 2011	
Prism 6	GraphPad Software Inc

[11] Available at www.meduniwien.ac.at/user/harald.noedl/malaria/hrp2sop.html (August 10th, 2015)

Equipment

Cell culture plates	Greiner bio one
EDTA tubes	BD Vacutainer, bd USA
ELISA plates	Greiner bio one
ELISA plate washer	Biotek
ELISA plate reader	Synergy 4, Gen 5
Heparin tubes	BD Vacutainer, BD USA
Incubator	Jencons, Nuaire
Microscope	Nikon Eclipse E200, China
Multi-channel pipette	Accu Jet Pro, Steinbrenner
Pipette tips	Neptunes, USA

4.2 Methods

4.2.1 Study site

The Pingilikani Dispensary (**Fig. 5**) is located on the Coast Province of Kenya in the Kilifi District, about 20 km south of Kilifi town. It is operated by the Ministry of Health and the **K**enya **M**edical **R**esearch **I**nstitute (KEMRI) / Centre for Geographic Medicine Research-Coast.

Fig. 5: **The Pingilikani dispensary.**[12]

[12] Author

A ward is located next to the dispensary where patients were admitted for three days after enrolment (**Fig. 6**).

Fig. 6: The Pingilikani ward.[13]

The clinical team in Pingilikani has been conducting research studies since 2005 (CATMAP study protocol 2011, v.1.3). Perennial transmission of malaria in the area has been reported with typical peaks within two rainy seasons throughout the year, one from April to June and the second from October to November (O'Meara, W. P. et al. 2008). The transmission rate for *P. falciparum* has been reported to range between 22 to 53 infective bites per person per year (Mbogo, C. M. et al. 2003). Bejon, P. et al. (2014) further investigated transmission in the Kilifi area and found hotspots which then again contained hotspots, sometimes narrowing down to one single high transmission household having an impact on the area surrounding it. They conclude that mosquito ecology as one determinant of malaria transmission could be influenced at a wide range of spatial scales in the area, ranging from macro- to micro-geographical characteristics.

[13] Author

4.2.2 Study design

Investigations were carried out as part of the “Confirmation of Artemisinin Tolerance in Malaria Parasites trial” (Study ID Number KEMRI_CT_2010/0013, SSC 1821; NCT Number NCT01190371[14]). The study was designed as a single-arm trial observing parasitological and clinical responses of *P. falciparum* to a 7-day mono-treatment with AS in order to answer the question if *Plasmodium falciparum* infections in Kenya have become less response to artemisinin treatment. Children who met the inclusion criteria (Appendix 1) were enrolled after obtaining written informed consent from a parent or a legal guardian. They were admitted to the paediatric ward in Pingilikani and treated orally with 2 mg / kg body weight AS for 7 days. Admission was planned for 3 days (day 0 – 3); from day 4 – 6 the children were seen and treated as out-patients. Follow-up was carried out on days 7, 14, 21, 28 and 42 including clinical and laboratory check-ups (CATMAP study protocol v1.3 2011, refer to **[14]**).

This work as part of the CATMAP study is about drug surveillance of artemisinin and its partner drugs with a more specific focused on the *ex vivo* data in comparison with previous data collected in the area. This will be supported by some of the collected *in vivo* data of the CATMAP trial.

4.2.3 Determination of parasite density in the field

Parasite density for the clinical outcomes was determined by an experienced laboratory technician according to the applicable Kilifi slide reading SOP. It was expressed as the number of asexual parasites per µl of blood by dividing the number of asexual parasites by the number of white blood cells counted. This number was multiplied by an assumed fixed white blood cell density of 8,000 / µl.

[14] As registered under clinicaltrials.gov, available under https://clinicaltrials.gov/ct2/show/record/NCT01190371?term=CATMAP&rank=1 (June 30th, 2016)

$$\text{Parasite density (per µl)} = \frac{\text{Number of parasites counted} \times 8{,}000}{\text{Number of leukocytes counted}}$$

The parasitaemia values calculated by this method were uploaded to the **W**orldwide **A**ntimalarial **R**esistance **N**etwork (WWARN) web-based tool for calculation of parasite clearance estimation.[15]

4.2.4 Determination of parasite density in the laboratory

Parasite density was calculated through inspection of 10 microscopic fields. The number of infected erythrocytes was divided by the number of total red blood cells to calculate the percentage of parasitaemia. This value was used for setting up the drug sensitivity assay.

4.2.5 Preparation of antimalarial drugs

All the drugs applied during the course of the *ex vivo* study were obtained readily dissolved as so called stock-solutions.
Prior to the set-up of the culture plate each drug had to be diluted down with RPMI 1640 to create a working solution. Those working-solutions were created as portrayed in the table below and stored at 4 °C.

Working solutions	**Medium (ml)**	**Drug stock solution (µl)**
CQ	4.991	9.259
MFQ	4.998	1.543
DHA	4.998	1.530
AS	4.998	1.530
LUM	4.990	10.285
PPQ	4.997	3.086

[15] Available under: http://www.wwarn.org/tools-resources/toolkit/analyse/parasite-clearance-estimator-pce (June 30th, 2016)

4.2.6 Handling of samples in the laboratory

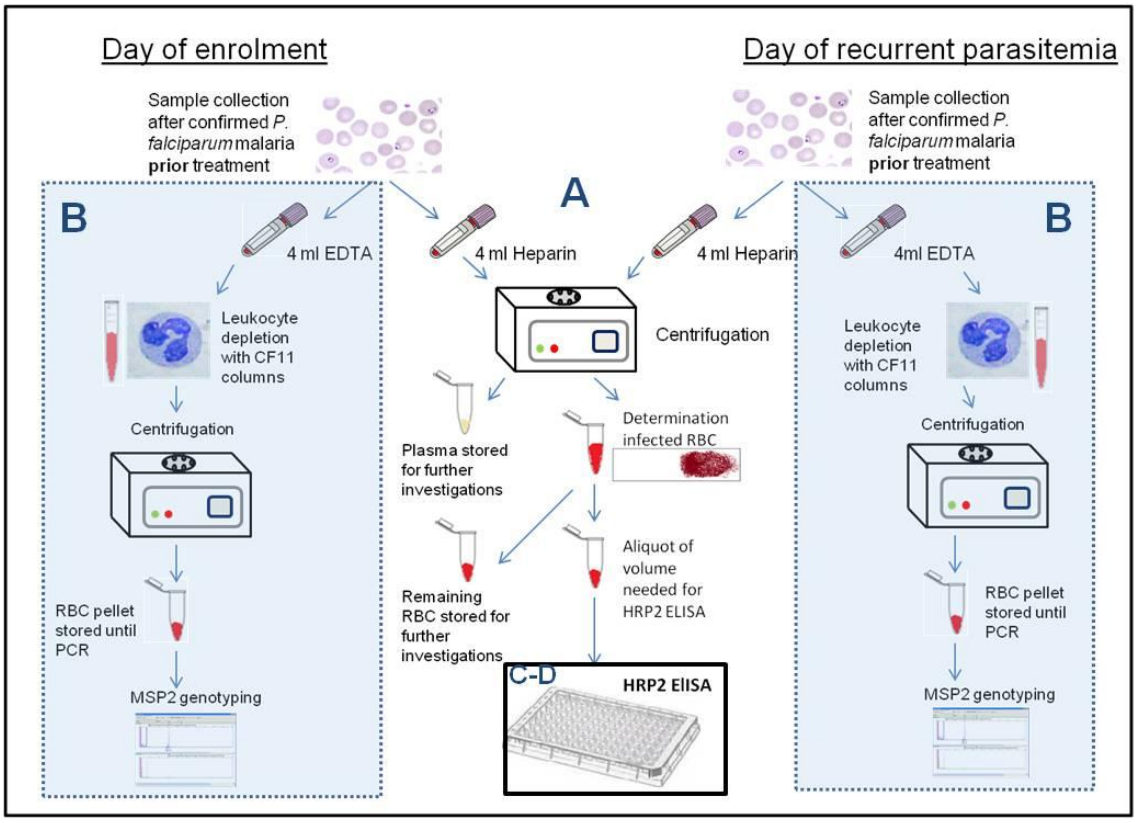

Fig. 7a: **Schematic of sample processing in the laboratory.**

A The blood sample for *ex vivo* investigation of the **i**nhibitory **c**oncentration **50 %** (**IC_{50}**) was collected from the study participants before administering the first dose of AS on day 0 as well as on the day of recurrent parasitaemia. 4 ml of venous blood was collected into a heparin tube and stored at 4 °C until cooled transport to the KEMRI Immunology laboratory (within 8 hours after collection). The sample was centrifuged at 2000 rpm for 5 min. The plasma was removed and stored separately (not necessary, removal done since further investigations within the CATMAP study were planned with it). The buffy coat was removed from the sample with a pipette and a thin slide was prepared. The slide was dried, fixed with methanol and stained with 10 % Giemsa for 15 minutes. Reading 10 microscopic fields under a light microscope (oil-immersion, 1000-fold magnification), the percentage of parasitaemia was determined. The volume of blood needed for the HRP2 assay was determined with regards to parasitaemia and an aliquot was made to set up the assay (**C-D**, see **Fig. 7b**). The rest of the sample was stored at -80 °C for other investigations within CATMAP.

B The blood sample for genotyping investigations was taken before the first treatment and on the day of recurrent parasitaemia. 4 ml venous blood was collected into an EDTA tube and stored at 4 °C until cooled transport to the KEMRI Immunology laboratory (within 8 hours after collection). The sample was immediately transferred into a 15 ml tube and the equal amount (1:1) of cold PBS was added before running the sample through a CF11 column for depletion of leukocytes and, consequently, human DNA. After centrifugation (2000 rpm for 5 min) an aliquot was made out of the pellet of favourably 200 µl for **p**olymerase **c**hain **r**eaction (PCR) / genotyping and stored at -80 °C until further processing.

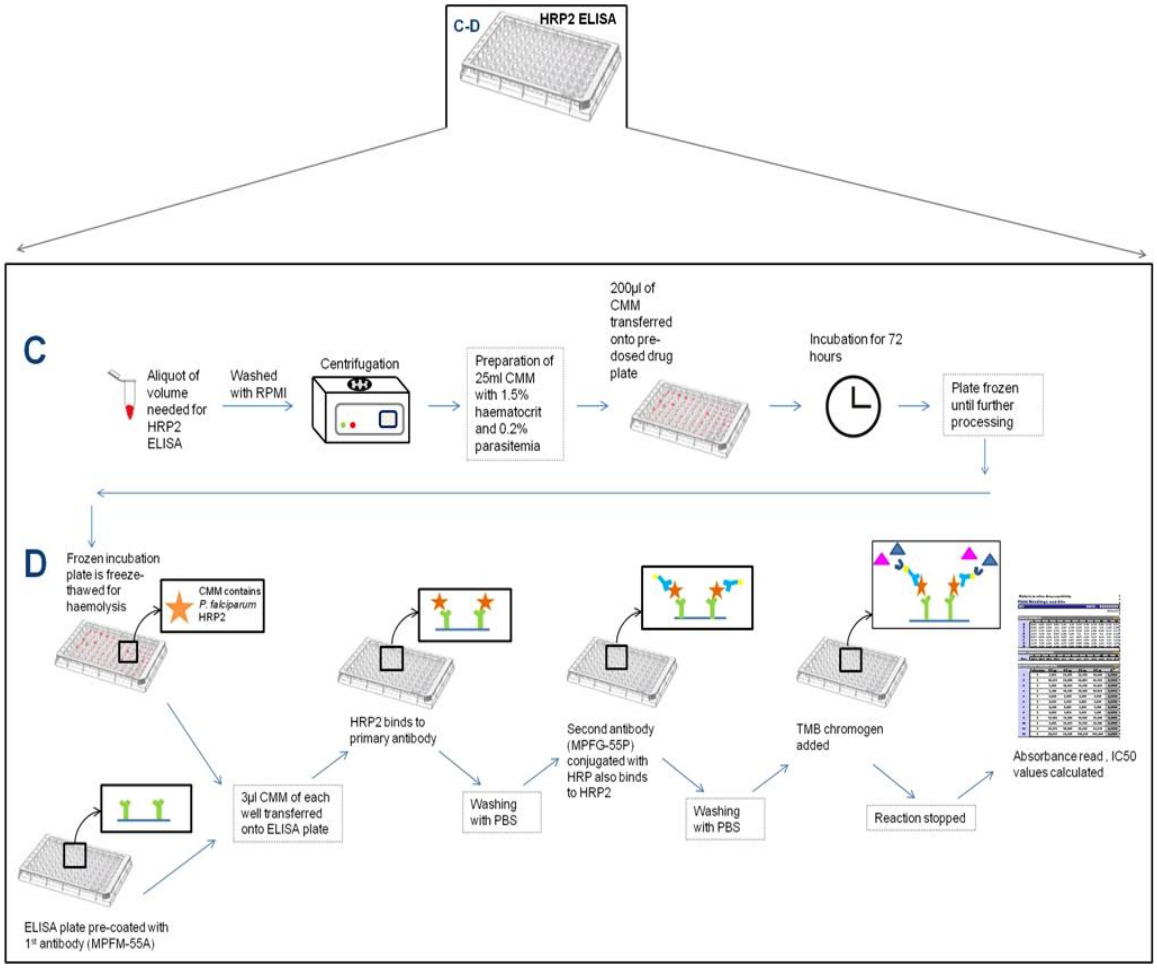

Fig.7b: Schematic of HRP2 ELISA.

C The aliquot mentioned under Fig. 7a A was washed with incomplete RPMI and centrifuged at 1800 rpm. Then the sample was adjusted to a **c**ell **m**edium **m**ixture (CMM) with a parasitaemia at 0.2 % and a haematocrit of 1.5 %. If the parasitaemia in the sample exceeded 0.2 %, it was diluted down using 0-negative red blood cells. 200 µl of this CMM was now transferred onto the pre-dosed drug exposure plate and put into an incubation chamber which al$_l$owed fo$_r$ daily app$_l$ication of gas consisting of a mixture of 3 % O_2, 5 % CO_2 and 92 % N_2. After 72 hours of incubation at 37 °C to allow for a full parasite life cycle, the culture was removed and frozen at -80 °C until further processing.

D For the ELISA test the incubated culture plates from C were removed from the freezer and haemolysed by further freeze-thawing. Then 3 µl of each well was transferred onto the pre-coated ELISA plate. After one hour of incubation contents were discarded, the plate washed three times with 200 µl / well washing solution and bang-dried. 100 µl working solution of the second antibody was transferred into each well followed by another hour of incubation. Afterwards the contents were discarded, each well was washed three times and the plate was bang-dried before adding 100 µl / well of TMB substrate. The plate was incubated in the dark and then 50 µl of stopping solution to each well was added. Absorbance was read in the ELISA plate reader at 450 nm. For more details of HRP2 ELISA refer to the main text.

4.2.7 Drug sensitivity testing *ex vivo* via HRP2 ELISA

The HRP2 ELISA (refer to **3.1.2**) was carried out on *ex vivo* samples to test drug sensitivity. The drugs chosen for testing were DHA, AS, LUM, MFQ, PPQ and CQ according to following rationale:

Artemisinin derivatives are considered the most efficient drugs for the treatment of *Plasmodium falciparum* malaria. Since monotherapies have been proven to encourage drug resistance, the artemisinins are combined with a partner drug. A total of six drugs were tested in the CATMAP study and all results will be reported here, while the main focus of this work will remain on the artemisinins, LUM and PPQ.
The IC50s for two artemisinins, namely **AS** and **DHA** were chosen to monitor the efficacy of artemisinins in Kenya. The main active metabolite of all artemisinins is DHA (Woerdenbag, H. J. et al. 1994; Morris, C. A. et al. 2011; Hall, A. J. 2013). The exposure to AS itself, consequently, is fairly low in comparison to DHA (Navaratnam, V. et al. 2000; Olliaro, P. L. et al. 2001; Morris, C. A. et al. 2011). Testing two artemisinins in the same assay makes it is also possible to use them as internal control.

LUM belongs to the aryl-amino-alcohol group of antimalarials and is the partner drug in the current ACT used as first-line treatment in uncomplicated *P. falciparum* malaria in Kenya. **MFQ** belongs to the same group as LUM and plays an important role in the global treatment and prevention of malaria. MFQ is used in combination with AS as ACT. Both compounds are thought to have similar mechanisms of action, supposedly due to the same basic chemical characteristic, consisting of a hydroxyl group near the ring which is hypothesized to be the key feature in antimalarial activity (Chien, P. L. and Cheng, C. C. 1976; Karle, J. M. and Karle, I. L. 1991). Therefore, similar IC50 values can be shown (Basco, L. K. et al. 1998).

PPQ is a bisquinoline (dimeric 4-aminoquinoline) and has been combined with DHA into an ACT as another option in treatment for *P. falciparum* malaria.

PPQ has been synthesised in order to conquer CQ resistance by adding structural modifications (Schlitzer, M. 2007). The proposal for the mechanism of action in the group of aryl-amino-alcohols as well as in the group of 4-aminoquinolines has been by interference with the haeme polymerization. In China cross-resistance between CQ and PPQ has been reported (Fan, B. et al. 1998; Yang, H. et al. 1999), but these results could not be reproduced e.g. in Cameroon (Basco, L. K. and Ringwald, P. 2003). To gather current data from Kenya, **CQ** as former first-line treatment was tested here as well.

4.2.7.1 Preparations for HRP2 ELISA

Drug exposure culture plate preparation and layout

On the drug exposure culture plate parasites were incubated under drug pressure before the ELISA (**Fig. 7b**, **C**).

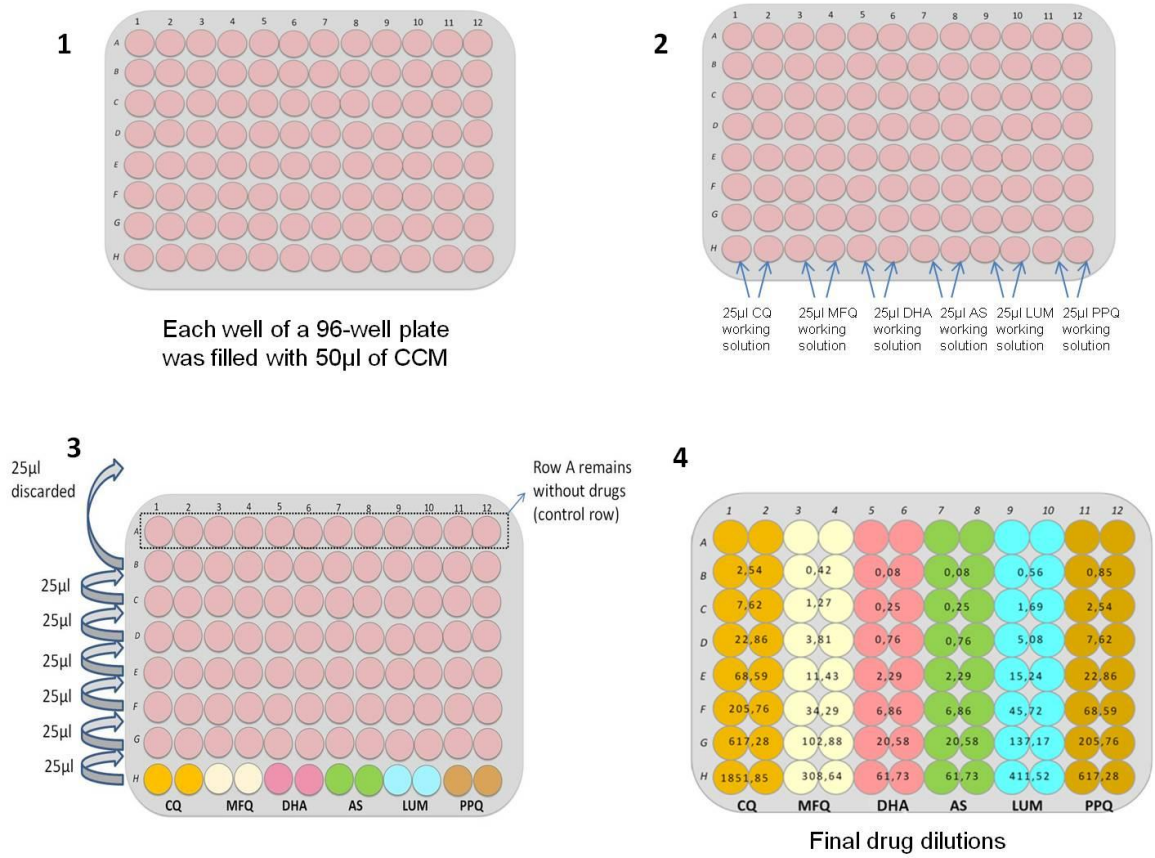

Fig. 8: Drug exposure plate layout for the inhibition assay. Drug sensitivity was measured in duplicates. The number in each well (**4**) displays the drug concentration in ng / ml.

When planning the set-up, in order to find the highest drug solution needed for full inhibition, the previously reported IC50 values for the area (Borrmann, S. et al. 2011) were used as reference, leading to a plate layout as shown in **Fig. 8**, **4**. To prepare for drug dilution, each well of a 96-well plate was filled with 50 µl of CCM (refer to **4.1**, solutions) (**Fig. 8**, **1**).

To each well in row H 25 µl working solution of the drug of interest was added in duplicates (**Fig. 8**, **2**). Using a multi-channel pipette starting in row H the solution was mixed by pipetting up and down and then 25 µl of the mixture was transferred from row H into row G. After mixing, now 25 µl was transferred from row G to F. This procedure was repeated for each row going up, resulting in a 3-fold dilution per row. 25 µl was discarded after row B to allow each row to contain the same amount of 50 µl (**Fig. 8**, **3**). Row A was skipped and functioned as positive control row without drug exposure.

Pre-coating of ELISA plates

For each ELISA plate the primary antibody was diluted to 1 µg / ml in PBS. 100 µl of this mixture was transferred into each well of the ELISA plate using a multi-channel pipette. The plate was then sealed and incubated overnight at 4 °C. The following day the contents were discarded and the plate bang-dried. To each well 200 µl of freshly prepared blocking solution was added. After an incubation time of 2 hours at room temperature the contents were discarded and the plate bang-dried a second time. Following that the plate was washed with 200 µl washing solution per well and bang-dried again before adding the sample.

4.2.7.2 The HRP2 ELISA

The HRP2 ELISA is a sandwich ELISA, whereas a primary antibody is coated onto the ELISA plate (refer to **4.2.7.1**, Pre-coating of ELISA plates) Then the sample is added and the protein of interest, here HRP2, binds specifically to the primary antibody. After incubation and removal of unbound material with washing solution the second antibody is added. This second antibody is conjugated to an enzyme, called **H**orse**r**adish **p**eroxidase (HRP). This second

antibody now also binds to HRP2 at a different site, thereby “sandwiching” it in between the primary and the second antibody. After another washing step a substrate is added. This substrate is a chromogen which changes colour due to the HRP linked to the second antibody. This colour change depends on the quantity of the second antibody, and, thus, also the quantity of HRP2. This colour change can then be measured in an ELISA plate reader to quantify exactly the presence of HRP2 and, consequently, parasite growth.

For the ELISA (**Fig. 7b**, **D**) the incubated drug exposure culture plates were removed from the freezer and haemolysed by further freeze-thawing until all wells looked clear. To ensure proper mixing, the content of each well was pipetted up and down multiple times before transferring 3 µl of each well onto the pre-coated ELISA plate with a multi-channel pipette. The remaining sample was frozen again. Allowing for incubation, the plate was left for one hour before discarding the contents and washing three times with 200 µl / well washing solution. The plate was then bang-dried.
Now, using a multi-channel pipette, 100 µl working solution of the second antibody was transferred into each well. This was followed by another hour of incubation. Afterwards the contents were discarded, each well was washed three times with 200 µl washing solution and the plate was bang-dried before adding 100 µl / well of TMB substrate. For seven minutes the plate was incubated in the dark before adding 50 µl of stopping solution (refer to **4.1**, solutions) to each well. Then absorbance was read in the ELISA plate reader at 450 nm.

Optical **d**ensity (OD) values were stored in an excel sheet and evaluated with regard to growth differences between the well with the highest drug exposure up to the lowest concentration and control wells. The values had to show at least a 2-fold growth in order to be included in the analysis. In some cases this limit was not reached, but the values were included in the analysis nevertheless because they showed a noticeable growth and a distinct half maximal inhibitory concentration.

All included samples were then manually transferred onto the HN-NonLin V1.1 sheet[16] provided to calculate IC50 values (ng / ml). The IC50 values were transferred into nM by using an excel sheet containing the needed formula for each drug (Appendix 2).

4.2.8 Genotyping of recurrence samples to distinguish between recrudescence and reinfection

DNA was extracted from matched pairs of parasite isolates from baseline and recurrence samples (**Fig. 7a**, **B**) with a commercial QIAGEN kit. Capillary electrophoresis analysis of fluorescence-labelled nested *msp2*-PCR products was done both in Kilifi and in Germany.

4.2.9 Statistical analysis

Fever was defined as temperature above 37.5 °C. Mean time to fever clearance was determined if the patient had fever within the first 24 hours. Time to fever clearance was then calculated from enrolment until two consecutive documentations of temperature below or equal to 37.5 °C. The parasite reduction ratio was defined as the parasite count at admission divided by the parasite count on the hour of interest. This value was then expressed as **log**arithm (log_{10}). When parasitaemia falls below the detection level in the clinical settings, the value 0 is stated by the microscopist. When excluding these values the PRR is underestimated. Therefore, to accommodate for the ongoing decline in parasite reduction, the value 0 at hour 12 was substituted by 1, at hour 18, at hour 12 by 0.5, at hour 24 by 0.1 etc.[17]. The ratios were determined by using parasite count at hour 0 as numerator and the hour of interest as denominator (parasite reduction ratio within the first 6 hours (PRR_{H6}), within 12 hours ($PRR_{H12)}$, within 24 hours (PRR_{H24}) and within 48 hours (PRR_{H48}).

[16] Available for download under http:\\www.malaria.farch.net (August 10th, 2015)

[17] Refer to http://www.statssa.gov.za/isi2009/ScientificProgramme/IPMS/0607.pdf (May 11th, 2014)

In order to determine the development of the reduction ratios as well, the parasite reduction ratio six hours before was subtracted from the hour of interest and divided by 6 to obtain a rate / hour for each six hourly interval. The correlation of patient age and initial parasitaemia with clinical treatment responses and other independent variables was evaluated by linear regression analysis. *Ex vivo* inhibitory concentrations were obtained by using logarithmic transformed drug concentrations in a polynomial non-linear regression model (automatically done when copying OD values into the HN-NonLin V.1.1 sheet) and expressed as geometric mean with the 95 % **C**onfidence **I**nterval (CI). Hints for cross-resistance were looked for using pair-wise correlation of Spearman (rho) (non-parametric test), coefficient of correlation of Pearson (r) (parametric test) including the coefficient of correlation (r^2). Wilcoxon´s paired nonparametric test was used to evaluate differences in the *ex vivo* drug responses between baseline and recurrent infections. Linear regression analysis was done to examine whether the time of drug storage influenced the IC50 values. When a possible influence was recognised, a non-paired, non-parametric t-test (between DHA and AS values in- and excluding the measurements made with the conspicuous drug solutions) was used to check whether this influence created a statistically significant difference. Numbers were rounded to two digits after the comma. In some cases, were necessary, rounding was done to three digits after the comma. Percentages were rounded to full numbers.

5. Results

5.1 Study cohort

One hundred and seventy five patients (n = 175) were recruited between April and December 2011 into the CATMAP study. The children were treated with AS monotherapy for 7 days (2 mg / kg body weight) and followed up for 42 days. Due to consent withdrawal the baseline sample was not taken from six children. One patient was withdrawn on day 4 due to persisting parasitaemia under treatment with AS and given rescue treatment. There was one consent withdrawal on day 14 and one on day 28. One patient was excluded on day 28 due to taking AL from a different drug dispensary. Three patients were lost in the follow- up (one on day 28, two on day 42). Seven patients were enrolled despite their parasitaemia exceeded the maximum enrolment parasitaemia of 300,000 / µl (302,505 – 566,470 / µl). The median patient age was 51 months and the median *P. falciparum* parasitaemia at enrolment was 95,200 / µl.
Further information on the baseline characteristics is provided in **Tab. 1**.

BASELINE CHARACTERISTICS	
Median age (range) in months	51 *(7 months – 10 years)*
Median axillary temperature (range) in °C	37.5 *(36.4 – 40.3)*
Median asexual *P. falciparum* density / µl (range)	95,203 *(16,579 – 566,470)*

Tab. 1: **Baseline characteristics of patients from CATMAP.**

5.1.1 Clinical treatment responses and analysis

The mean overall parasite clearance time (n = 168) was 30.5 hours. The median PCT was 30 hours for patients with a parasitaemia below 10^5 / µl and 36 hours for patients with a parasitaemia above 10^5 / µl (p = 0.001).

Tab. 2 displays the clinical and parasitological results.

Clinical and Parasitological Results		
Parasite clearance time in hours	Median (range)	30.5 *(12 – 54)*
For patients with baseline parasite density < 10^5 / µl	Number of patients	88 / 168 (52 %)
	Median PCT (range) in hours	30 *(12 – 54)*
For patients with baseline parasite density > 10^5 / µl	Number of patients	80 / 168 (48 %)
	Median PCT (range)	36 *(12-54)*
Parasite reduction ratio	Median (range) $\log_{10}$ parasite reduction ratio$_{H6}$	0.2 *(-1.14 – 2.11)*
	Median (range) $\log_{10}$ parasite reduction ratio$_{H12}$	1.07 *(-0.31 – 5.25)*
	Median (range) $\log_{10}$ parasite reduction ratio$_{H24}$	2.5 *(0.02 – 6.05)*
	Median (range) $_{\log10}$ parasite reduction ratio $_{H48}$	5.97 *(1.89 – 6.75)*
Patients presenting with fever (> 37.5 °C)	Baseline / Day 0	83
	Mean time to fever clearance time (95 % CI) in hours	24.42 (20.37 – 28.48)
Patients with recurrent parasitaemia		56 / 168 *(33 %)*
	< Day 28	32 / 168 *(19 %)*
	> Day 28	24 / 168 *(14 %)*

Tab. 2: Clinical and parasitological results in the study patients. (n = 168, one did not clear parasites and six withdrew consent right after enrolment)

On day of enrolment elevated body temperature (> 37.5 °C) was recorded in 83 patients, mean time to fever clearance was 24 hours (95 % CI 20 – 28 hours).

On the first day (24 hours) after initiation of treatment there were 117 / 168 patients (Day 1 Parasite Prevalence Rate PPR_{D1} 70 %) with residual parasitaemia. By hour 48, only 3 patients had residual parasitaemia (PPR_{D2} 2 %) and by hour 54 all patients were cleared of parasitaemia except of one patient, who was later excluded and given rescue treatment for suspected clinical treatment failure.

Most parasites were killed between 6 and 12 hours after the treatment dose. The increase in parasite reduction was high between hour 6 and 12 after initiation of treatment (reduction rate rose from 0.2 to 1.07, therefore, from 0.03 / hour to 0.14 / hour). It then decreased until reaching the time of second artesunate treatment dose at hour 24. After the second dose it increased rapidly within six hours between hour 24 and 30 (0.08 / hour to 0.55 / hour) to then slow once again (**Fig. 9**).

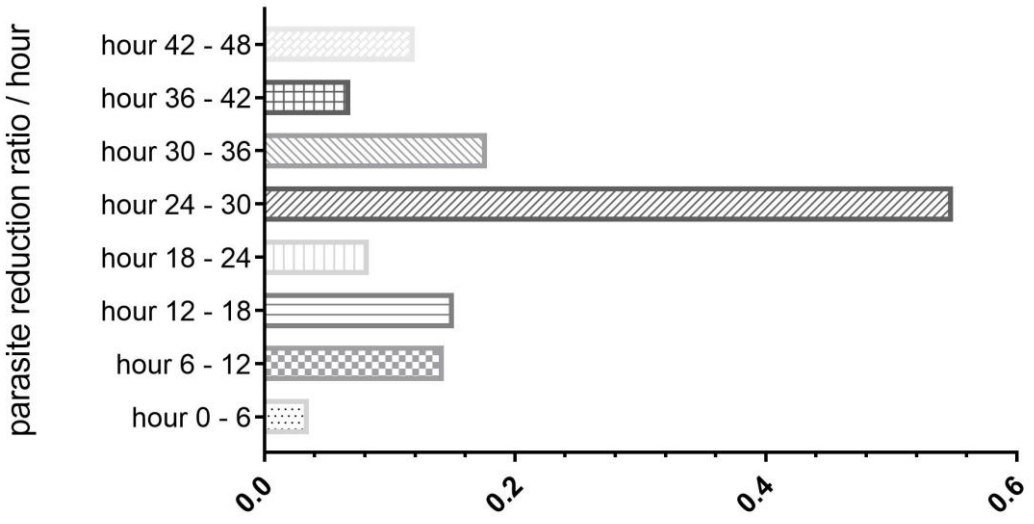

Fig. 9: Median parasite reduction ratios per hour in six hourly intervals for the first 48 hours after initiation of treatment.

Fig. 10 shows the reduction in total mean parasitaemia levels over time.

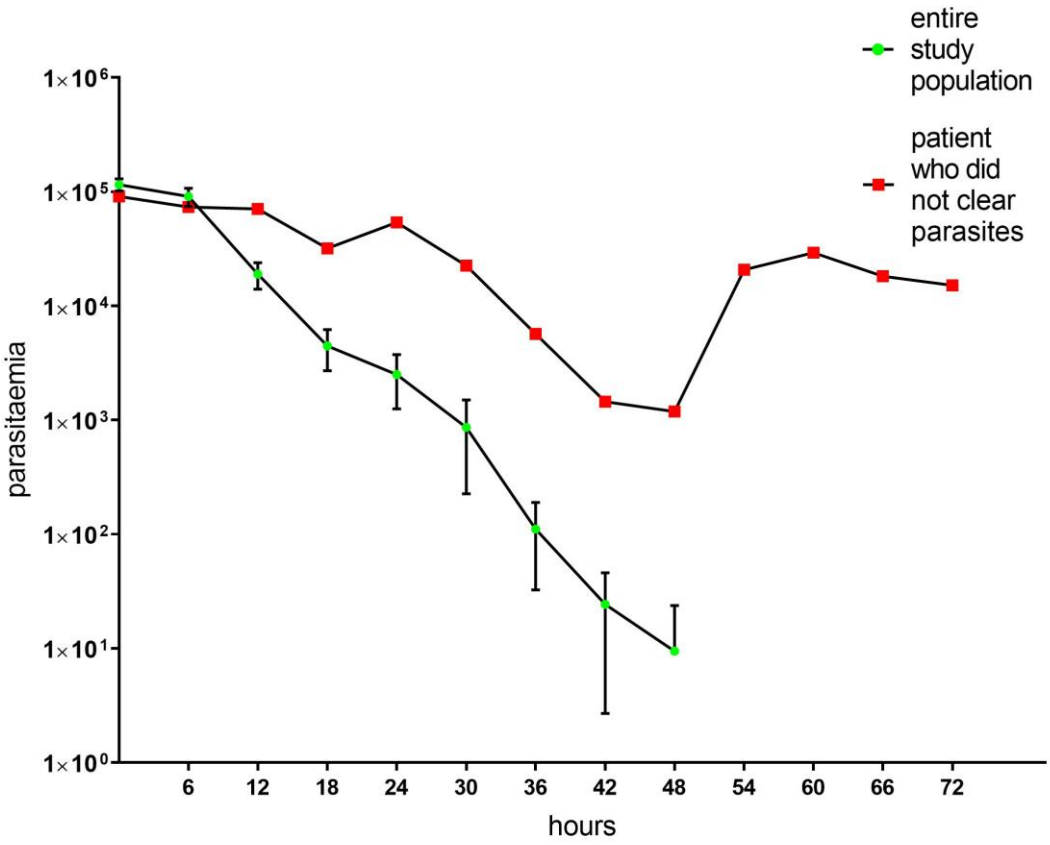

Fig. 10: Reduction in mean parasitaemia levels over hours. Green dots display mean parasitaemia in overall study population over time. Red dots display parasitaemia in one single patient not clearing parasites. Bars show 95 % CI of mean parasite counts.

The parasite clearance estimation was obtained by entering the data into the web based tool as mentioned above (refer to [15]).

An overview of the results is displayed in **Tab. 3.**

PCE		
Clearance rate constant (/ hr)	Median	0.29
	Geometric mean	0.30
Slope half life (hrs)	Median	2.37
	Geometric mean	2.33
Lag phase (hrs)	Median	11.93
	Geometric mean	11.72
Parasite clearance 50 (PC50) (hrs)	Median	4.67
	Mean	5.69
Parasite clearance 90 (PC90) (hrs)	Median	10.80
	Mean	11.18
Parasite clearance (PC99) (hrs)	Median	18.76
	Mean	19.41

Tab. 3: Parasite clearance estimation values as calculated by web-based tool provided by WWARN (refer to [15])**.**

As mentioned before (refer to **3.1.1**), the PCT has been reported to correlate with initial parasitaemia. Baseline parasitaemia counts did show a significant positive correlation with the initial parasitaemia at enrolment ($p < 0.001$) (**Fig. 11**).

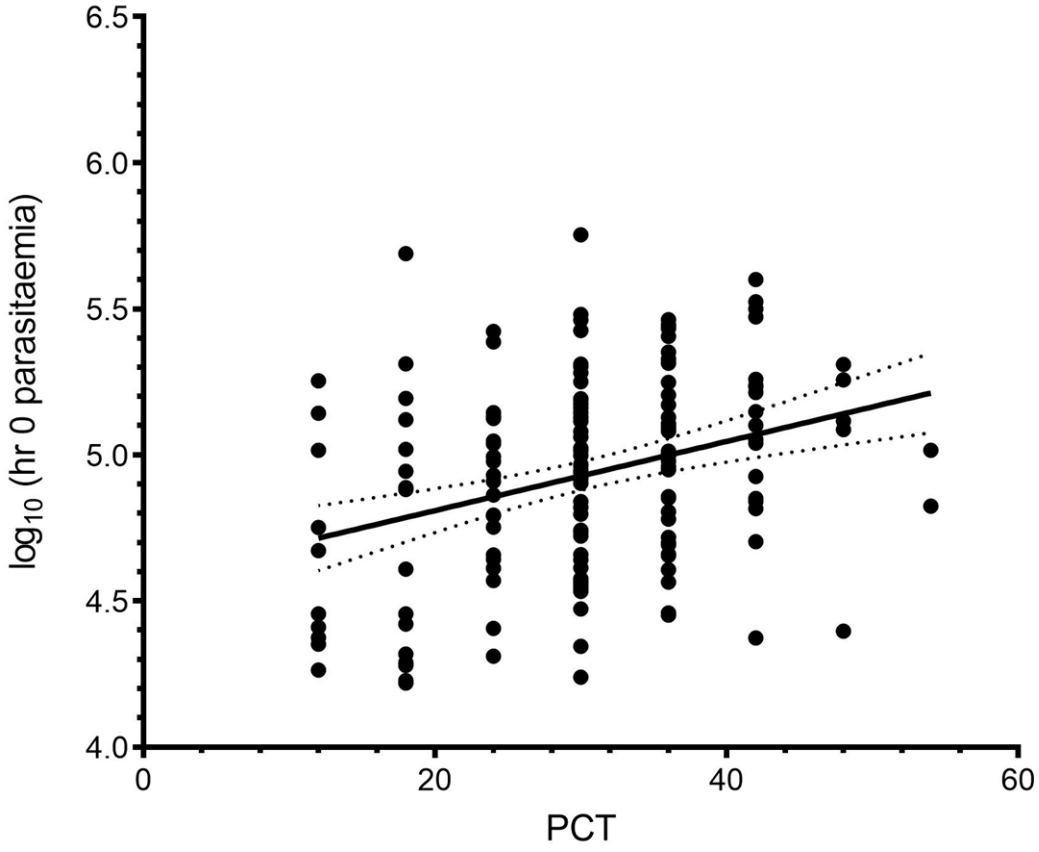

Fig. 11: Correlation of PCT and initial parasitaemia. Significant positive correlation; linear regression analysis showed a significant deviation from zero for the slope ($p < 0.0001$); dotted line shows 95 % CI.

No significant correlation could be found between PCT and the age of the patient (**Fig. 12**).

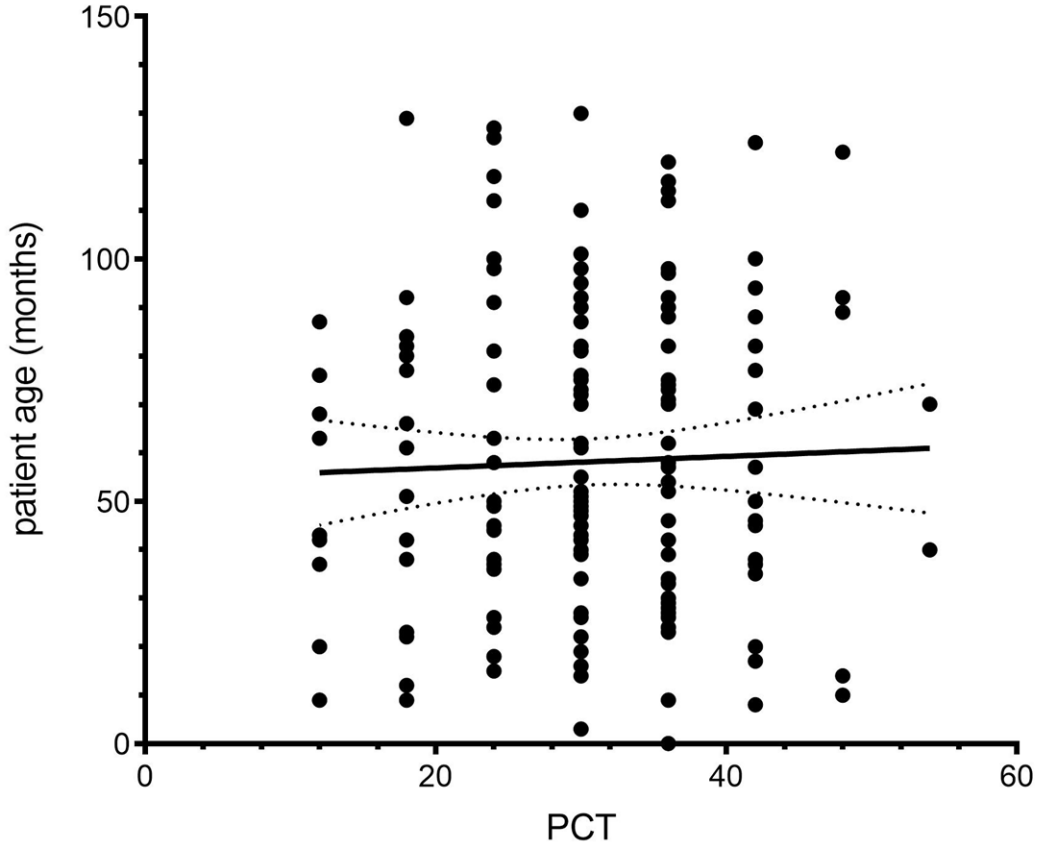

Fig. 12: **Correlation of PCT and patient age.** No significant correlation; dotted line shows 95 % CI.

Using the PCE values as obtained from the web-based tool on the other hand, no significant correlation could be found between the clearance rate constant and initial parasitaemia (**Fig. 13**).

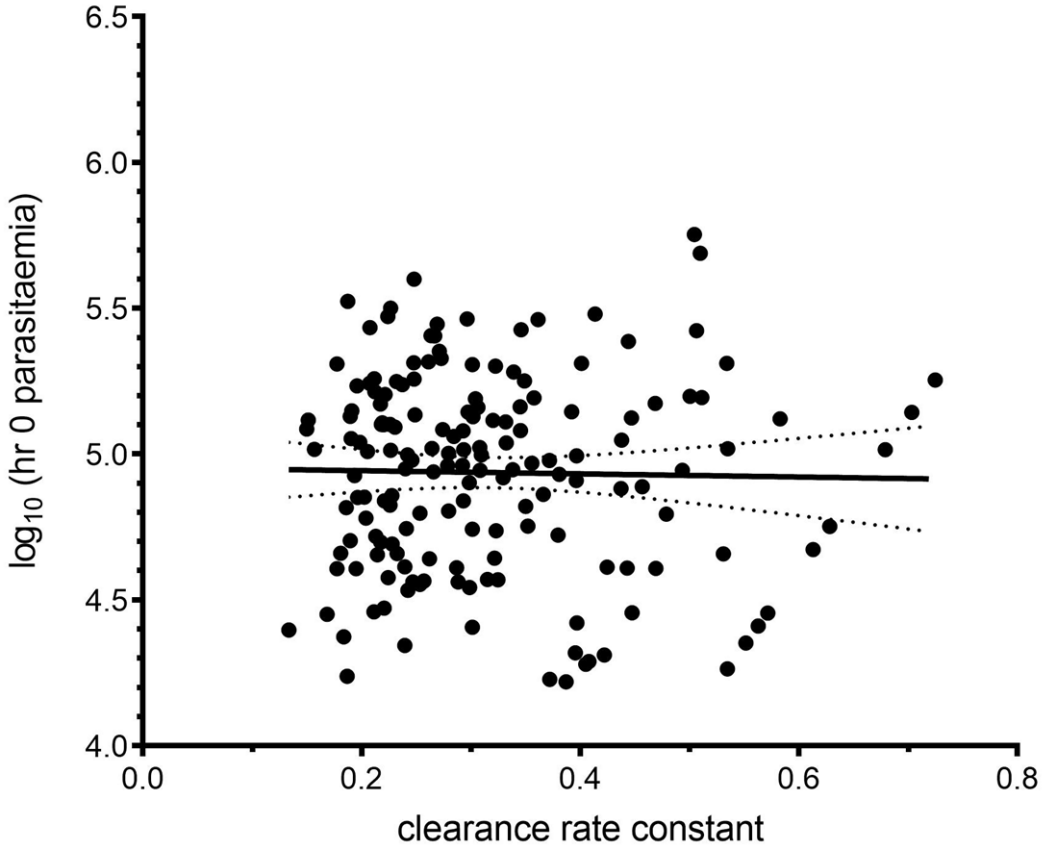

Fig. 13: Correlation of clearance rate constant and initial parasitaemia. No significant correlation; linear regression analysis showed a perfect line for clearance rate constant and $\log_{10}$ initial parasitaemia; dotted lines show 95 % CI.

Also, the age of the patient did not show a significant correlation with the clearance rate constant (**Fig. 14**).

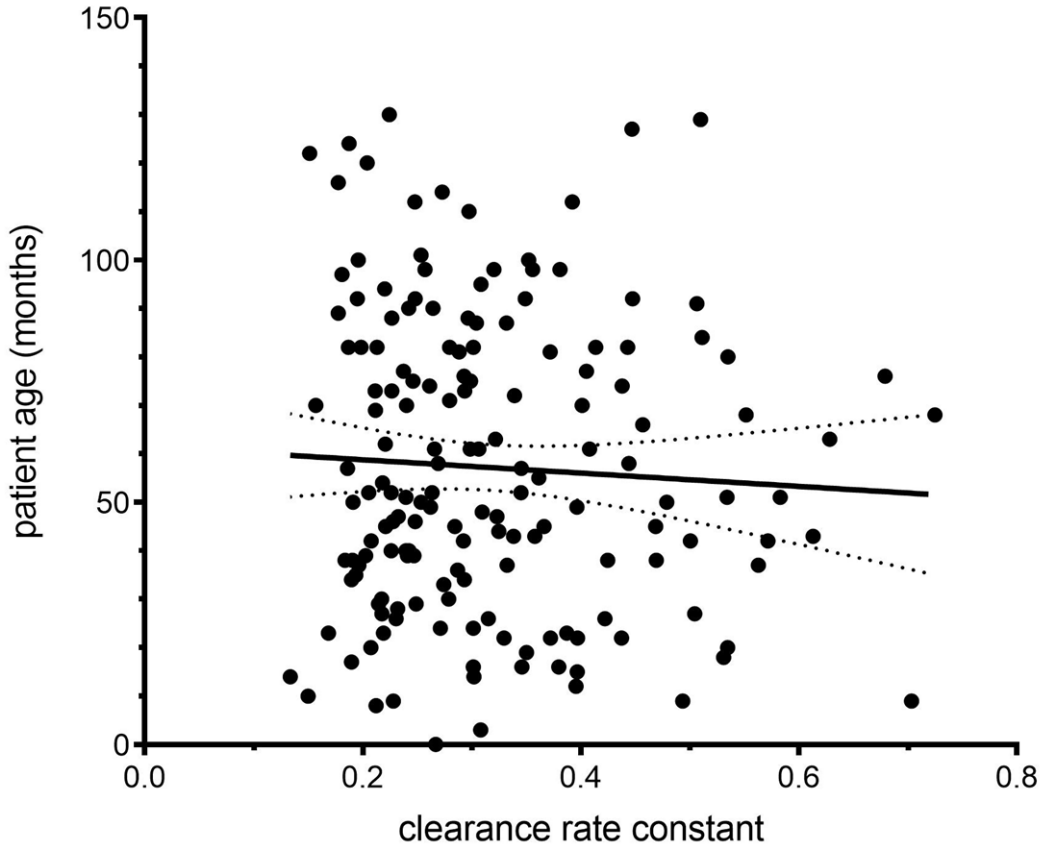

Fig. 14: Correlation of clearance rate constant and patient age. No significant correlation; linear regression analysis stated a non-significant deviation from 0 for the slope; dotted lines show 95 % CI.

The same applied for the slope half life (**Fig. 15**).

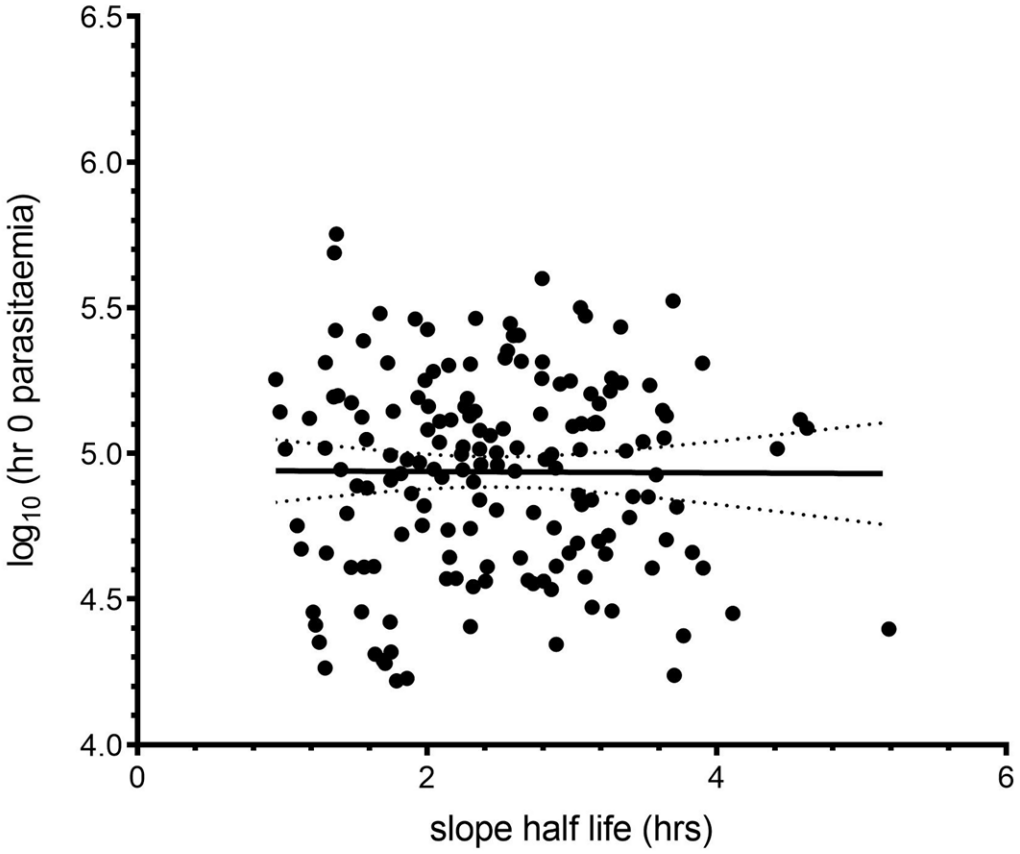

Fig. 15: Correlation of slope half life and initial parasitaemia. No significant correlation; linear regression analysis stated a non-significant deviation from 0 for the slope; dotted lines show 95 % CI.

No correlation was seen between the slope half life and the patient age (**Fig. 16**).

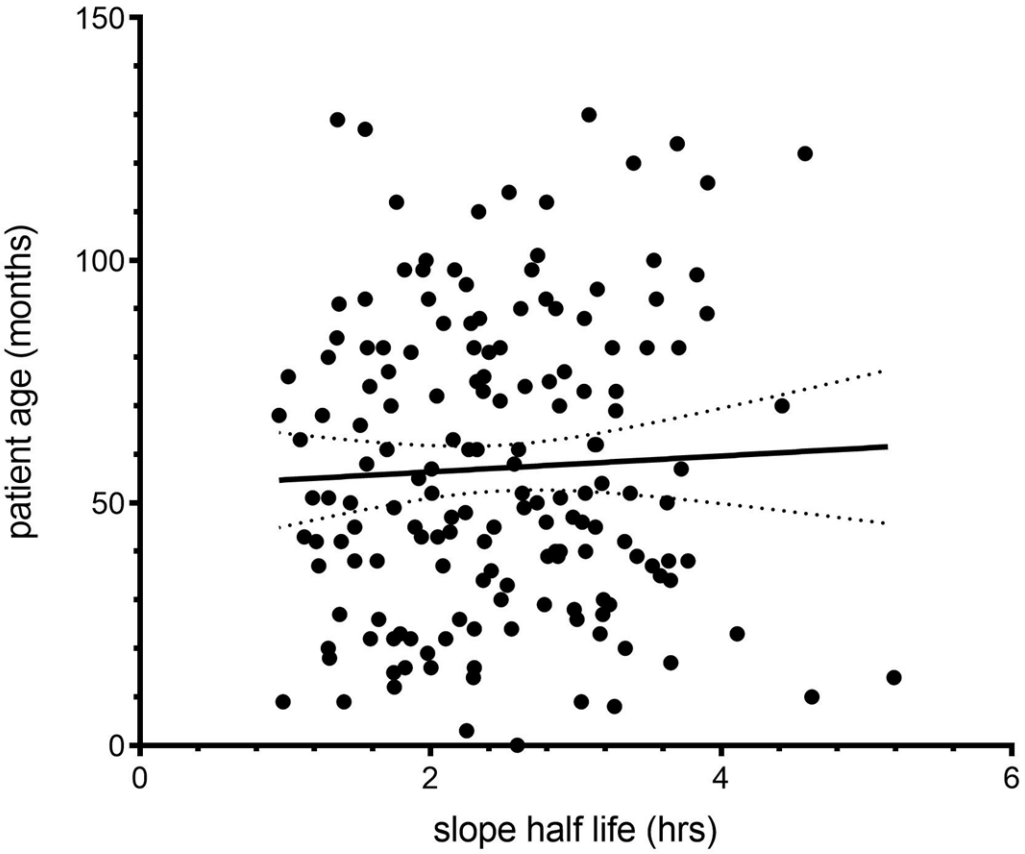

Fig. 16: Correlation of slope half life and patient age. No significant correlation; linear regression analysis stated a non-significant deviation from 0 for the slope; dotted lines show 95 % CI.

5.1.2 Recurrent parasitaemia

A total of 56 / 168 patients (33 %) showed recurrent parasitaemia during the follow-up. Up to day 28 we recorded 32 / 168 patients (19 %) with recurrent parasitaemia. Median parasitaemia at presentation of recurrent parasitaemia was 75,800 parasites / µl.

Analysis of the genotyping data confirmed recrudescence in 8 / 168 patients (5 %), whereas 5 / 168 patients (3 %) had recrudescent parasites within 28 days.

5.1.3 *Ex vivo* drug responses and analysis

Out of 175 patients that were recruited for the CATMAP study, 6 patients withdrew consent before having the baseline blood samples taken. From 145 successfully set up assays, 111 showed adequate growth and inhibition against at least one drug and were included in the final analysis (77 %). The intra-sample correlation between the duplicates as a measure of reproducibility was high (median 0.94, range 0.91 – 0.98).

Baseline *ex vivo* IC_{50} drug responses were determined using the established HRP2 ELISA. The median **AS** IC50 value was 6.6 nM, the geometric mean 5.8 nM. Median and geometric mean for **DHA** were 4.5 nM and 4.8 nM. For **LUM** the median and geometric mean IC50 values were 13.7 nM and 12.1 nM; median and geometric mean for **PPQ** were 7.9 nM and 8.0 nM.

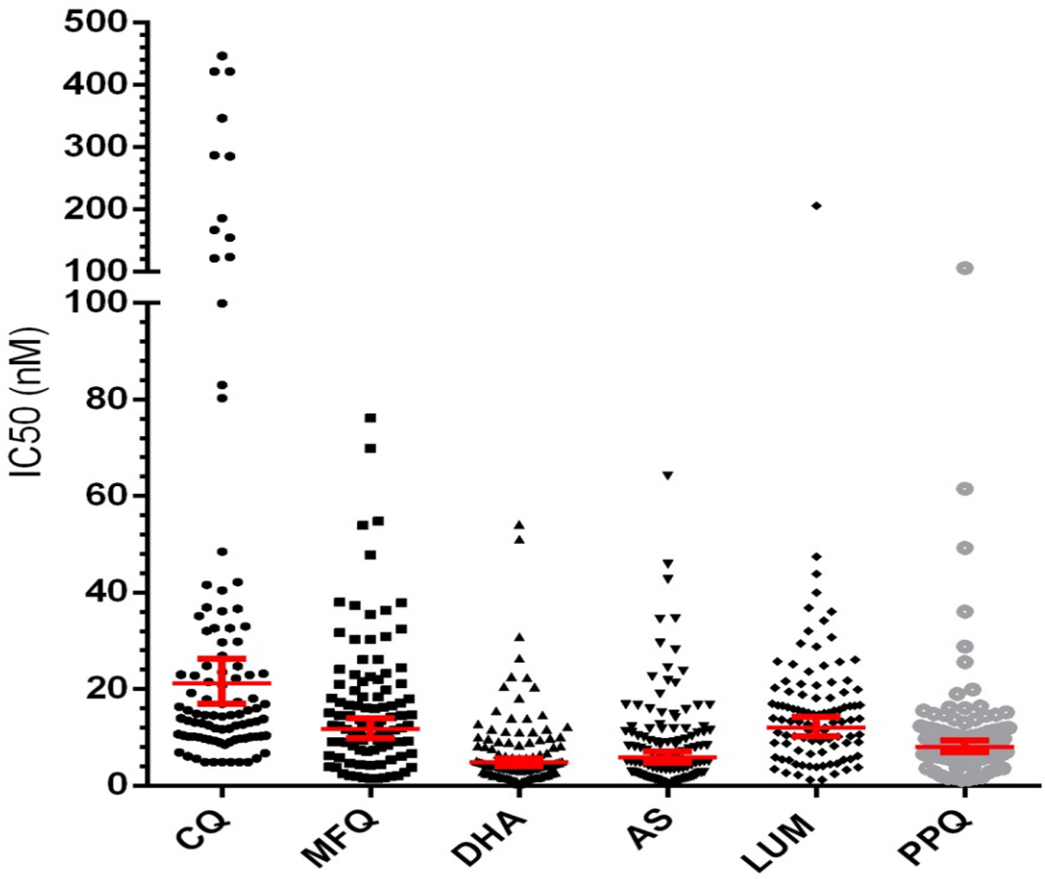

Fig. 17: Distribution of baseline IC50 values. Red bars show geometric mean with 95 % CI.

All IC50 values calculated are also presented in the above **Fig. 17**. + **Tab. 4**.

***Ex vivo* susceptibility**	**CQ**	**MFQ**	**DHA**	**AS**	**LUM**	**PPQ**
Number of values	97	99	111	111	99	98
Minimum	4.93	1.48	0.42	0.30	1.25	1.25
25 % Percentile	10.22	7.36	2.80	2.82	8.26	5.65
Median	15.63	12.59	4.52	6.59	13.71	7.88
75 % Percentile	32.64	21.12	8.99	11.84	19.54	11.85
Maximum	446.80	76.19	54.17	64.19	206.2	106.50
Geometric mean	21.15	11.76	4.84	5.82	12.10	8.03
Lower 95 % CI of geo. mean	16.98	9.86	4.10	4.77	10.31	6.91
Upper 95 % CI of geo. mean	26.35	14.03	5.70	7.12	14.20	9.34

Tab. 4: *Ex vivo* susceptibility in nM of isolates of *Plasmodium falciparum* to AS, DHA, CQ, MFQ, LUM and PPQ.

Pair-wise correlation analysis was done in order to look for indications of cross-resistance (**Tab. 5** + **Fig. 18** - **Fig. 32**).

A significant positive correlation was shown between DHA and AS (Rho = 0.77, $p < 0.0001$), but also between DHA and PPQ (Rho = 0.54, $p < 0.0001$), DHA and LUM (Rho = 0.46, $p < 0.0001$) and DHA and MFQ (Rho = 0.49, $p < 0.0001$). Likewise did AS show a significant positive correlation with PPQ (Rho = 0.41, $p < 0.0001$), with LUM (Rho = 0.42, $p < 0.0001$) and with MFQ (Rho = 0.44, $p < 0.0001$).

Susceptibility to PPQ correlated with LUM (Rho = 0.31, $p = 0.0018$) and MFQ (Rho = 0.22, $p = 0.0324$), and LUM additionally correlated with MFQ in a statistically significant way (Rho = 0.62, $p < 0.0001$).

A negative correlation was found between LUM and CQ (Rho = -0.24, $p = 0.02$).

Correlations	CQ	DHA	AS	PPQ	LUM
DHA					
Rho	0.002				
p-value	0.98				
r^2	0.005				
AS					
Rho	0.04	**0.77**			
p-value	0.70	**< 0.0001**			
r^2	0.008	**0.58**			
PPQ					
Rho	0.09	**0.54**	**0.41**		
p-value	0.40	**< 0.0001**	**< 0.0001**		
r^2	0.003	**0.02**	**0.03**		
LUM					
Rho	**-0.24**	**0.46**	**0.42**	**0.31**	
p-value	**0.020**	**< 0.0001**	**< 0.0001**	**0.002**	
r^2	**0.006**	**0.01**	**0.03**	**4.929e^{-006}**	
MFQ					
Rho	0.04	**0.49**	**0.44**	**0.22**	**0.62**
p-value	0.7	**< 0.0001**	**< 0.0001**	**0.0324**	**< 0.0001**
r^2	0.002	**0.04**	**0.10**	**0.006**	**0.05**

Tab. 5: Spearman (rho) and Pearson (r^2) correlation of *ex vivo* responses. Significant correlations in bold.

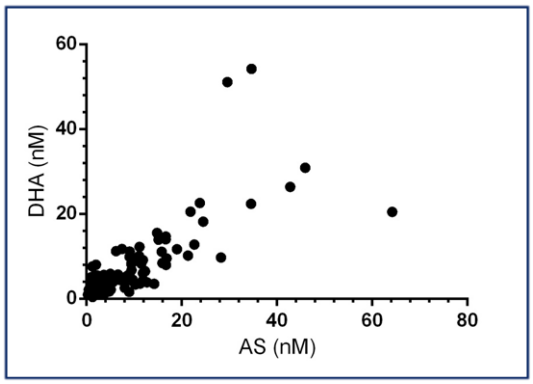

Fig. 18: Scatter plot of IC50 values of AS and DHA. Rho = 0.77, p < 0.0001.

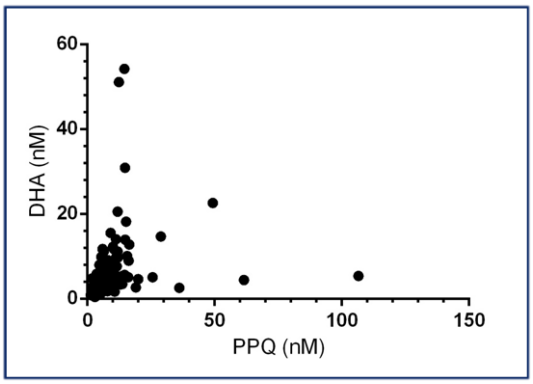

Fig. 19: Scatter plot of IC50 values of PPQ and DHA. Rho = 0.54, p < 0.0001.

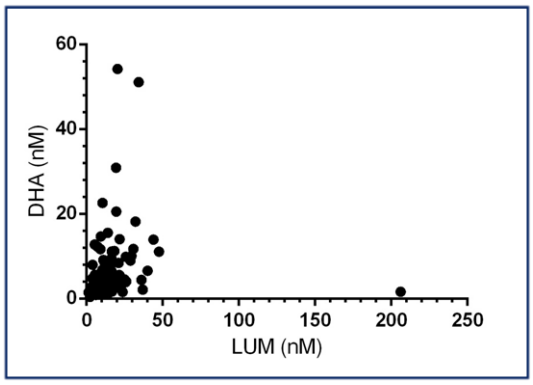

Fig. 20: Scatter plot of IC50 values of LUM and DHA. Rho = 0.46, p < 0.0001.

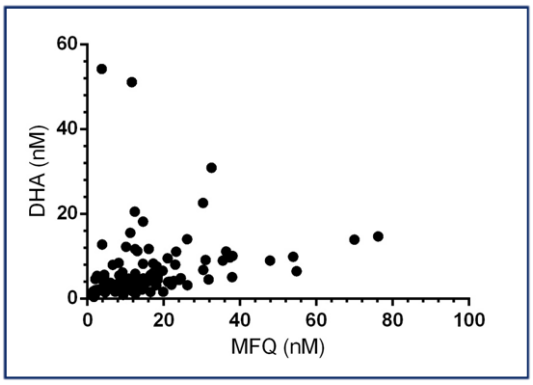

Fig. 21: Scatter plot of IC50 values of MFQ and DHA. Rho = 0.49, p < 0.0001.

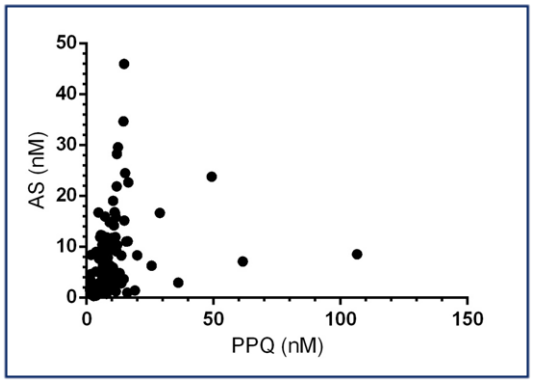

Fig. 22: Scatter plot of IC50 values of PPQ and AS. Rho = 0.41, p < 0.0001.

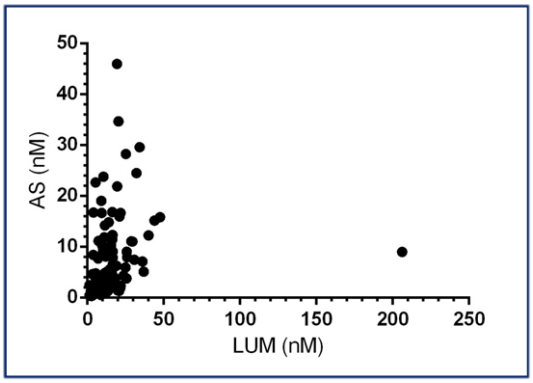

Fig. 23: Scatter plot of IC50 values of LUM and AS. Rho = 0.42, p < 0.0001.

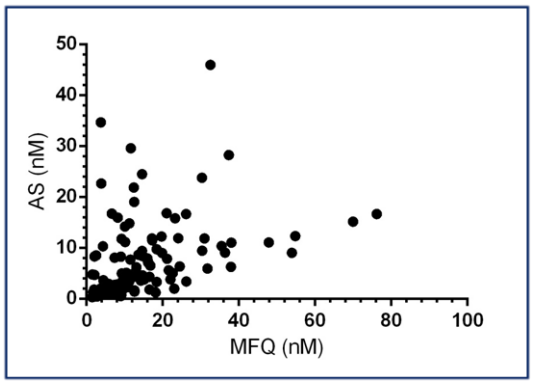

Fig. 24: Scatter plot of IC50 values of MFQ and AS. Rho = 0.44, p < 0.0001.

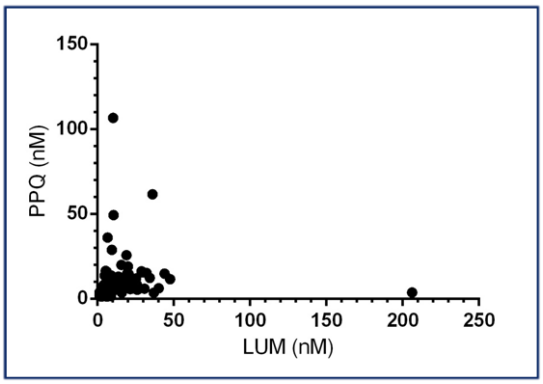

Fig. 25: Scatter plot of IC50 values of LUM and PPQ. Rho = 0.31, p = 0.0018.

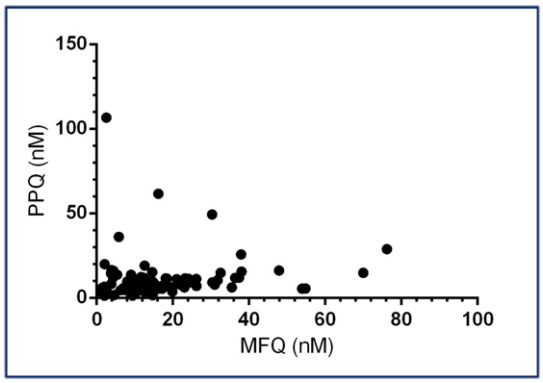

Fig. 26: Scatter plot IC50 values of MFQ and PPQ. Rho = 0.22, p = 0.0324.

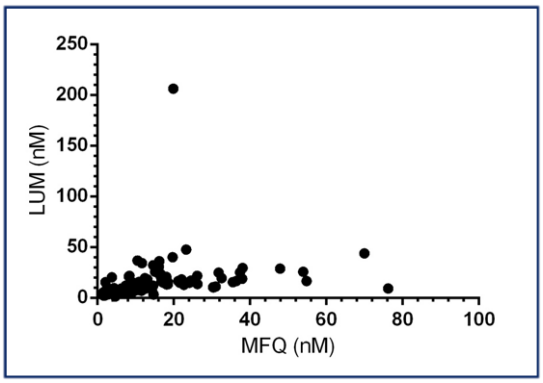

Fig. 27: Scatter plot of IC50 values of MFQ and LUM. Rho = 0.62, p < 0.0001.

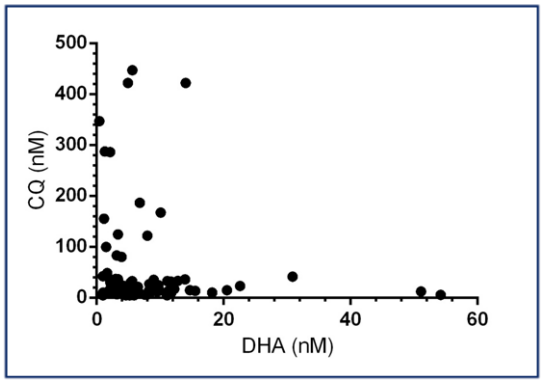

Fig. 28: Scatter plot of IC50 values of DHA and CQ.

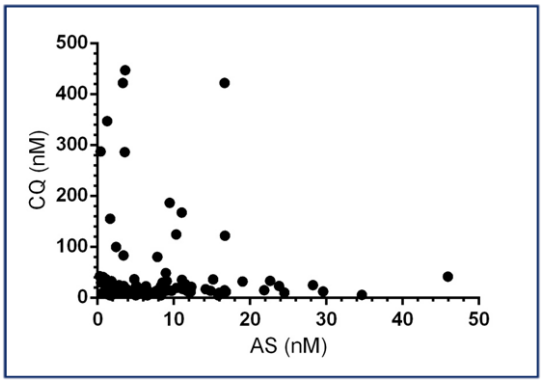

Fig. 29: Scatter plot of IC50 values of AS and CQ.

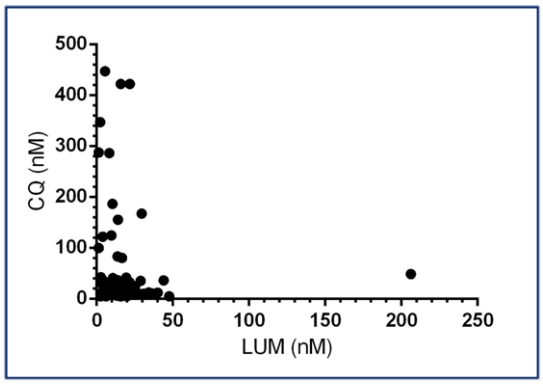

Fig. 30: Scatter plot of IC50 values of LUM and CQ. Rho = -0.24, p = 0.0202.

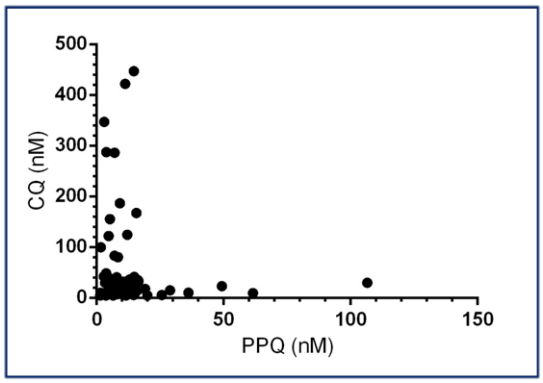

Fig. 31: Scatter plot of IC50 values of PPQ and CQ.

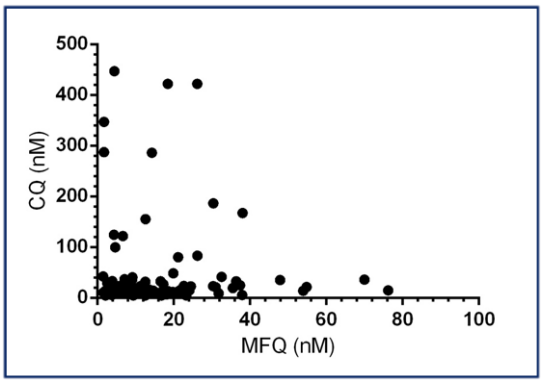

Fig. 32: Scatter plot of IC50 values of MFQ and CQ.

Multiple drug solutions were used in the study (refer to **5.2.1**) which differed in their responses. The correlation analysis was also done for each drug solution separately to further look at the ranges and to detect smaller correlations which might not be significant in the overall analysis but have been shown in other studies before (Appendix 3).

5.2 Potential confounding variables on the *ex vivo* analysis

When appraising the *ex vivo* drug susceptibility of the isolates, a range of potential confounding variables needs to be considered for a correct interpretation of results. The assay was set up in a controlled manner and experiment conditions did not change over time to reduce possible confounders to a minimum.

However, there were two factors that need further consideration which could have altered the results apart from a change in susceptibility of the parasite isolate and need to be taken into account:

- The drugs were stored as stock solutions. The working solutions used during the assay were prepared seven times in total over the course of the study. The steps taken and instruments used in the preparation were always the same. However, a slight change in concentration due to pipetting mistakes can never be completely ruled out.

- Over the course of the study 175 patients were recruited within a time frame of 9 months (April – December 2011). The patients were distributed over these 9 months due to multiple reasons (no malaria cases, refusals of enrolment as well as amendments in the study protocol resulting in a break in enrolment for about 6 weeks). Hence, some working solutions of the drugs were stored only for a short time when recruitment was proceeding quickly; others were stored over a longer period. Since storage conditions remained unaltered, the time of storage of drugs was plotted against the IC50 value to check for dependence.

5.2.1 Drug working solutions

The *ex vivo* drug susceptibility assays were performed with seven working solutions of the different drugs. This might account for different results among different solutions. Therefore, each working solution was also looked at individually concerning the distributions of IC50 (AS **Fig. 33** + **Tab. 6**, DHA **Fig. 34** + **Tab. 7,** LUM **Fig. 35** + **Tab. 8,** MFQ **Fig. 36** + **Tab. 9,** CQ **Fig. 37** + **Tab. 10,** PPQ **Fig. 38** + **Tab. 11**).

5.2.1.1 Artesunate solution I – VII

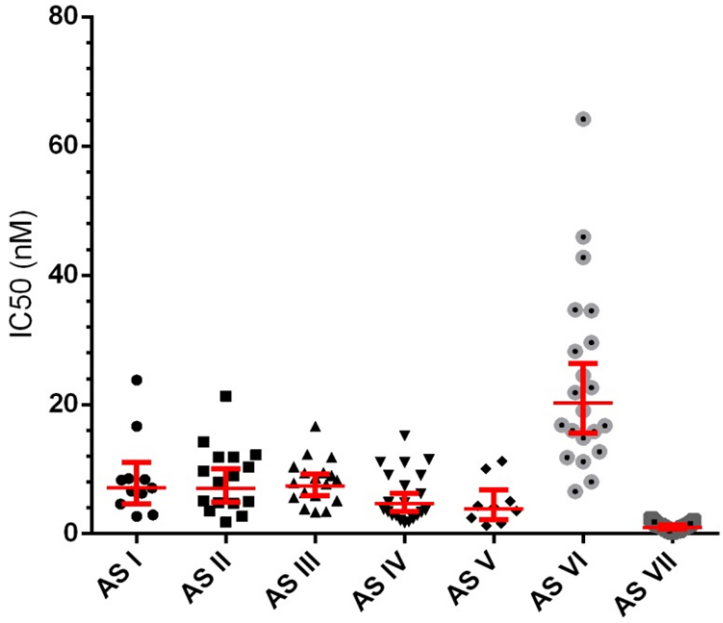

Fig. 33: Distribution of AS IC50 values among prepared drug solutions. Red lines show geometric mean with 95 % CI.

AS	I	II	III	IV	V	VI	VII
Number of values	11	16	18	21	9	21	15
Minimum	2.68	1.78	3.33	1.65	1.26	6.53	0.30
25 % Percentile	4.60	4.72	5.45	2.80	2.08	13.76	0.50
Median	7.10	8.46	8.06	3.75	4.14	19.02	1.06
75 % Percentile	8.52	11.88	9.67	9.05	7.54	32.06	1.54
Maximum	23.79	21.32	16.66	15.14	11.27	64.19	2.30
Geometric mean	7.14	7.04	7.36	4.62	3.84	20.23	0.96
Lower 95 % CI of geometric mean	4.62	4.93	5.89	3.40	2.17	15.53	0.68
Upper 95 % CI of geometric mean	11.05	10.05	9.2	6.28	6.77	26.35	1.35

Tab. 6: AS IC50 values in nM among prepared drug solutions.

5.2.1.2 Dihydroartemisinin solution I – VII

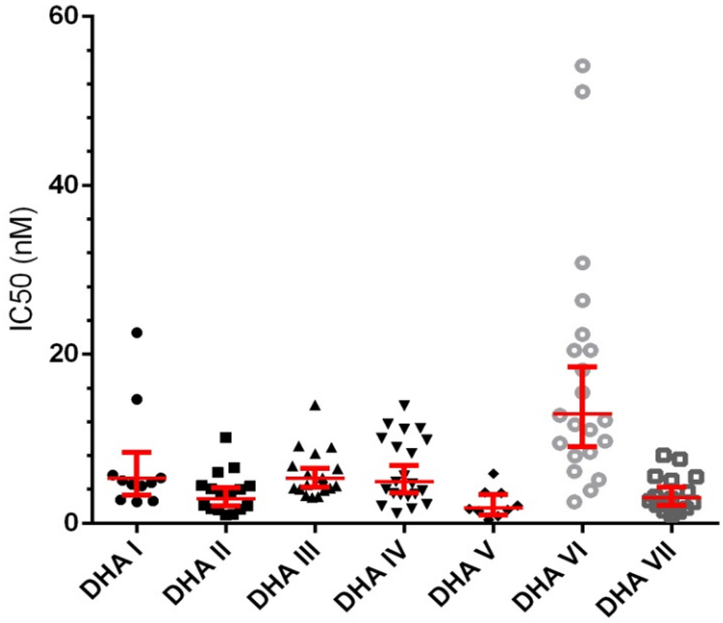

Fig. 34: Distribution of DHA IC50 values among prepared drug solutions. Red lines show geometric mean with 95 % CI.

DHA	I	II	III	IV	V	VI	VII
Number of values	11	16	18	21	9	21	15
Minimum	2.55	0.97	3.04	1.16	0.42	2.56	0.98
25 % Percentile	2.80	1.66	4.01	3.28	1.16	8.19	1.76
Median	4.80	3.44	5.68	4.72	1.69	12.20	2.97
75 % Percentile	5.72	4.46	7.15	9.98	3.62	21.44	5.47
Maximum	22.59	10.18	14.02	13.93	5.87	54.17	8.03
Geometric mean	5.32	2.93	5.30	4.94	1.83	12.98	3.01
Lower 95 % CI of geometric mean	3.37	2.05	4.30	3.58	0.99	9.10	2.10
Upper 95 % CI of geometric mean	8.40	4.21	6.52	6.83	3.39	18.51	4.31

Tab. 7: DHA IC50 values in nM among prepared drug solutions.

5.2.1.3 Lumefantrine solution I – VII

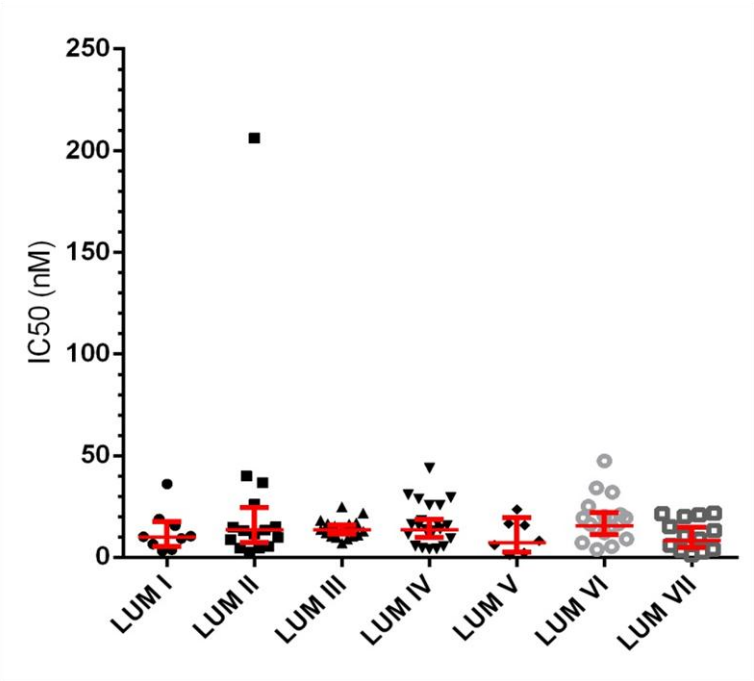

Fig. 35: Distribution of LUM IC50 values among prepared drug solutions. Red lines show geometric mean with 95 % CI.

LUM	I	II	III	IV	V	VI	VII
Number of values	9	15	17	21	7	17	13
Minimum	3.31	2.50	6.99	4.15	1.28	3.93	1.25
25 % Percentile	5.25	5.45	10.29	7.47	2.27	10.57	3.74
Median	10.19	13.28	13.68	15.75	8.26	16.61	10.68
75 % Percentile	17.25	26.10	16.83	25.71	16.72	23.13	20.40
Maximum	36.08	206.20	24.90	43.90	23.66	47.51	21.71
Geometric mean	9.88	13.45	13.49	13.60	7.13	15.66	8.40
Lower 95 % CI of geometric mean	5.53	7.37	11.37	9.87	2.60	11.18	4.80
Upper 95 % CI of geometric mean	17.65	24.54	16.00	18.76	19.53	21.93	14.72

Tab. 8: LUM IC50 values in nM among prepared drug solutions.

5.2.1.4 Mefloquine solution I – VII

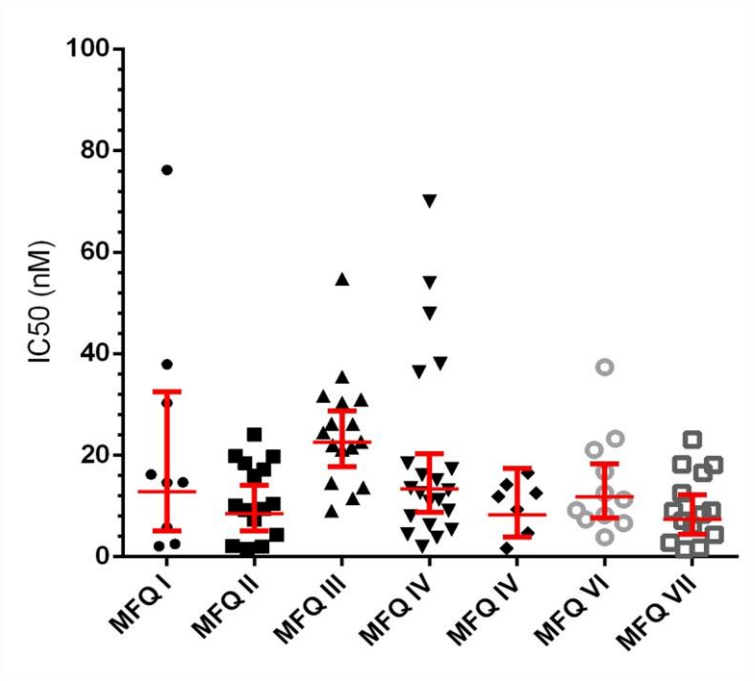

Fig. 36: Distribution of MFQ IC50 values among prepared drug solutions. Red lines show geometric mean with 95 % CI.

MFQ	I	II	III	IV	V	VI	VII
Number of values	9	15	16	21	7	11	14
Minimum	2.09	1.53	9.04	1.95	1.69	3.87	1.47
25 % Percentile	4.18	4.29	16.22	7.14	4.61	7.36	3.96
Median	14.68	10.09	23.56	13.10	11.92	11.26	8.66
75 % Percentile	34.10	18.44	30.81	27.38	14.25	21.05	16.92
Maximum	76.19	24.10	54.79	69.95	16.54	37.37	23.05
Geometric mean	12.83	8.510	22.58	13.33	8.24	11.79	7.38
Lower 95 % CI of geometric mean	5.06	5.14	17.74	8.76	3.88	7.61	4.45
Upper 95 % CI of geometric mean	32.52	14.08	28.76	20.29	17.46	18.27	12.22

Tab. 9: MFQ IC50 values in nM among prepared drug solutions.

5.2.1.5 Chloroquine solution I – VII

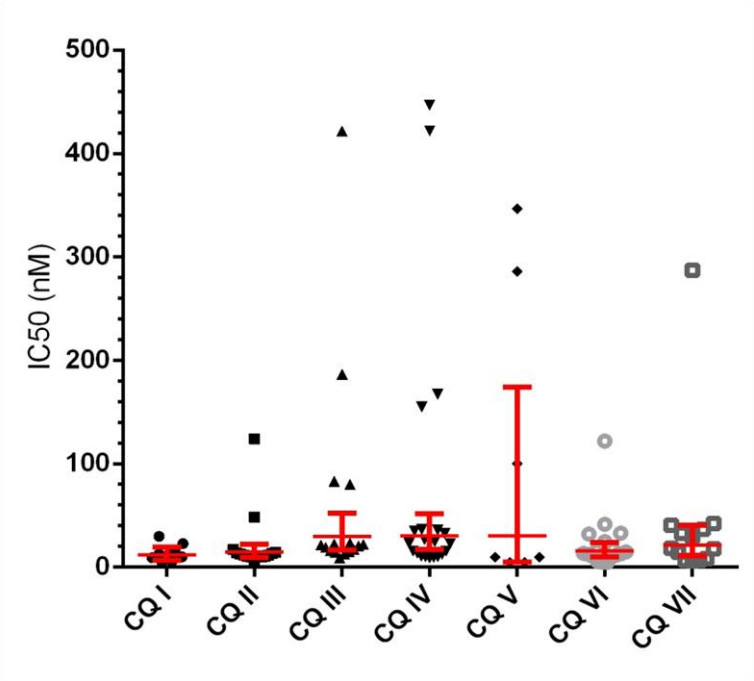

Fig. 37: Distribution of CQ IC50 values among prepared drug solutions. Red lines show geometric mean with 95 % CI.

CQ	I	II	III	IV	V	VI	VII
Number of values	8	15	16	21	7	17	13
Minimum	4.92	4.92	9.00	9.06	4.92	4.92	6.13
25 % Percentile	6.60	10.18	15.17	11.71	4.92	10.13	7.75
Median	11.62	11.87	21.19	23.29	9.81	13.48	18.12
75 % Percentile	20.96	14.67	66.12	36.39	285.9	28.41	38.71
Maximum	29.87	124.30	421.70	446.80	346.70	121.90	287.20
Geometric mean	11.70	14.49	29.71	30.09	30.13	15.68	21.47
Lower 95 % CI of geometric mean	6.93	9.53	16.88	17.47	5.21	10.29	11.34
Upper 95 % CI of geometric mean	19.74	22.04	52.29	51.82	174.30	23.90	40.65

Tab. 10: CQ IC50 values in nM among prepared drug solutions.

5.2.1.6 Piperaquine solution I – VII

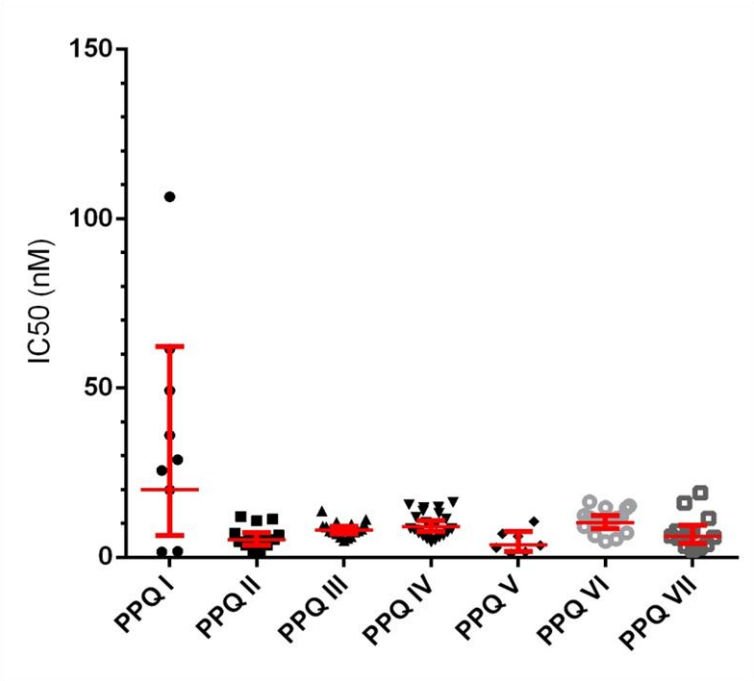

Fig. 38: Distribution of PPQ IC50 values among prepared drug solutions. Red lines show geometric mean with 95 % CI.

PPQ	I	II	III	IV	V	VI	VII
Number of values	9	15	17	21	7	17	12
Minimum	1.63	1.34	4.91	4.58	1.25	4.71	2.12
25 % Percentile	10.88	3.67	6.64	6.52	1.64	8.23	3.44
Median	28.87	5.74	7.87	8.56	3.60	11.02	6.11
75 % Percentile	55.40	7.20	9.49	13.39	7.02	13.45	10.64
Maximum	106.50	12.04	13.73	16.23	10.67	16.43	19.00
Geometric mean	19.95	5.21	8.04	9.11	3.73	10.21	6.22
Lower 95 % CI of geometric mean	6.39	3.67	7.02	7.62	1.81	8.48	4.04
Upper 95 % CI of geometric mean	62.29	7.39	9.21	10.90	7.71	12.30	9.57

Tab. 11: PPQ IC50 values in nM among prepared drug solutions.

5.2.2 Drug sensitivity over time of drug storage

Due to the fluctuation of patients recruited within a time span, some of the drug solutions were stored longer than others. To check if and how the storage time of the drug might influence the IC50 value, the measured inhibitory concentrations were plotted against the time of drug storage and analysed by linear regression. The time of drug storage did show a significant positive correlation with the IC50 values determined of AS ($p < 0.0001$) (**Fig. 39**) and DHA ($p < 0.0001$) (**Fig. 40**).

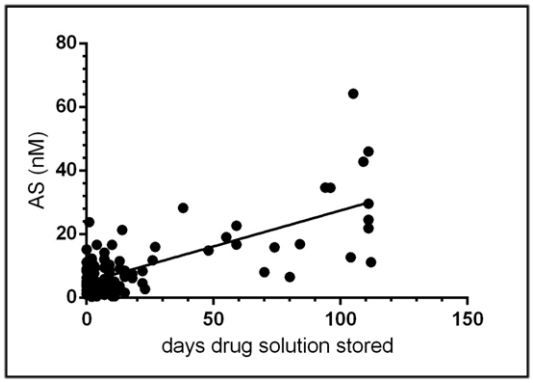

Fig. 39: Correlation between days of drug storage and AS IC50 values. $p < 0.0001$.

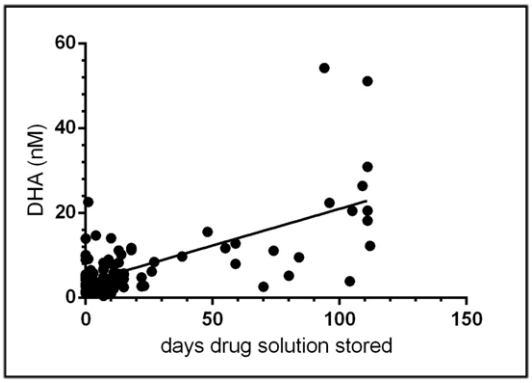

Fig. 40: Correlation between days of drug storage and DHA IC50 values. $p < 0.0001$.

All the other drugs did not show an indication for a decrease in efficacy over time of drug storage (**Fig. 41** – **Fig. 44**).

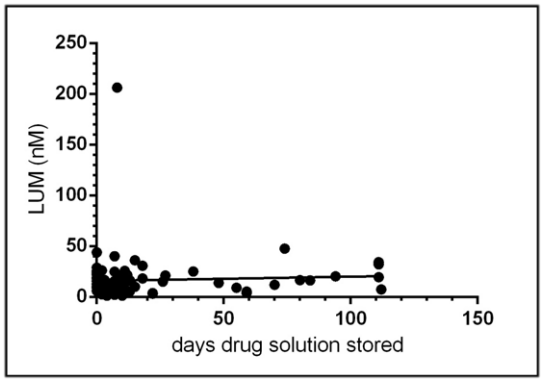

Fig. 41: Correlation between days of drug storage and IC50 values of LUM.

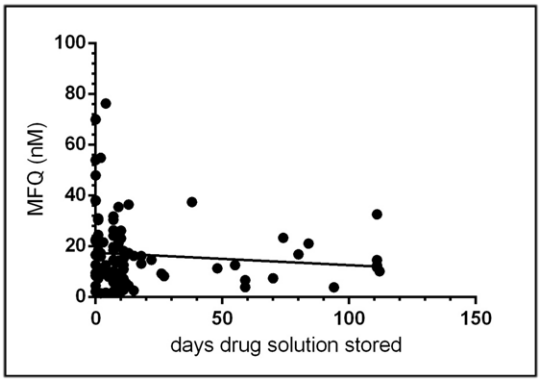

Fig. 42: Correlation between days of drug storage and IC50 values of MFQ.

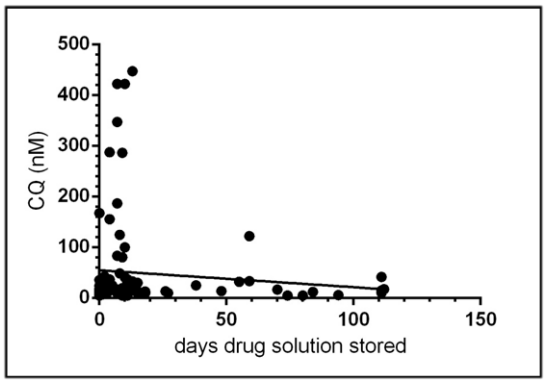

Fig. 43: Correlation between days of drug storage and IC50 values of CQ.

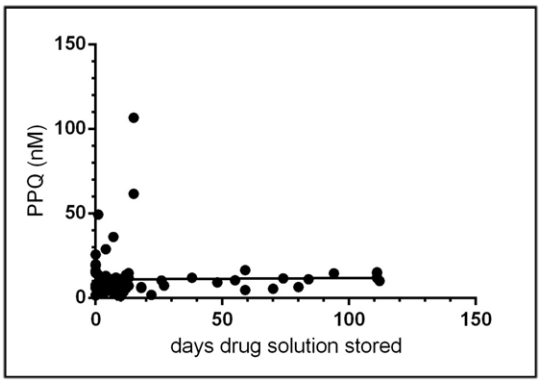

Fig. 44: Correlation between days of drug storage and IC50 values of PPQ.

Highlighting the plots of the IC50 values in different colours according to their drug solutions, measurements from the longest stored drug solution (AS VI) produced almost exclusively values of high mean inhibitory concentrations (**Fig. 45**, encircled).

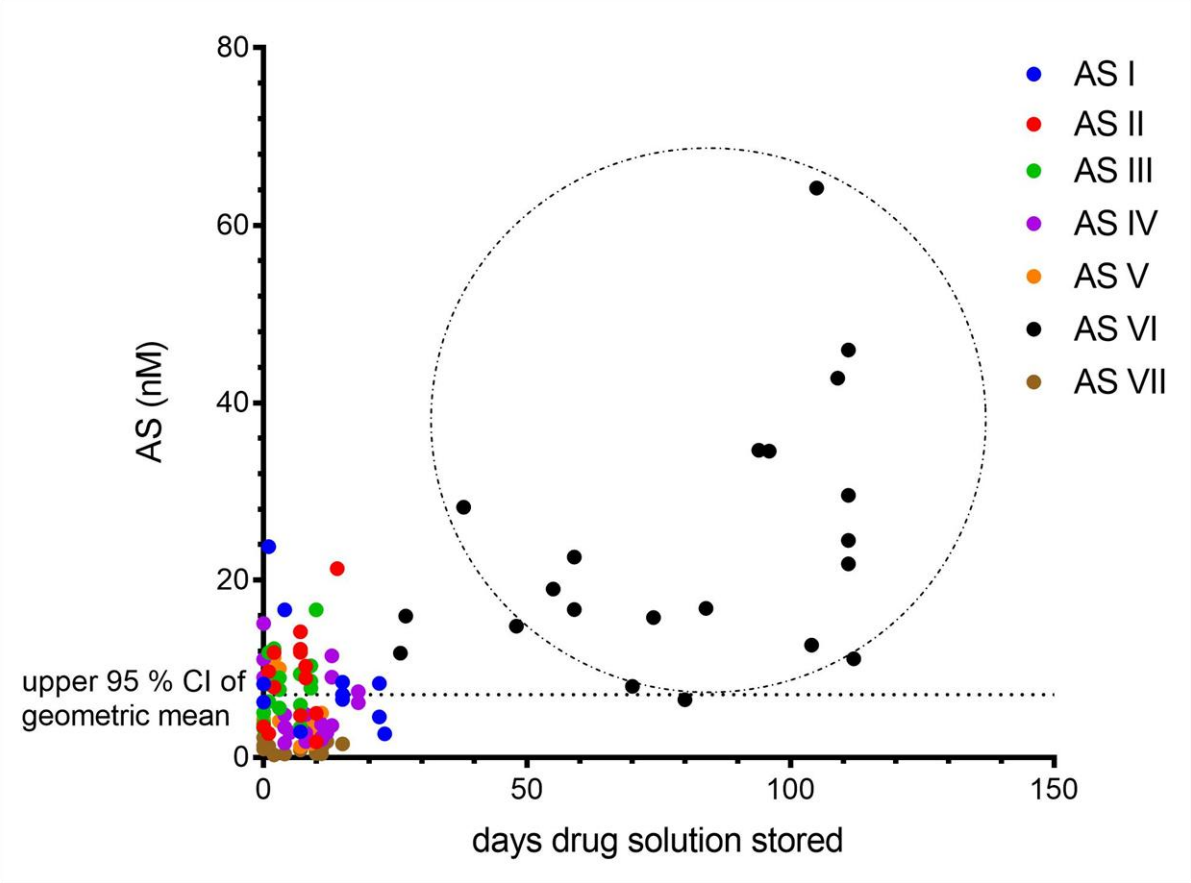

Fig. 45: Scatter plot of AS IC50 values dependent on time of drug storage and drug working solution.

Also the coloured plot of DHA according to drug solution and time of storage (**Fig. 46**, encircled) showed the same as above mentioned for AS.

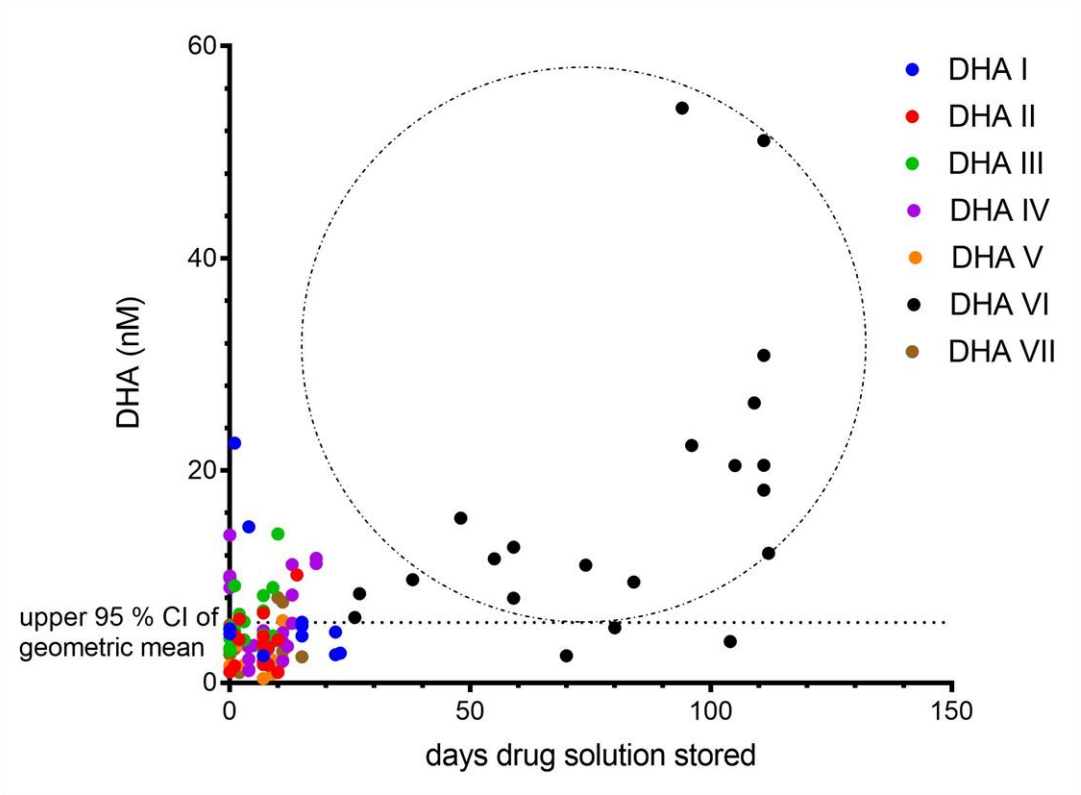

Fig. 46: Scatter plot of DHA IC50 values dependent on time of drug storage and drug working solution.

This one drug solution was stored over a long period of time. That indicates that ex vivo IC50 values of the artemisinins are highly influenced by the time of drug storage. The efficacy decreases with time and in order to produce reliable results drug storage time has to be strictly observed and controlled.

Already the analysis of individual drug solutions of the artemisinins (refer to **5.2.1.1** and **5.2.1.2**) did show that the distribution of IC50 values in drug solution VII differed between AS and DHA. To re-evaluate this finding, the ratio between DHA and AS IC50 was calculated as quotient (IC50 DHA / IC50 AS) and plotted with regards to drug solution and time of drug storage (**Fig. 47**). It was seen that the ratio was not influenced by time of drug storage, indicating that both drugs degrade at similar rates over time. But, as suspected, did drug solution VII show a high variety of ratios differing significantly from the rest ($p < 0.0001$). Since this was suspicious only in that one set of drug solution it is likely that another confounder, e.g. pipetting inaccuracy etc. influenced the results.

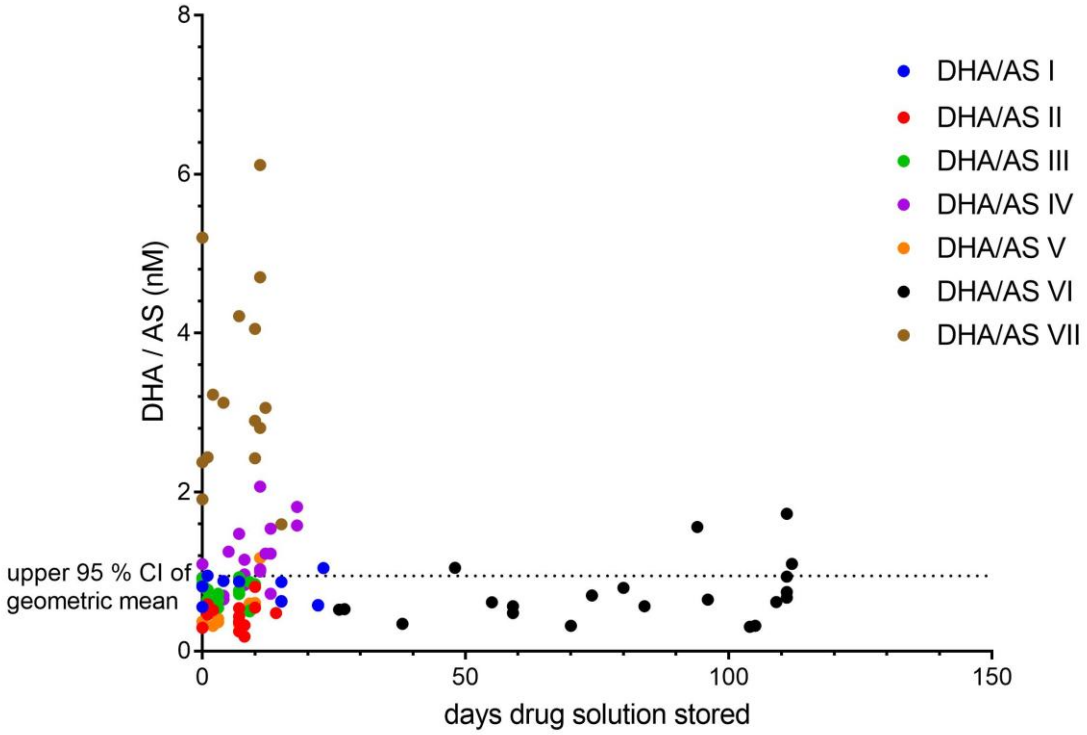

Fig. 47: Scatter plot of ratio between DHA and AS IC50 values dependent on time of drug storage and drug working solution.

5.2.3 Corrected analysis by accounting for the effect of drug batches

To account for the confounder time of drug storage as well as an unknown one (solution VII) the analysis was repeated excluding the sixth and seventh drug solution. **Tab. 12** shows IC50 values for DHA and AS in- (DHA, AS) and excluding (DHA*, AS*) those drug solutions in comparison.

***Ex vivo* susceptibility**	**DHA***	**DHA**	**AS***	**AS**
Number of values	75	111	75	111
Minimum	0.42	0.42	1.26	0.30
25 % Percentile	2.66	2.80	3.50	2.82
Median	4.17	4.52	6.26	6.59
75 % Percentile	6.02	8.99	9.72	11.84
Maximum	22.59	54.17	23.79	64.19
Geometric mean	4.04	4.84	5.89	5.82
Lower 95 % CI of geometric mean	3.41	4.10	5.06	4.76
Upper 95 % CI of geometric mean	4.71	5.70	6.86	7.12

Tab. 12: *Ex vivo* susceptibility (in nM) of AS and DHA when excluding (AS*, DHA*) the working solutions that were potentially confounded.

The correlation analysis to check for cross-resistance was repeated as well for the concerned pairs (**Tab. 13**). The IC50 values for DHA and AS were tested using unpaired nonparametric t-test to check for differences when excluding the values from the potentially degraded drug solution VI and the other suspicious drug solution VII. The overall analysis was not significantly influenced by the discussed confounders (AS vs. AS* p = 0.72, DHA vs. DHA* p = 0.23). Interestingly, the corrected correlation analysis did show a main difference for the correlation between the artemisinins and MFQ. Before the correction the correlation between DHA and AS and MFQ was significant but with only a coefficient of determination of r^2 = 0.04 and 0.10, respectively. After the correction this coefficient of determination increased to 0.41 and 0.36, respectively.

The corrected analysis does now indicate cross resistance between MFQ and the artemisinins. Other correlations did not change significantly.

Correlations	CQ	DHA*	AS*	PPQ	LUM
DHA*					
Rho	0.15				
p-value	0.23				
r^2	0.003				
AS*					
Rho	0.04	**0.74**			
p-value	0.79	**< 0.0001**			
r^2	0.02	**0.61**			
PPQ					
Rho	0.09	**0.46**	**0.35**		
p-value	0.40	**< 0.0001**	**0.004**		
r^2	0.003	**0.08**	**0.08**		
LUM					
Rho	**-0.24**	**0.45**	**0.46**	**0.31**	
p-value	**0.02**	**< 0.0001**	**< 0.0001**	**0.002**	
r^2	**0.006**	**0.001**	**0.03**	**$4.929e^{-006}$**	
MFQ					
Rho	0.04	**0.63**	**0.63**	**0.22**	**0.62**
p-value	0.70	**< 0.0001**	**< 0.0001**	**0.0324**	**< 0.0001**
r^2	0.002	**0.41**	**0.36**	**0.006**	**0.05**

Tab. 13: Pair-wise Spearman (Rho) and Pearson (r^2) correlation matrix of *ex vivo* responses to different compounds after exclusion of potentially confounded working solutions VI and VII. Significant correlations in bold.

5.3 Drug sensitivity in recurrent parasitaemia samples

Recurrent parasitaemia was recorded in 56 / 168 patients over the course of the follow-up (33 %). Before or on day 28 after enrolment, 32 patients (19 %) had recurrences and 24 (14 %) after day 28. From 39 (70 %) of these 56 total recurrent parasitaemia patients another sample for drug sensitivity testing was obtained before giving treatment.

The drug-sensitivity assay was successful in 35 of these samples (90 %). The distributions of the IC50 values among all the recurrence samples are shown below (**Fig. 48**).

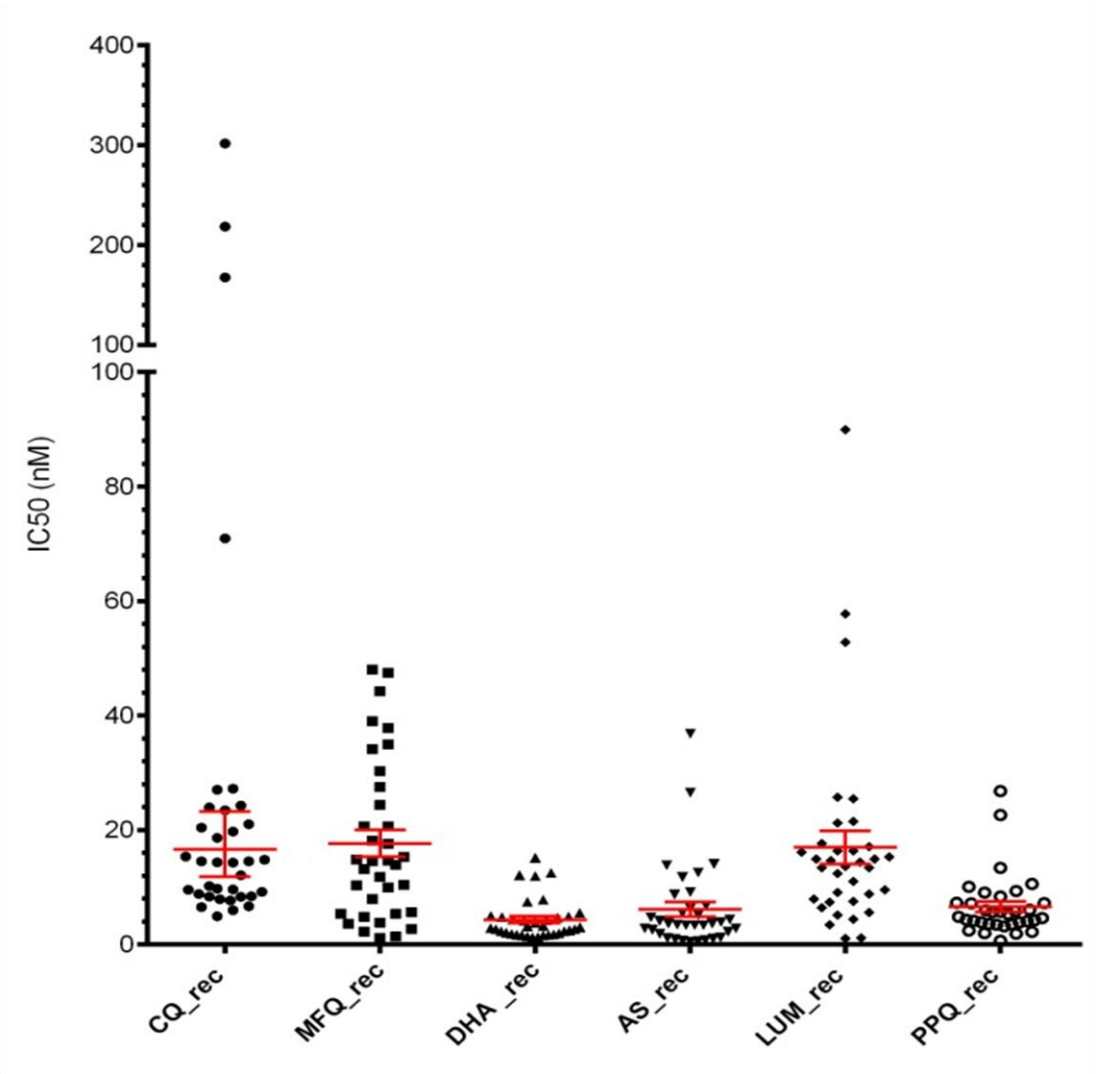

Fig. 48: Distribution of IC50 values determined in isolates obtained from recurrent infections.

Results are displayed without regards to the used drug solution. IC50 distributions in recurrent parasitaemia isolates (as shown in **5.2.1** for baseline samples) can be found as additional information in the Appendix (Appendix 4). **Tab. 14** shows the IC50 values of recurrent samples in detail.

IC50 of isolates from recurrent infections	CQ	MFQ	DHA	AS	LUM	PPQ
Number of values	35	35	35	35	35	34
Minimum	4.93	1.19	1.29	0.47	1.07	0.67
25 % Percentile	8.41	5.38	1.86	1.86	7.49	3.57
Median	14.35	14.57	3.03	3.70	13.66	5.08
75 % Percentile	23.45	27.48	4.91	6.83	17.11	7.56
Maximum	301.60	47.99	15.14	36.83	89.92	26.81
Geometric mean	16.62	11.95	3.37	3.62	11.76	5.17
Lower 95 % CI of geo. mean	11.89	8.47	2.66	2.52	8.61	4.04
Upper 95 % CI of geo. mean	23.23	16.86	4.27	5.199	16.07	6.61

Tab. 14: *Ex vivo* susceptibility of isolates obtained from recurrent infections.

A total of 18 matched pairs of samples obtained from baseline and recurrent infections for *ex vivo* drug susceptibility testing were collected.

Comparing these 18 pairs using Wilcoxon´s paired nonparametric test, the IC50 values between the baseline sample and recurrence sample did not differ significantly besides a slightly significant lower value of DHA in the isolates from recurrent infections. (p = 0.02) (**Fig. 49**). This may be difficult to explain but importantly, drug selection did not lead to **elevated** values.

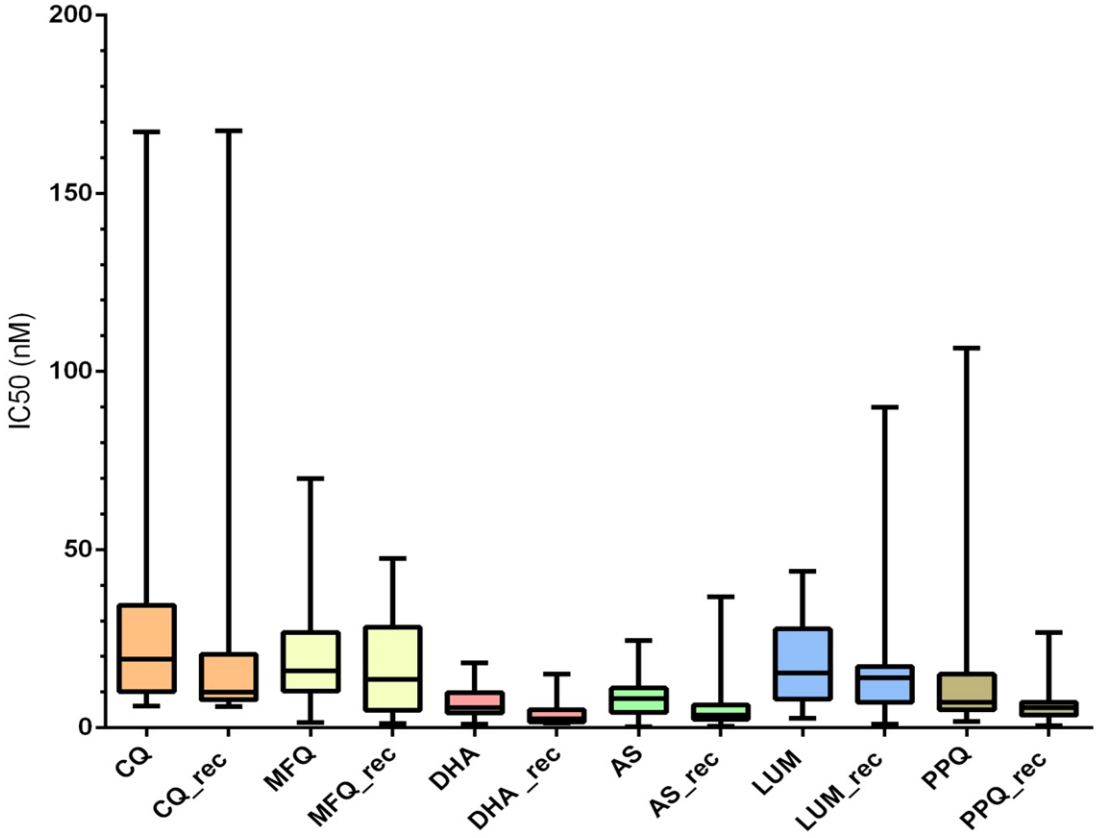

Fig. 49: Whisker plots showing *ex vivo* drug responses of matched baseline-recurrence isolates. No significant differences in responses between baseline and recurrence samples except a significant lower value in the isolates of recurrent infections towards DHA (p = 0.02).

6. Discussion

The continued and uncontrolled use of monotherapies against malaria parasites has led to widespread reduced susceptibility of almost all known antimalarials. The introduction of combination therapies was hoped to prevent or at least delay the emergence of reduced susceptibility to the fastest acting antimalarial drugs these days, artemisinin derivatives. Drug surveillance studies monitor drug efficacy and effectiveness to be able to detect and react to declining responsiveness of malaria parasites as fast as possible.

This chapter will first discuss the *in vivo* parameters and their limitations before comparing results obtained here with previous data. Afterwards *ex vivo* drug susceptibility testing and its general limitations will be debated. The results gathered here will be also compared to previous findings. While completing this work an important study was published reporting a correlation between *in vivo* parasite clearance and *in vitro* artemisinin susceptibility using an assay called RSA^{0-3} (Witkowski, B. et al. 2013a). The HRP2 ELISA is accordingly not the correct tool to identify artemisinin resistance. Nevertheless does, considering the results from Borrmann, S. et al. (2013), the approach via the HRP2 assay allow identification of artemisinin drug susceptibility phenotypes. In consideration of the recent developments regarding artemisinin drug tolerance the role of the results and the concluded need for further surveillance will be underlined.

6.1 Artemisinin drug susceptibility *in vivo*

Parameters such as parasite **clearance time, parasite reduction ratio and parasite prevalence rate** can indicate the effectiveness of a drug applied *in vivo* by providing values to compare how fast parasites fall under the microscopic threshold after initiation of treatment.

In 2009, the first artemisinin-resistant malaria episodes were reported in Cambodia (Dondorp, A. M. et al. 2009) with a prolonged mean parasite clearance time of 84 hours in Pailin, Cambodia and of 50 hours in Wang Pha, Thailand. Following that the longitudinal study by Borrmann, S. et al. (2011) in the coast of Kenya showed a rise in mean PCT from 37.8 (2005 – 2006) to 46.0 (2007 – 2008) hours after treatment with DHA-PPQ and from 45.0 (2005 – 2006) to 49.8 (2007 – 2008) hours after treatment with ART-LUM. Here, after treatment with the single drug AS at a dose of 2 mg / kg body weight, the mean PCT was 30.5 hours at the same study site.

The PCT is mostly determined by microscopic evaluations of blood smears and therefore, limited by the microscopically detectable parasite biomass, ($\leq 10^8$ parasites). A patient might be declared parasite-free when actually parasitaemia was just reduced below that threshold. In this case the efficacy of the drug would be overestimated, leading to wrong implications for further drug usage. The determination of parasitaemia in the field using an average leukocyte count of 8,000 / µl (refer to **4.2.3**) leads to inaccuracy. Especially in children the leukocyte counts can vary within a wide range, often depending on the age. Here the white blood cell count had a median of about 8,600 / µl (range 2,900 – 21,700 / µl). The average leukocyte count of 8,000 / µl as an overall value was, thus, close to the median, but an underestimation of parasitaemia in patients with higher leukocytes and overestimation in the ones with lower white blood cell count have to be considered and the possibility of inaccurate clearance and reduction ratios kept in mind. Besides microscopic threshold and counting errors, the PCT has been shown to be influenced by factors such as initial parasitaemia, the stage of development of the parasite, the frequency of sampling and host defence (immunity and, therefore, also the age of the patient in high transmission areas) (White, N. J. 2011). Also do different drugs act at different stages during the parasite life cycle (refer to **2.5**). A drug that is not exactly administered at the point of highest vulnerability of the parasite might suggest a PCT and PRR that are prolonged or reduced. This might also lead to falsely drawn conclusions regarding drug efficacy.

Parasite clearance is regarded as a first-order-process; higher initial parasitaemia, consequently, lead to longer clearance times. The PCT did show a correlation with the initial parasitaemia (**Fig. 11,** $p < 0.0001$). About half of the patients (52 %) had a parasitaemia at enrolment below 10^5 parasites / µl, the other half (48 %) above. For the first group the PCT was 30 hours, for the second 36 hours ($p = 0.001$). Comparing the results of this study with the previous study by Borrmann, S. et al. (2011) CATMAP enrolled patients with initial asexual parasitaemia between 10,000 – 300,000 / µl; the previous study included patients with lower parasitaemia (2,000 – 200,000 / µl).

Median asexual *P. falciparum* density was lower in the last study (34,600 – 57,600 parasites / µl); here the median parasitaemia was 95,200 parasites / µl. Therefore, in comparison to PCTs of 38 and 50 hours in 2005 – 2008, for now no further indication for a decline of responsiveness *in vivo* to artemisinin could be found.

Another confounder often mentioned is the immunity of the patient against malaria acquired in high transmission areas. The age group most at risk for severe infections are children up to 5 years of age. After that, they are considered to have built up a level of immunity. Hence, age can be used as an indicator for immunity. However, no correlation was seen between the patients' age and the PCT in this study. It is possible that due to a general decline in malaria transmission in the area (O´Meara, W. P. et al. 2008) the level of immunity has decreased, thus, not showing a correlation here.

The parasite prevalence rate on the first day after initiation of treatment (PPR_{D1}) with an ACT is mainly determined by the artemisinin partner (White, N.J. 2004). From 2005 – 2006 to 2007 – 2008, an increase in the PPR_{D1} from 55 % to 87 % in patients receiving treatment with DHA-PPQ and from 81 % to 95 % in the AL group was noted, meaning that after initiation of treatment with an ACT the number of patients with remaining parasitaemia 24 hours later rose in both study arms. The hypothesis drawn from this drop in early treatment response was a declined sensitivity to the artemisinin-derivate as the fast acting

component of the ACT. Comparison of the PPR_{D1} of 70 % (117 / 168) as observed after monotherapy with AS in CATMAP shows a faster clinical response than to ART-LUM. The PPR_{D1} here also places itself in between the 2005 – 2006 and 2007 – 2008 recorded values for DHA-PPQ. This again did not raise further concerns of overall reduced drug susceptibility to artemisinins at the time CATMAP was carried out. Moreover, when comparing the parameter for patients that still had detectable parasitaemia on the second day after initiation of treatment (PPR_{D2}), it was much lower (2 %) than the 4 % (DHA-PPQ) / 7 % (ART-LUM) in 2005 / 2006 and the 5 % (DHA-PPQ) / 13 % (ART-LUM) in 2007 / 2008 in the same study area.

Another parameter, the parasite reduction ratio is defined as the factor by which parasitaemia declines over time and is, therefore, describing how fast the antimalarial agent reduces parasite burden. Median parasite reduction ratio on the first day (PRR_{H24}) for DHA-PPQ fell from 2.5 to 1.8 and for AM-LUM from 1.9 to 1.4 in the previous study in Kenya- thus, resembling the observed incline in detectable parasitaemia on Day 1. Here the median PRR_{H24} was fairly high with 2.5. Parasite reduction rates increased quickly within 6 – 12 hours after each treatment dose, whereas the parasite reduction rate rose from 0.03 / hour to 0.14 / hour between hour 6 and 12. After the second treatment dose this reduction rate increased even further within the same time span (from 0.08 / hour to 0.55 / hour). Also this clinical parameter still indicates a high susceptibility to Artemisinins with rapid onset of parasite reduction.

Due to multiple potential confounders of the PCT, a new method, the PCE, was proposed (Flegg, J. A. et al. 2011). In summary, there is only one segment in the parasite clearance curve which is robust against the above mentioned confounder. The PCE excludes the two segments that are easily influenced, the lag phase and the tail, and also identifies outliers. From the robust segment the parasite clearance values are calculated. Here, for wider comparison with the results from different studies from regions in artemisinin resistant areas as well as the study by Borrmann, S. et al. (2011) which used different methods, PCE and PCT values were shown.

As stated before, the PCT did indeed show a significant correlation with the initial parasitaemia, but not with the age of the patient. The PCE values however, specifically the clearance rate constant and the slope half life showed no significant correlation with these variables, suggesting them to be indeed more independent values for discussing drug sensitivity.
The mean slope half life of 2.37 hours indicated no overall reduced susceptibility compared to other studies where AS-monotherapy was given at the same dose– mean of half life in Pursat (artemisinin resistant area) 5.85 hours, in Wang Pha (artemisinin responsive area) 2.16 hours (Amaratunga, C. et al. 2012), mean half life in western Thailand 3.7 hours (Phyo, A. P. et al. 2012)– or with even higher doses (4 mg / kg body weight)– in Mali mean half life 1.9 hours (Lopera-Mesa, T. M. et al. 2013). PC90 in the artemisinin resistant parasites in Pailin (Dondorp, A. M. et al. 2009) was as high as 23 hours, in Wang Pha on the other hand 12 hours.
In Coastal Kenya an estimated PC90 of 11.18 hours, therefore, also does not indicate slowed parasite clearance. The median time of the lag phase was 11.93 hours which confirms above mentioned drastic rise in PRR between hour 6 and 12. **Fig. 10** visualizes this lag phase by the first linear part of the clearance curve (green dots) between hour 0 and 6 before parasite biomass reduces.

As one last clinical aspect, fever clearance happened within about 24 hours, which is slightly faster than in the previous study in Kilifi (27 – 30 hours).

Borrmann, S. et al. (2011) already suggested that the drop reported in clinical response might not be due to less sensitivity to the artemisinins, but due to a change in transmission (O´Meara, W. P. et al. 2008). Less transmission results in lower immunity of the children, leading to higher parasitaemia at admission and in conclusion to prolonged clinical clearance times. The initial parasitaemia in this study was raised compared to initial parasitaemia from the last study in the same study area, hinting towards a further decline in immunity against *P. falciparum* infections. Parasite clearance and reduction ratios though showed high susceptibility. Describing *in vivo* observations there are variables within the

patient that might have an impact on clinical outcomes (Amaratunga, C. et al. 2012). Consequently, changes in values as PCT and PPR cannot singularly be assumed to be a result of changes in drug response. Nevertheless, the larger impact comes from the genetics of the parasite (Anderson, T. J. C. et al. 2010), still making the description of these parameters a useful tool to monitor drug efficacy *in vivo*. The PCE provides a robust tool to uniform *in vivo* surveillance, which cannot be replaced by *ex vivo* and *in vitro* tests but should complement each other.

Altogether high susceptibility to artemisinins *in vivo* in the coast of Kenya can still be assumed. Looking at individual patients though, there was one patient in particular to point out: This patient still had parasitaemia on the fourth day after initiation of treatment Pharmacokinetic analysis revealed a lower level of drug exposure in the patient's blood (personal communication with Prof. Dr. S. Borrmann). This could have been the main reason for not responding adequately to the treatment and, thus, not meeting the WHO criteria for drug resistance (refer to **3**).

Recurrent parasitaemia can occur due to recrudescence of the same parasite or reinfection with a different strain. To distinguish between these two cases, PCR-based techniques and genotyping of baseline and recurrence samples are undertaken. For instance, high polymorphism has been shown for *msp1*, *msp2* and *glurp* genes (Babiker, H. A. et al. 1997; Felger, I. et al. 1999; Snounou, G. et al. 1999; Magesa, S. M. et al. 2001; Aubouy, A. et al. 2003) which, therefore, have been proposed as means by which one could distinguish between recrudescence and newly acquired infection (Kain, K. C. et al. 1996).
Even with optimal experimental conditions there are sources for mistakes though. First, the allele diversity of genes in a parasite population is high, but not indefinite.

Second, in patients with multiplicity of infection, the PCR-bias[18] might lead to non-detection of a recrudescence. The possibility of being reinfected with a strain possessing an identical genotype has been reported as low, but not impossible (Snounou, G. and Beck, H. P. 1998)– thus, imposing a bias in the conclusions drawn.

Here a total of 56 patients presented with recurrent parasitaemia during the follow-up. Out of these eight patients (5 %) had PCR confirmed recrudescence, five (3 %) within 28 days. The null-hypothesis stated for the CATMAP study was a reinfection adjusted failure rate of ≤ 5 % within 28 days after artesunate monotherapy. With 3 % recrudescences within 28 days the null hypothesis cannot be proven false, but undermines importance of further surveillance.

Pharmacokinetic tests carried out in the CATMAP study are unpublished to this date but will reveal further information on whether sufficient drug levels were reached in all these patients with recrudescent parasitaemia.

6.2 Drug susceptibility testing *ex vivo*

In support of monitoring *in vivo* parameters, drug sensitivity testing is also carried out *ex vivo* or *in vitro*. E*x vivo* tests are performed on parasites which were freshly obtained from the patient. Any preservation in the laboratory might lead to an alteration in the genome expression of the parasite, as shown for genes related to virulence (Peters, J. M. et al. 2007; Zhang, Q. et al. 2011; Fowler, E. V. et al. 2006) and possibly also to alteration in drug responses or to selection of certain parasites (Brockelman, C. R. et al. 1981; Le Bras, J. et al. 1983, Chaorattanakawee, S. et al. 2015).

[18] Alleles might be different in length. The time that the enzymes need to amplify the shorter allele is consequently reduced compared to a longer allele. While the reaction is running, there is more time to amplify the short allele and that one will overrun the longer one in quantity.

Here, to determine *ex vivo* drug susceptibility the established HRP2 ELISA was chosen. It determines growth via measurement of HRP2, a stable protein that is produced throughout all stages in the life cycle of *P. falciparum*, making it a reliable marker for multiplication. The culture conditions were controlled and all the samples were tested *ex vivo*. That means only ring-stage parasites were used without prior culturing or manipulation by sorbitol treatment for synchronization. The increase of HRP2 was determined per single column between concentration in the well with the highest drug concentration (full inhibition, in this case wells in row H) and the control, which was calculated automatically as the geometric mean in the well without drug and the well with the lowest drug concentration (full growth, in this case row A and B). Therefore, each parasite strain was functioning as its own control. Parasite growth was given out in ranges. All the drugs were run in duplicates for verification of a measured inhibition (intra-sample correlation as a measure for reproducibility). Results were checked for adequate parasite growth on each culture plate and excluded if not fitting the needed criteria.

Considering limitations of the assay, it is first of all possible that a patient can be infected with more than one parasite strain. Without genotyping one cannot exclude that a patient had multiplicities of infection at the time the sample was taken. An example of the effect on the performed assay would be a sample with one isolate with reduced and a second isolate with high drug susceptibility. If drug resistance is accompanied by a loss of fitness, it is possible that the resistant strain does not grow under the culture conditions as effortlessly as the sensitive one. If this is the case, the inhibitory assay would mainly show the IC50 of the sensitive strain and the resistant one could possibly not be detected. Consequently, the conclusion would be a good susceptibility to the drug leading to wrong implications in treatment policies.
Another limitation is the stage-specific action of antimalarial drugs. All drugs used here are acting against the asexual blood-stages of *Plasmodium*. There are differences in the sensitivity of the parasite to the drug applied in the course of its life cycle (Geary, T. G. et al. 1989; Wilson, D. W. et al. 2013) and,

therefore, the timing of the onset of drug exposure can lead to alterations in the outcome of the assay. The samples were collected and stored cool until transport to the laboratory. Nevertheless, the time until exposure to the drug could have varied. A sample that was processed quicker and where the set-up of the drug exposure plate happened during the early ring-stage might, thus, possibly show different drug responses than one of which processing took longer and was set up in the late ring stage.

Regarding the performance of the assay an important limitation was the absence of a reference. In the set-up and the performances of the sensitivity tests there might be inter-individual variances. Additionally, the preparation and dilution of the drugs could include pipetting errors– leading to a slightly divergent drug concentration than proposed does exist. A culture adapted reference strain (for example 3D7 or W2) which is known to be sensitive to all monitored drugs should be tested in each run to standardize the drugs and function as a reference for sensitivity. That way results will be easier and more reliable to interpret rather than just determining the absolute value (Rutsivisuttinunt, W. et al. 2012).

Despite these general limitations a high quality of assays could be achieved here. A median intra-sample correlation between the duplicates of the tested drugs of 0.94 (range 0.91 – 0.98) indicated high reproducibility of the results. The scatter plot of the two artemisinins DHA and AS (**Fig. 18)** and the correlation matrix (**Tab. 5**) show how well the results for the two artemisinin compounds correlate ($p < 0.0001$). In a well performed assay, a parasite that is less susceptive against AS should show a higher tolerance to exposure to DHA just as it was seen here. The coefficient of determination of $r^2 = 0.58$ even rose up to 0.61 after excluding the two drug solutions with suspicious results. 58 % (61 %) of the differences in the response to DHA can, therefore, be related to differences in the response to AS. This result is comparable to a report by Pascual, A. et al. (with $r^2 = 0.68$) and similar to correlations found by Borrmann, S. et al. (2013). All together this supports the overall quality of the *ex vivo* results displayed here.

Specific potential confounders in the analysis concern the use of seven different working solutions. Due to known decreasing efficacy of some drugs over time and the fluctuation in enrolment of patients it was not possible to prepare one dilution of each drug and use it for all samples. Differences in values determined can be due to multiple reasons such as unequal distribution of sensitive and less sensitive strains between drug solutions, time of drug storage, inter-individual differences in sample processing etc. The artemisinins specifically showed a correlation between the IC50 values with the time of drug storage. When marking the plot of IC50 values for each drug solution against time (**Fig. 45** + **Fig. 46)** in different colours it became obvious that most of the higher IC50 values were obtained from one drug solution that was stored over a longer period of time (up to 7.5 times longer, 141 % – 750 % of the average storage time), making it very possible that the age of the solution lead to a decrease in the efficacy of the drug. Statistically this correlation between IC50 value obtained and the time of drug storage was highly significant ($p < 0.0001$). The ratio between IC50 values for DHA and AS (**Fig. 47**) indicated similar degradation of the two drugs over time.

Accordingly it is correct to question the reproducibility of the results obtained when using freshly prepared drug solutions. This assay was set up from *ex vivo* samples, therefore, the only way to test these isolates again is by culturing and repeating the drug sensitivity testing *in vitro*. This, on the other hand, would be accompanied by culture adaptation and a restriction in comparison with the other values. Additionally, the display of results from each drug solution (refer to **5.2.1**) did show a deviation in the distribution of values for AS and DHA in the seventh drug solution (**Fig. 33** and **Fig. 34**). This is not in accordance with the high correlation between the IC50´s for artemisinins in general and gave rise to analyse also drug solution VII further in order to include all possible confounders. In this case the ratio between the measured IC50 for AS and DHA (**Fig. 47**) confirmed the deviation between AS and DHA for solution VII (almost all values far above the upper level of the 95 % CI of the median). Due to the wide distribution of ratios it can be assumed that the cause of this was not in the drug solution itself but rather in the set-up of the individual assays.

An analysis excluding these suspicious results (refer to **5.2.3**) however did not reveal a significant influence of these confounders on the overall results. The median IC50 of DHA did change from 4.52 nM (geometric mean 4.84 nM, 95 % CI 4.10 – 5.70 nM) to 4.17 nM (geometric mean 4.04 nM, 95 % CI 3.41 – 4.71 nM) ($p = 0.23$) and for AS from 6.59 nM (geometric mean 5.82 nM, 95 % CI 4.77 – 7.12 nM) to 6.26 nM (geometric mean 5.89 nM, 95 % CI 5.06 – 6.86 nM) ($p = 0.72$) when excluding both drug solutions. When only excluding the sixth solution with a definite reason for degradation, AS differed slightly significant ($p = 0.04$) and DHA showed the trend to significance ($p = 0.08$). All together this does imply a trend of higher IC50 values over time of drug storage and the high importance of controlling for this confounder.

An overall slightly higher value for AS could possibly be explained by hydrolyzation of AS into DHA, the active metabolite, and succinic acid in aqueous solutions (Rutvisuttinunt, W. et al. 2012), whereas DHA already is an active metabolite.
When testing isolates from patients with recurrent parasitaemia, drug selection did not lead to elevated IC50 values (**Fig. 49**).

In comparison with previous studies in the same study population (Borrmann, S. et al. 2011 and 2013), the IC50 for DHA now is statistically significantly higher ($p < 0.001$) but remains on a low level. Another study by Borrmann, S. et al. (2013) showed a median IC50 of 2 nM (range 0.5 – 4 nM) for DHA. In these studies the ^{3}H-hypoxanthine uptake assay (Desjardins, R. E. et al. 1979) was used to test drug susceptibility. Thus, it can be argued, that the difference is caused by inter-test variability. The HRP2 ELISA though has been shown to produce similar results when compared directly to other assays, including the isotopic method (Noedl, H. 2002a).
New developments in the research field of artemisinin resistance also have to be taken into account. Though, considering recent reports as Witkowski, B. et al. (2013a), the results obtained here are not qualified to detect artemisinin resistance, they do show the various phenotypes of artemisinin drug responses in *P. falciparum* in Coastal Kenya. **Fig. 17** displays the variety in artemisinin

susceptibility in all isolates. Different phenotypes like these have been connected to SNPs by Borrmann et al. 2013, including SNPs on chromosome 13. This is of particular interest since chromosome 13 has been discussed before in the context of artemisinin susceptibility (Cheeseman, I. H. et al. 2012) and remains a candidate of high attentiveness (Ariey, F. et al. 2014; Mbengue, A. et al. 2015, Straimer, J. et al. 2015, Mita, T. et al. 2016). Since artemisinin susceptibility phenotypes have been correlated in Kenya with chromosome 13 despite no reports of artemisinin resistance in this area it is crucial to carry out further drug surveillance and to investigate the importance of already existing molecular markers and possibly even identify others. Borrmann, S. et al. (2013) argued, that the molecular markers might not show signatures of recent positive selection since by that time artemisinins were only introduced 2 – 3 years before the sampling started. The development of this finding has to be followed over time of artemisinin usage and connected with clinical treatment responses. Equally important is the RSA testing in the area to investigate a correlation with parasite clearance in this area and identify its importance in further surveillance in Kenya.

Apart from artemisinin susceptibility it is of interest, since treatment guidelines nowadays abandon the use of monotherapies in *P. falciparum* malaria, to take a closer look at the drug susceptibility to the ACT partner drugs. The combination of AL is the first line-treatment for malaria in Kenya, DHA-PPQ the second line-treatment (Ministry of Public Health and Sanitation, National Guidelines for the Diagnosis, Treatment and Prevention of Malaria in Kenya, 2010). Median IC50 values for LUM were 13.71 nM (geometric mean 12.10 nM, 95 % CI 10.31 – 14.20 nM) and for PPQ 7.88 nM (geometric mean 8.03 nM, 95 % CI 6.91 – 9.34 nM). Both did not show any indication for a decrease in efficacy *ex vivo* since the last studies (Borrmann, S. et al. 2011 and 2013– where LUM IC 50 median was 42.04 nM and 69.67 nM (2011) as well as 17 nM (2013), PPQ 27.83 nM and 19.67 nM (2011) as well as 49 nM (2013)). Both drugs can, consequently, as far as *ex vivo* data is concerned, be considered a safe choice as partner in artemisinin combination therapy.

A correlation matrix was calculated in order to check for hints of cross-resistances between drugs. The observed patterns were consistent with previously reported findings (Wrongsrichanalai, C. et al. 2002; Sidhu, A. B. et al. 2006; Borrmann, S. et al. 2013).

The artemisinins did show significant correlations with LUM, PPQ and MFQ ($p < 0.0001$). For PPQ and LUM, the coefficients of determinations were small, ranging from $r^2 = 0.01$ to 0.03. *In vitro* responses between PPQ and artemisinin drugs have been reported indifferent or antagonistic (Davis, T. M. E. et al. 2006; Fivelman, Q. L. et al. 2007), but proven safe and efficient for the treatment of malaria in Africa (Bassat, Q. et al. 2009; Nambozi, M. et al. 2011; Adam, I. et al. 2010), South America (Grande, T. et al. 2007) and Asia (Valecha, N. et al. 2010). Looking at this analysis in more detail (see Appendix 3), PPQ actually did show higher r^2 (up to 0.5) with DHA. Since this is an effect produced *ex vivo*, with very few isolates as well, and clinically the drug combination has been proven safe and efficient the relevance for this effect is unclear. Additionally, one of these solutions was the solution shown to be influenced by time of drug storage. The result should, therefore, be verified.

Also LUM did show such a high coefficient of determination with the artemisinins (up to 0.69, refer to Appendix 3) in single drug solutions, making PPQ not inferior to LUM.

One highly interesting finding related to the corrected analysis was the correlation between the artemisinins and MFQ. Before these drugs did correlate (rho = 0.49 and 0.44 for MFQ and DHA and AS, respectively) but with fairly low coefficients of determination ($r^2 = 0.04$ and 0.10 for MFQ and DHA and AS, respectively). In the corrected analysis the correlation became more obvious by rho = 0.63 for each drug. Coefficients of determination rose up to $r^2 = 0.41$ and 0.36 for MFQ and DHA and AS, respectively. This correlation was also reported by Borrmann, S. et al. (2013). Considering the fact that earlier reports investigated *in vitro* drug responses in association with *pfmdr1* gene amplification (Sidhu, A. B. et al. 2006) and found that not only MFQ drug susceptibility was correlated inversely with *pfmdr1* copy numbers but also other drug responses– including the drug response to artemisinin–

this finding is alarming. Price, R. N. et al. (2004) had before presented *pfmdr1* amplification as the cause for MFQ resistance *in vitro* and *in vivo.* This was also correlated with decreased artemisinin susceptibility. These findings all indicate that the found *in vitro* and *ex vivo* correlation of MFQ and artemisinins could be of clinical importance (Noedl, H et al. 2001).

Though *pfmdr1* has an importance in South East Asia, it used to be rare in the African region. But lately a rise in *pfmdr1* copy numbers has been reported after the introduction of ACTs in Kenya (Gadalla, N. B. et al. 2012; Duah, N. et al. 2013; Ngalah, B. S. et al. 2015) underlining how rapidly and suddenly changes in the genome of *P. falciparum* occur. This, together with the findings of SNPs on chromosome 13 by Borrmann et al. (2013) is significant despite– or maybe especially because of– the absence of reports of artemisinin resistance in Kenya so far since molecular markers for the track of resistance are the most useful when established before the onset of resistance. Only then it can be detected as fast as possible and its spread actually be prevented.

Looking at possible cross-resistances between the partner drugs LUM and PPQ and other drugs, especially CQ with worldwide resistance, neither PPQ nor LUM showed alarming results. Former reports of cross-resistance between CQ and PPQ from China (Fan, B. et al. 1998; Yang, H. et al. 1999) could not be reproduced in African isolates from Cameroon (Basco, L. K. and Ringwald, P. 2003). It has been proposed by Mwai, L. et al. (2009) that sensitivity to CQ has increased again since its withdrawal in Kenya, proposing a cut-off point for CQ-resistance at 25 nM. Here an overall median inhibitory concentration of 15.63 nM, geometric mean 21.15 nM can be reported. Inhibitory tests from 2005 – 2008 showed a median of 36.8 nM and mean 33.64 nM towards CQ. This supports the findings by Mwai, L. et al (2009). In accordance with the results from Cameroon, also in this analysis here does PPQ not show decreased sensitivity in isolates resistant to CQ in Kenya. For example, highly CQ-resistant strains IC50 values of 124.29 nM, 186.49 nM, 421.74 nM, 422.05 nM did show high sensitivity to PPQ with IC50s of 12.04 nM, 9.14 nM, 11.21 nM and 11.41 nM, respectively.

Interestingly, LUM even showed a negative correlation with CQ– indicating that isolates resistant to CQ might be even more susceptible to LUM. MFQ was included in the drug testing due to its continued role in treatment and prevention of malaria. Median and mean values of these two drugs were very similar here (median LUM 13.71 nM, mean 12.10 nM; median MFQ 12.59 nM, mean 11.76 nM). MFQ and LUM are both aryl-amino-alcohols and share the same basic characteristic– a hydroxyl group near the ring in their structure that has been hypothesized to be the key structure in the antimalarial activity of synthetic amino-alcohols (Chien, P. L. and Cheng, C. C 1976; Karle, J. M. and Karle, I. L 1991; Basco, L. K. and Le Bras, J. 1993). The similar structure might explain the highly positive correlation ($rho = 0.62$, $p < 0.0001$) and the similar IC50 values.

6.3 Clinical implications and the need for further drug surveillance

In 2005 the WHO published an implementation guide for changing malaria treatment to artemisinin-based combinations (Rational Pharmaceutical Management Plus 2005, Center for Pharmaceutical Management 2005). To prevent or delay the emergence of resistance against artemisinin derivatives they have been combined with partner drugs with different mechanisms of action and a longer half-life.

Regarding clinical aspects, malaria treatment needs to be prompt and effective. One practical aspect regarding the complexity of this is access to treatment. Since AL was adapted as first-line treatment of uncomplicated *Plasmodium falciparum* malaria in Kenya there have been reports of stock-outs and inaccessibility (Sudoi, R. K. et al. 2012). This is often leads to the use of monotherapies in malaria, which should be avoided in order to prevent drug-resistance. Another study in two districts in Kenya showed, from a perspective of wanting to prevent drug resistance, disastrous findings. Just about 60 % of the patients were adherent to the AL. Besides knowledge of the dosing regimen the amount of tablets was criticized (Lawford, H. et al. 2011). AL has to be taken twice a day for 3 days in a row under a strict dosing-regimen

(at hours 0, 8, 24, 36, 48 and 60). Depending on the age and weight of the patient these doses can consist of a total of 6 – 24 tablets (Ministry of Public Health and Sanitation, National Guidelines for the Diagnosis, Treatment and Prevention of Malaria, 2010). This amount of tablets and the strict regimen for dosing can easily lead to non-adherence and in the long run imposes a threat on drug-susceptibility in malaria parasites. The rationale for combining two drugs with different modes of actions and accordingly different mechanisms of drug resistance was explained in detail by Nosten and White (Nosten, F. and White, N. J. 2007). In combination therapy the artemisinin-derivative is eliminated quickly from the patient, always being protected by the partner drug against possible resistant parasites. But when the artemisinin derivative is not present anymore, the partner drug with a longer half-life is slowly reduced in the concentration in the blood of the patient. First this might work as prevention against reinfection, but when the drug concentration falls below a certain threshold there is a time-span in which the drug is concentrated too low to kill parasites due to a new infection. This would lead to parasites being exposed to low concentrations of the drugan a raise in possibility of creating resistant parasites. That is a disadvantage of drugs with longer elimination half-time. Therefore, not only drug susceptibility to the fast-acting drug artemisinin need to be under surveillance, but also the partner drugs.

The main partner drugs of ACT-regimens used in Kenya, LUM and PPQ, both showed high *ex vivo* efficacy. Until new antimalarials are discovered, working with what we have at the most efficient level is crucial, which includes avoidance of monotherapies. Since no inferiority of DHA-PPQ to AL was shown in other studies and data from 2005 - 2008 did even show better clearance data after treatment with DHA-PPQ, the question arises if DHA-PPQ should possibly gain more clinical importance in Kenya (so far it is only second-line treatment). A Cochrane review from 2009 comparing the ACTs stated the overall well performance of DHA-PPQ when looking at (PCR adjusted) cure rates, serious adverse events, gametocyte-development, anaemia and vomiting (Sinclair, D. et al. 2009). The treatment with DHA-PPQ even showed a significantly lower rate

of new infections over the 42-day follow up period, which demonstrates the good post-treatment prophylactic effect of PPQ due to its long elimination half time (Bassat, Q. et al. 2009). Several clinical studies have reported non-inferiority of DHA-PPQ compared to AL in multiple African countries such as Zambia (Nambozi, M. et al. 2011), Uganda (Yeka, A. et al. 2008) in a multicentre-trial carried out in Cameroon, Côte d´Ivoire and Senegal (Yavo, W. et al. 2011) and in Kenya (Bassat, Q. et al. 2009).

This study has presented data which overall did not confirm a decreased *in vivo* responsiveness towards artemisinins in the area as reported before. *Ex vivo* drug responses identified different artemisinin susceptibility phenotypes and indicated cross-resistances with so far mainly unclear clinical importance. Especially MFQ could possibly impact artemisinin susceptibility in high transmission areas (Noedl, H. et al. 2001), and the shown correlation in this analysis together with previous works underlines the necessity for surveillance of drug usage and susceptibility. Recent studies showed much progress regarding drug susceptibility surveillance via the RSA (Witkowski, B. et al. 2013a and b). This assay also needs to be implemented for surveillance in Africa. Molecular markers for resistance like the K13-propeller polymorphism and *pfmdr1* have to be under observation and / or other markers for the African region have to be established.

Apart from these treatment considerations, the main and most important conclusion drawn from this work though has to be the need for further continuous drug surveillance. Both aspects, *in vivo* as well as *ex vivo* / *in vitro* susceptibility have to be monitored to detect any changes in parasite drug susceptibility in appropriate time. At the beginning of this work it was stated that the first onset of upcoming resistance is often overlooked by clinicians since patients first appear to improve clinically. Fever clearance remains quick and patients are considered to be cured. In the case of detection of recurrent parasitaemia within 28 days after a malaria treatment though, clinicians have to be alarmed. Also during the initial treatment parameters such as parasite reduction ratios can easily be calculated in order to check whether parasites

clear the patients´ blood as expected or if treatment response is inadequate (e.g. no adequate parasite reduction after 12 hours, since PRR here increased highly per hour between hour 6 and 12). Besides the attention of each clinician treating malaria patients, there is a high need for sentinel sites across Africa in order to monitor drug susceptibility continuously. The major public health importance of malaria explained in the beginning of this work in addition to the history of previous emergence of antimalarial resistance justifies and requires continuous supervision of antimalarial treatment. The responsibility lies with national as well as international facilities. Sentinel sites are usually able to obtain data of higher quality and in more detail, but each health facility should feel responsible to monitor drug susceptibility, inform patients on the importance of therapy adherence and report suspicious events. Drug efficiency has to be transparent, for example through an open database. In addition to selected sentinel sites at areas of high transmission and with many malaria cases, studies like CATMAP with preferably a standard protocol at different study sites can function as additional support to the continuous drug surveillance. They can however not replace it since changes might be detected too late and resistance spreads fast once established.

All together it can be said that artemisinins for now remain highly effective against *P. falciparum* malaria in Coastal Kenya. *In vivo* data suggested fast overall parasite clearance after treatment with artesunate. *Ex vivo* drug susceptibility tests identified different phenotypes but need to be investigated further, e.g. correlation with molecular markers and RSA testing. A high need for continuous drug surveillance in Kenya as well as the whole African region is given due to the enormous public health importance of malaria, the spontaneous occurrence of drug resistance and its fast spread.

7. Summary

7.1 English

Background: Malaria remains one of the major causes of morbidity and mortality worldwide. Artemisinin-based combination therapies are used as first-line treatment in all endemic countries for uncomplicated *P. falciparum* malaria. artemether-lumefantrine was introduced as first-line treatment in Kenya in 2006. Since the first report of artemisinin-resistant *P. falciparum* malaria in 2009 on the Thai-Cambodian border, concerns about declining responsiveness to artemisinins have also been expressed in Kenya, stressing the need for continued drug-surveillance studies.

Methods: In a single-armed trial children between the ages of 6 months and 10 years with microscopically detected *P. falciparum* malaria were treated with a 7-day artesunate monotherapy regimen (2 mg / kg per body weight per day as a single dose) and followed up for 42 days. Data on parasite clearance parameters was used in order to either substantiate or refute the previously reported decline in artemisinin responses. Additionally, susceptibility to lumefantrine and piperaquine, the two most commonly prescribed artemisinin-based combination therapy partner drugs in Kenya, was tested *ex vivo* via Histidine-rich protein 2 Enzyme-linked-immunosorbent-assay. To distinguish between reinfection and recrudescence, Merozoite-surface-protein 2 - genotyping was carried out.

Results: 175 patients were recruited between April and December 2011. Mean overall parasite clearance times were 30.5 hours and median slope half life was 2.4 hours, with a median clearance rate constant of 0.29 / hour. The highest reduction in parasites took place between hour 6 and hour 12 after initiation of treatment (parasite reduction ratio increased from 0.03 / hour to 0.15 / hour). *Ex vivo* drug susceptibility showed geometric means of 3.8 nM and 4.2 nM for dihydroartemisinin and artesunate, respectively.

Geometric mean IC50 for lumefantrine and piperaquine were 12.1 nM and 8.0 nM, respectively. An indication for cross-resistance between artemisinins and mefloquine was found (r^2 = 0.41 and 0.38 $p < 0.0001$ for each value). Out of a total of 56 (33 %) total recurrent parasitaemia cases, 32 (19 %) happened within 28 days after initiation of treatment. Eight patients had PCR-confirmed recrudescence (5 %), 5 of those (3 %) within 28 days. Tested recurrence samples did almost exceptionally not differ significantly from baseline values regarding their IC50 ($0.02 \leq p \geq 0.96$).

Conclusion: For now overall artemisinin susceptibility in Kenya remains high, clinically as well as when testing *ex vivo*. In order to prevent resistance and to respond adequately in the case of it, new drugs and combinations have to be studied. PPQ proved to be highly efficient *ex vivo.* This suggests a high importance as alternative to the first-line combination therapy in Kenya. All together it is inevitable to further continue close drug surveillance. High drug pressure is present due to the frequent use of artemether-lumefantrine in Kenya and mutations that could affect drug susceptibility arise spontaneously. By closely monitoring drug effectiveness, a decline in response of *P. falciparum* towards artemisinins can be detected and contained early.

7.2 German

Hintergrund: Malaria stellt weltweit noch immer eine wesentliche Ursache für Morbidität und Mortalität dar. Kombinationstherapien mit Artemisinin-Derivaten sind seit einigen Jahren in allen endemischen Gebieten als Goldstandard etabliert. Artemether-Lumefantrin bildet in Kenia seit 2006 den Grundstein der Therapie. Seit dem ersten Report über Artemisinin-resistente *P. falciparum* Malaria an der thailändisch-kambodschanischen Grenze im Jahr 2009 wird auch aus Kenia über abnehmende Sensibilität gegenüber Artemisinin berichtet. Daraus resultiert die Dringlichkeit weiterer Studien zur Medikamentensensitvität von *P. falciparum* in diesem Land.

Methoden: In einer einarmigen Studie wurden im Kinder zwischen 6 Monaten und 10 Jahren mit mikroskopisch verifizierter *P. falciparum* Malaria mit einer 7-tägigen Artesunat Monotherapie (2 mg / kg Körpergewicht) behandelt und über einen 42-tägigen Zeitraum beobachtet. Parasiten Eliminations-Parameter und *ex vivo* Medikamentensensitivität dienten als Indikatoren um eine zuvor publizierte abnehmende Wirksamkeit von Artemisinin in Kenia zu verifizieren oder zu widerlegen. Die *ex vivo* Medikamentensensitivität wurde anhand eines antikörperbasierten Nachweisverfahrens von Histidin-reichem-Protein 2 bestimmt. Zusätzlich wurden die *ex vivo* Sensitivitäten von Lumefantrin und Piperaquin, den beiden am häufgsten genutzten Kombinationspartnern der Artemisinine, getestet. Um zwischen einer Rekrudeszenz und einer Reinfektion zu unterscheiden, wurde eine Genotypisierung anhand des Merozoiten-Oberflächen-Proteins 2 durchgeführt.

Resultate: 175 Patienten wurden zwischen April und Dezember 2011 rekrutiert. Die mittlere Parasiten Eliminations-Zeit betrug 30,5 Stunden, mediane Steigungs-Halbzeit 2,37 Stunden. Die höchste Parasitenreduktion fand zwischen 6 und 12 Stunden nach der ersten Medikamenteneinnahme statt (Anstieg der Rate von 0.03 / Stunde auf 0.15 / Stunde). Die *ex vivo*-Inhibitionskonzentration betrug im geometrischen Mittel 3,8 nM gegenüber Dihydroartemisinin und 4,2 nM gegenüber Artesunat. Gegenüber Lumefantrin

und Piperaquin wurden Werte von 12,1 nM und 8,0 nM bestimmt. Statistisch gab es durch Errechnung des Determinationskoeffizienten r^2 Hinweise auf Kreuzresistenz zwischen den Artemisinen und Mefloquin (r^2 = 0.41 und 0.38, p jeweils < 0.0001). Von insgesamt 56 (33 %) wiederkehrenden Parasitämie-Fällen traten 32 (19 %) innerhalb von 28 Tagen nach Beginn der Therapie mit Artesunat auf. 8 Patienten hatten PCR- bestätigte Rekrudeszenz (5 %), 5 davon (3 %) innerhalb von 28 Tagen nach Therapiebeginn. Die Inhibitionskonzentrationen bei wiederkehrender Parasitämie unterschieden sich nahezu ausschließlich nicht signifikant von denen vor Behandlungsbeginn ($0.02 \leq p \geq 0.96$).

Schlussfolgerung: Die Artemisinin-Wirksamkeit gegenüber *P. falciparum* in Kenia erweist sich klinisch und auch *ex vivo* als noch immer hoch. Um Resistenzen zu verhindern und gegebenenfalls rasch und adäquat handeln zu können, sind Untersuchungen neuer Medikamente und Kombinationen unabdingbar. PPQ zeigt eine hohe Effektivität *ex vivo* und könnte daher eine Alterative zum Goldstandard in Kenia darstellen. Trotzdem ist es unerlässlich weiterhin kontinuierlich Medikamentenwirksamkeitsstudien durchzuführen. Die häufige Verwendung von Artemether-Lumefantrin bedingt einen hohen Selektionsdruck der Parasiten. Zudem treten Mutationen, welche die Medikamentensensitivität beeinflussen können, sehr spontan auf. Durch engmaschige Kontrollen der Medikamentenwirksamkeit lässt sich deren Abnahme früh detektieren und ihre weitere Ausbreitung eindämmen.

8. References

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9. Ehrenerklärung

Ich erkläre, dass ich die der Medizinischen Fakultät der Eberhard Karls Universität Tübingen zur Promotion eingereichte Dissertation mit dem Titel “Surveillance of artemisinin and partner drug efficacy in uncomplicated *Plasmodium falciparum* malaria in Coastal Kenya” im Kenya Medical Research Institute (KEMRI, Kilifi) mit Unterstützung der Arbeitsgemeinschaft um Prof. Dr. med. Steffen Borrmann (damals Universität Heidelberg) ohne sonstige Hilfe durchgeführt und bei der Abfassung der Dissertation keine anderen als die aufgeführten Hilfsmittel benutzt habe.

Die Studie wurde durch Herrn Prof. Dr. med. Steffen Borrmann konzipiert, welcher ebenso die Betreuung der Versuche und der gesamten Arbeit übernommen hat. Die HRP2 Versuche wurden durch mich, Maria Rebekka Marschallek, durchgeführt und teilweise durch einen weiteren Doktoranden der Universität Heidelberg, Paul Wettzel, ergänzt. Die PCR Genotypisierungen wurden einmal durch mich in Kenia durchgeführt und im Anschluss vervollständigt und verifiziert durch die Arbeitsgemeinschaft Borrmann an der Universität Heidelberg. Die klinischen Daten der CATMAP Studie wurden durch das Studienteam in Kenia gesammelt. Die hier aufgeführte Recherche erfolgte ausschließlich durch mich, ebenso die statistische Analyse.

Bei der Abfassung der Dissertation sind Rechte Dritter nicht verletzt worden.

Ich habe diese Dissertation bisher an keiner in– oder ausländischen Hochschule zur Promotion eingereicht.

Neubrandenburg, den 19. Januar 2017

Maria Rebekka Marschallek

10. Danksagung

Ein großes Dankeschön gilt den kleinen Patienten, ohne die diese Studie nicht hätte durchgeführt werden können. Zudem gilt mein Dank meinem Betreuer, Prof. Dr. med. Steffen Borrmann, der es mir ermöglicht hat an diesem Projekt mitzuarbeiten. Außerdem den Mitgliedern seiner Arbeitsgruppe in Heidelberg, die mich tatkräftig unterstützt haben. Ebenso Herrn apl. Prof. Dr. Jude Przyborski und seinen Mitarbeitern in Marburg für die Starthilfe bei der Laborarbeit. Und natürlich allen Mitarbeitern in KEMRI, die immer ein offenes Ohr für meine Anliegen hatten.

Ich danke jedem einzelnen meiner Geschwister für die gezeigte Geduld und meinen Eltern, die mir immer wieder die Welt zu Füßen legen, damit ich sie erobern kann.

11. Appendices 1 – 4

Appendix 1[19]

Criteria for inclusion:

- Age between 6 months to 10 years, inclusive
- Mono-infection with P. falciparum detected by microscopy
- Parasitaemia of 10,000 – 300,000 / µl asexual forms
- Presence of axillary temperature ≥ 37.5 °C or history of fever during the past 24 hrs
- Ability to swallow oral medication
- Ability and willingness to comply with the study protocol for the duration of the study and to comply with the study visit schedule
- Informed consent from a parent or guardian

Criteria for exclusion:

- Presence of clinical danger signs: not able to drink or breast-feed, vomiting (> twice in 24 hrs), recent history of convulsions (> 1 in 24 hrs), unconscious state, unable to sit or stand
- Mixed or mono-infection with another Plasmodium species detected by microscopy
- Presence of severe acute malnutrition defined as weight for height < 70 % of the median National Center for Health Statistics / WHO
- Presence of febrile conditions due to diseases other than malaria (e.g. measles, acute lower respiratory tract infection, severe diarrhoea with dehydration) or other known underlying chronic or severe diseases (e.g. cardiac, renal and hepatic diseases, Human Immunodeficiency Virus / Aquired Immune Deficiency Syndrome)
- Regular medication, which may interfere with antimalarial pharmacokinetics or pharmacodynamic assessments (e.g., antibiotics with known antimalarial activity)
- History of hypersensitivity reactions or contraindications to any of the medicine(s) being tested or used as alternative treatment(s)

[19] CATMAP study protocol v1.3, 2011

Appendix 2

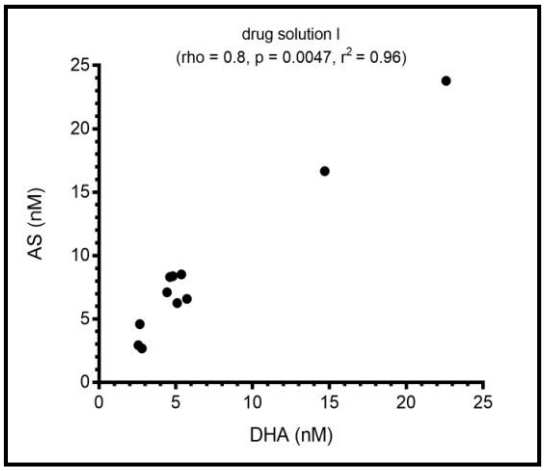
The absorbance read during the performance oft he HRP2 ELISA resulted in OD values which were transferred into the HN-NonLin V1.1 sheet to calculate IC50 values (ng / ml). The IC50 values were transferred into nM by using an excel sheet containing the needed formula for each drug.

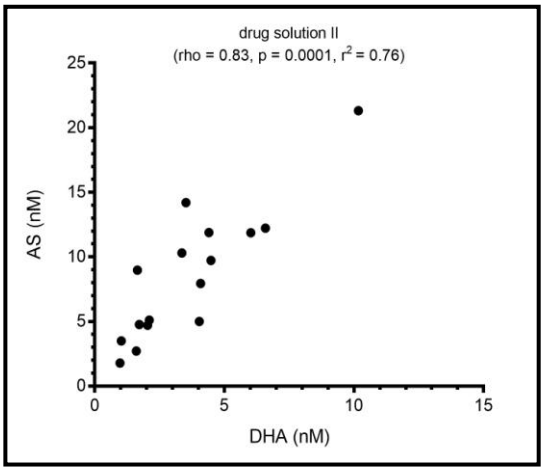
AS	nM = (ng / ml)*1000 / 284.3
CQ	nM = (ng / ml)*1000 / 515.87
DHA	nM = (ng / ml)*1000 / 284.3
LUM	nM = (ng / ml)*1000 / 528.939
MFQ	nM = (ng / ml)*1000 / 414.77
PPQ	nM = (ng / ml)*1000 / 927.5

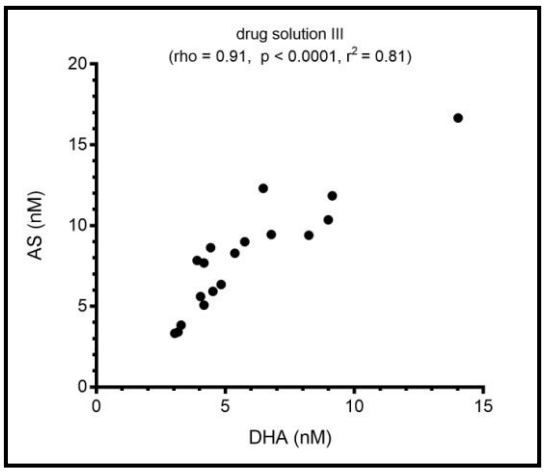
Appendix 3

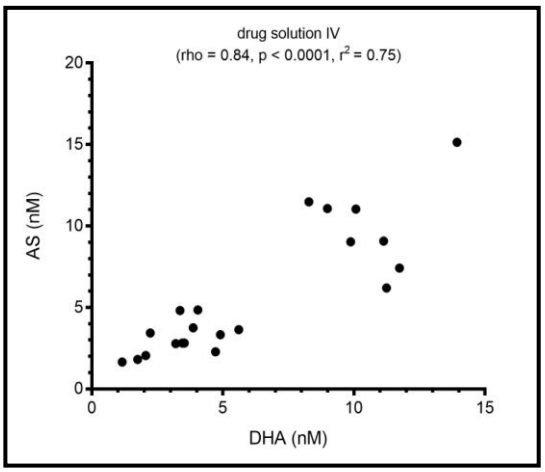
Correlations artesunate and dihydroartemisinin

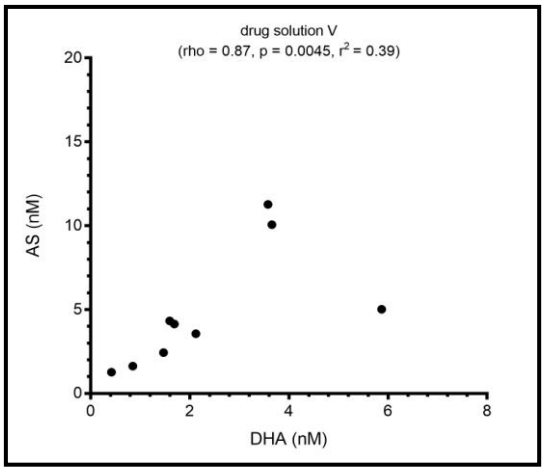
AS and DHA are both derivatives of artemisinin, whereas DHA is the main active metabolite *in vivo*. One expects a highly significant positive correlation between these two drugs, also in favor of the reliability of the assay. Here in all drug solutions a significant positive correlation was shown.

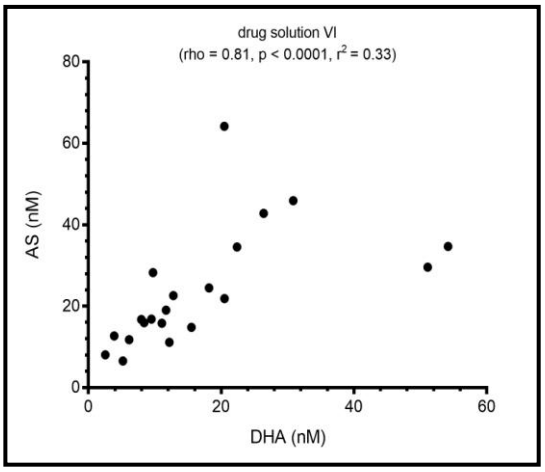


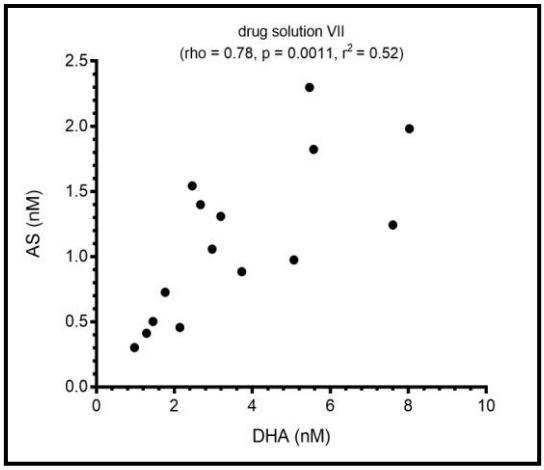






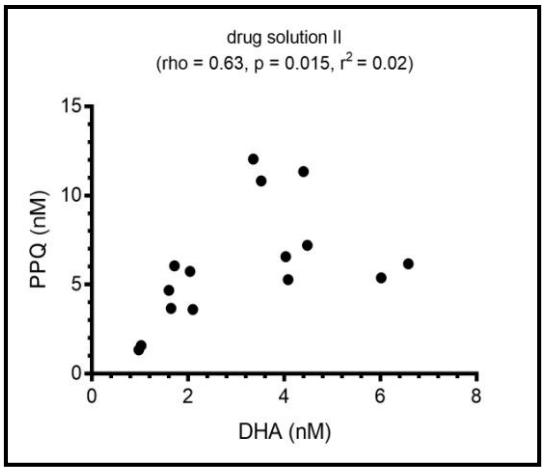

drug solution V
(rho = 0.87, p = 0.0045, r2 = 0.39)
AS (nM)
DHA (nM)
0
5
10
15
20
0
2
4
6
8

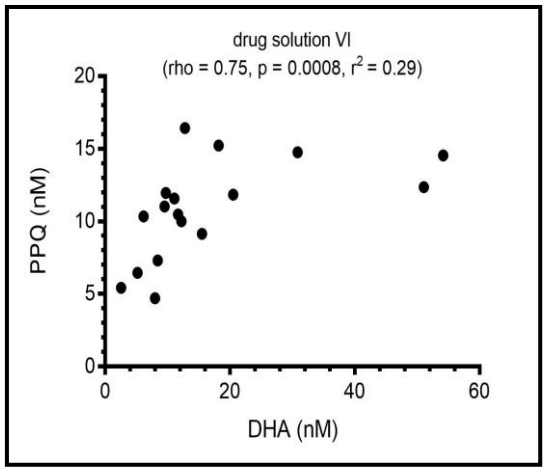

drug solution VI
(rho = 0.81, p < 0.0001, r2 = 0.33)
AS (nM)
DHA (nM)
0
20
40
60
80
0
20
40
60


drug solution VII
(rho = 0.78, p = 0.0011, r2 = 0.52)
AS (nM)
DHA (nM)
0.0
0.5
1.0
1.5
2.0
2.5
0
2
4
6
8
10

Correlations dihydroartemisinin and piperaquine

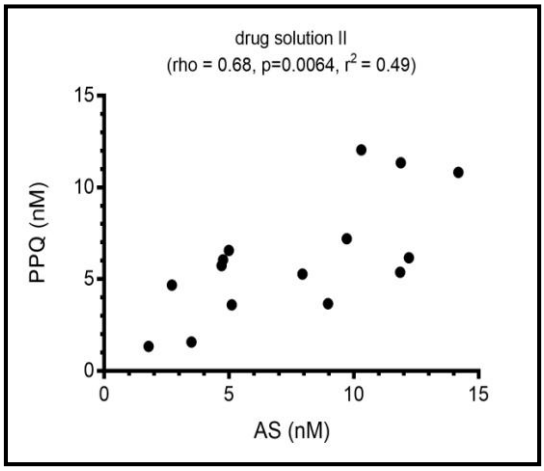
In the analysis including all drug solutions a positive significant correlation between DHA and PPQ was found. When analyzing drug solutions separately, two out of the seven solutions did show a significant positive correlation between those two drugs.

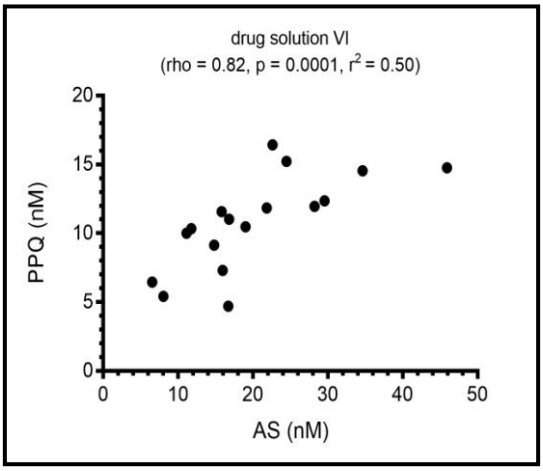




Correlations artesunate and piperaquine

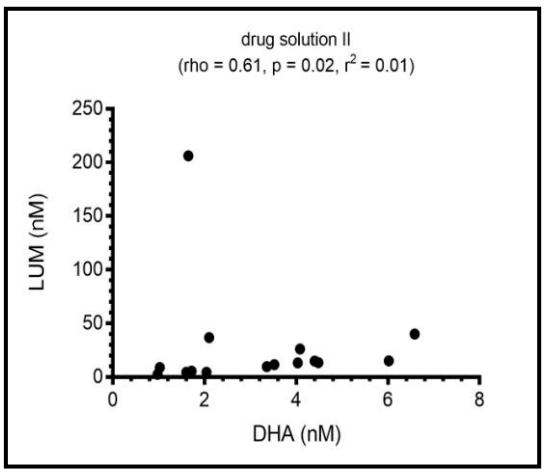
Between AS and PPQ there were two working solutions that showed significant positive correlations [(rho=0.6821, p=0.0064, r^2=0.4919), (rho= 0.8162, p=0.0001, r^2=0.5017)]. Those were the same two drug solutions that also showed a significant correlation between DHA and PPQ, the coefficient of determination was much higher though.

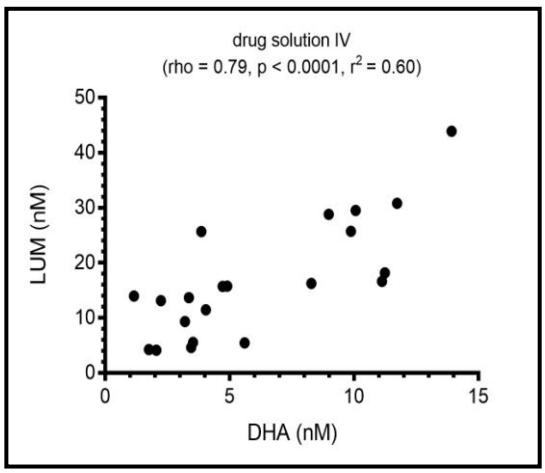


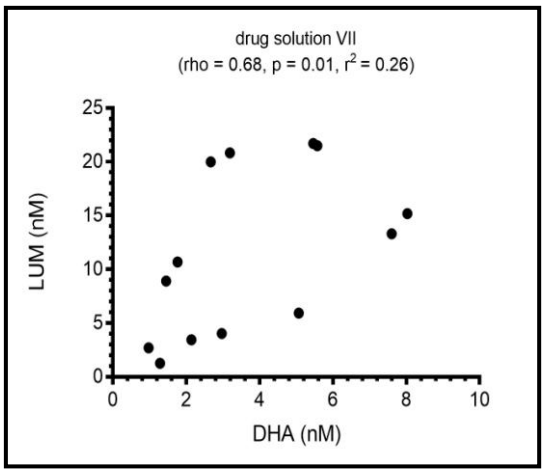


Correlations dihydroartemisinin and lumefantrine

The analysis among all drug solutions showed a significant positive correlation between DHA and LUM. When analyzing this again for each single drug solution the correlation was found in three drug solutions.

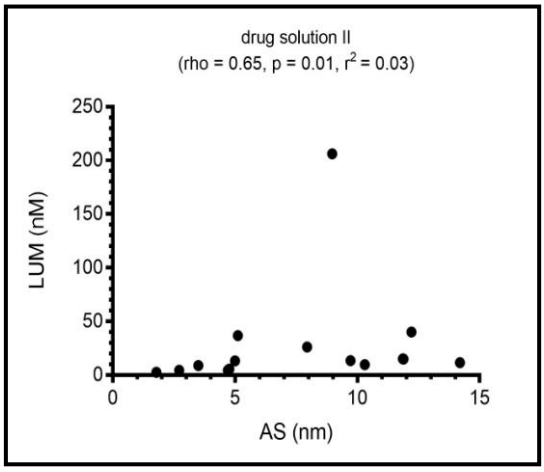


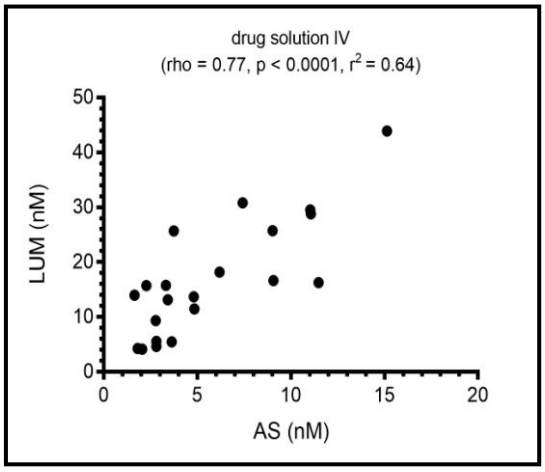


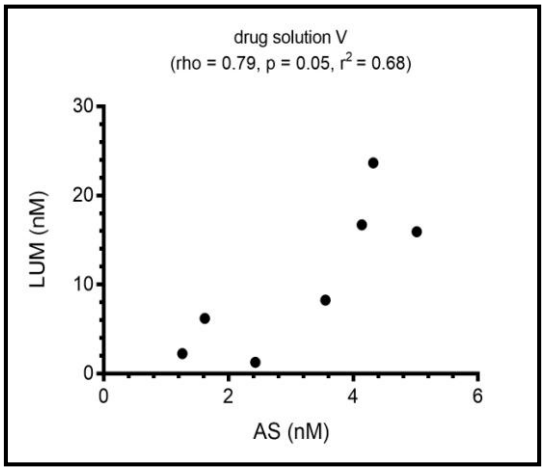


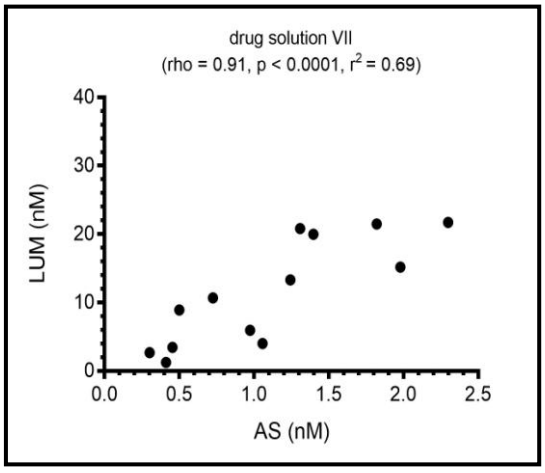
Correlations artesunate and lumefantrine

Also AS did show a significant positive correlation with LUM in four drug solutions.



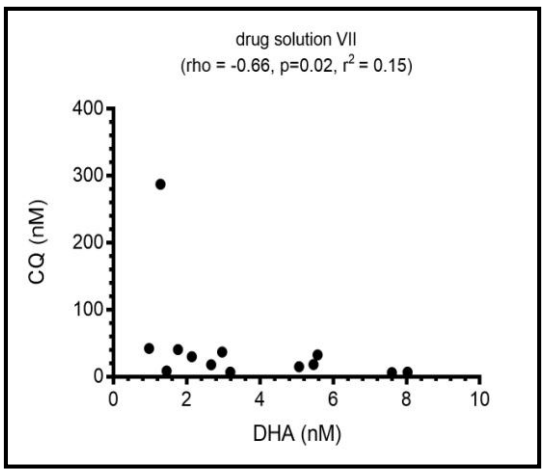


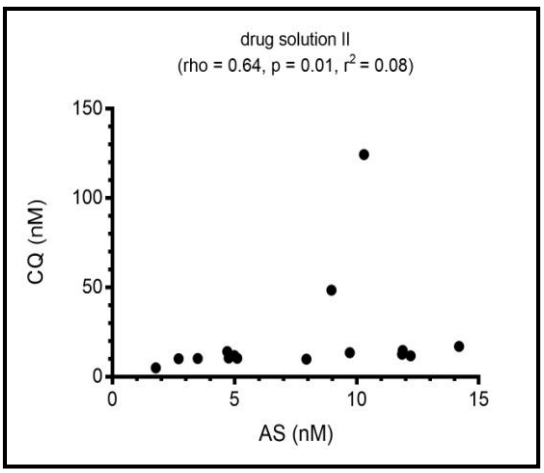


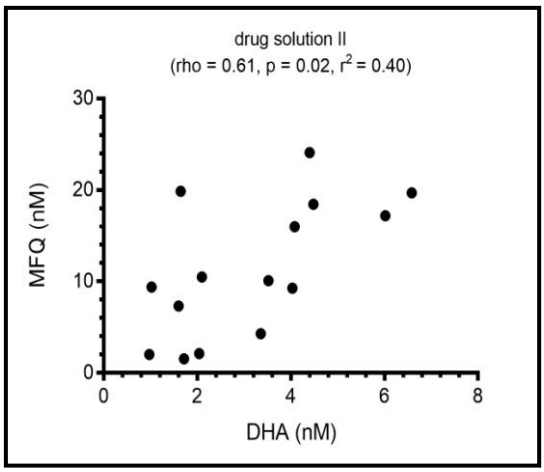


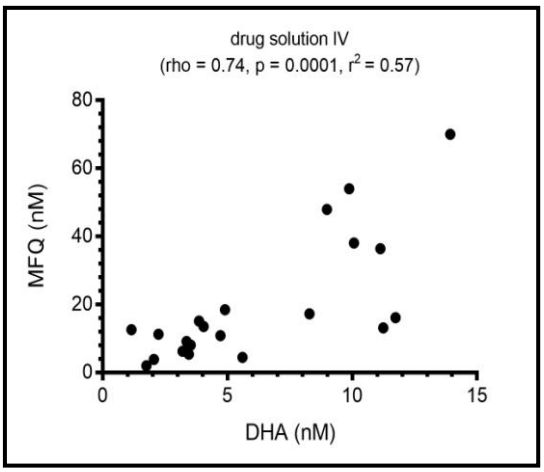
Correlations artemisinins and chloroquine and mefloquine

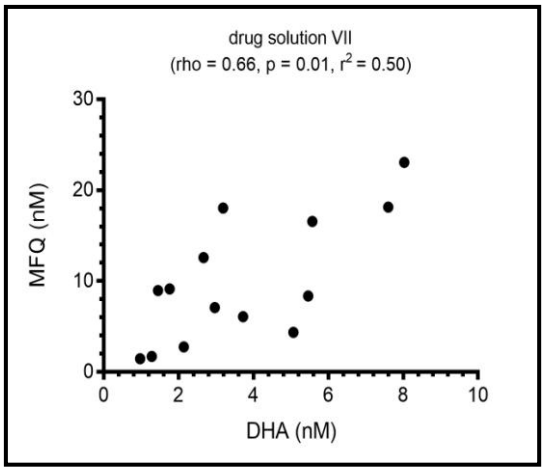
There were several different significant positive correlations between the artemisinin derivatives and CQ as well as MFQ when analyzing each working solutions individually.

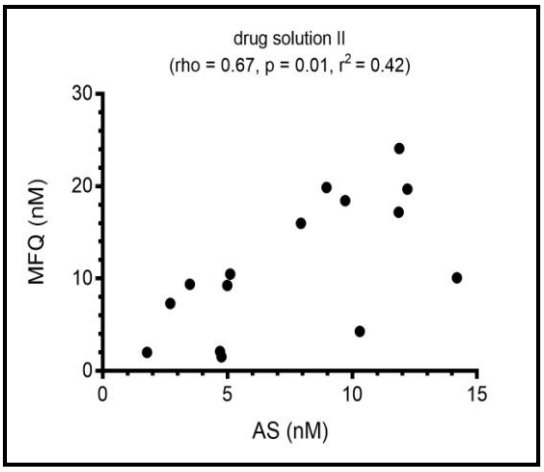


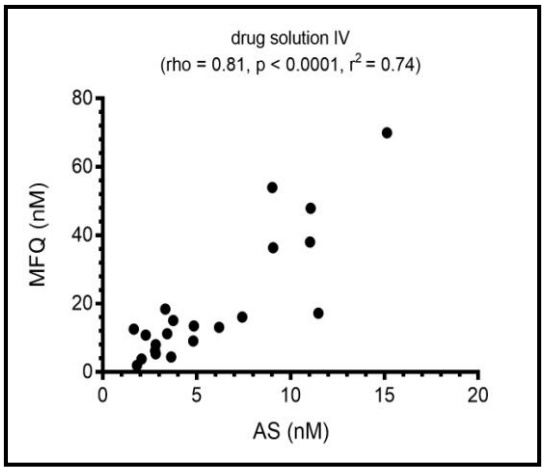


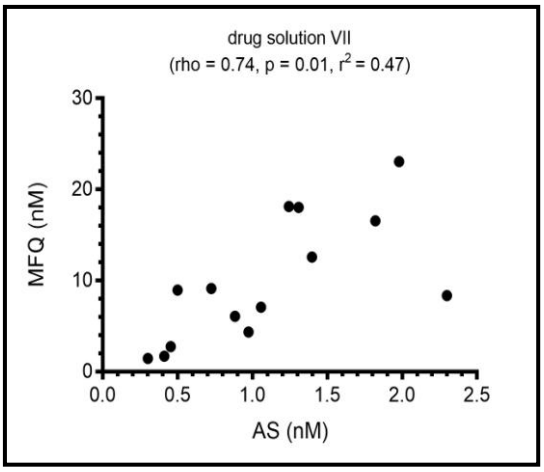






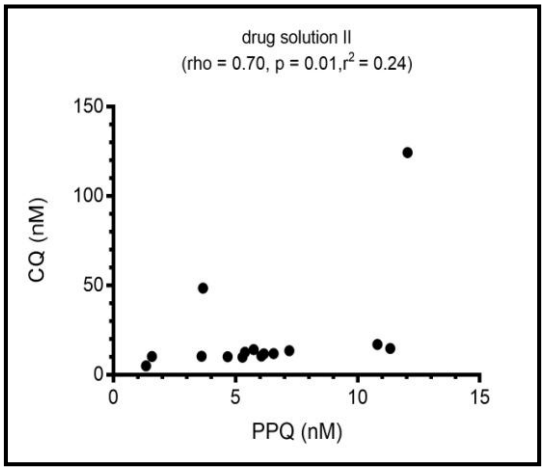


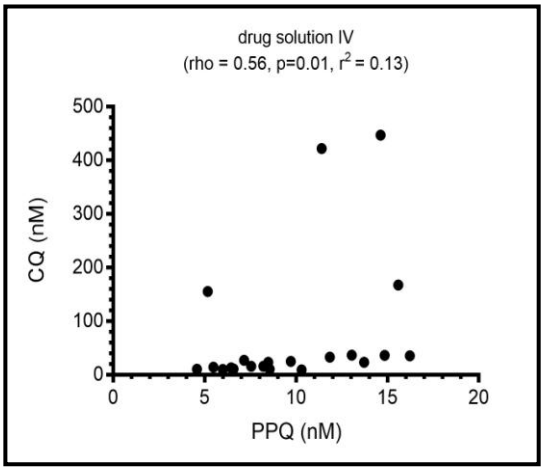




Correlations piperaquine and chloroquine, mefloquine and lumefantrine

PPQ did not show any correlation with CQ in the analysis including all drug solutions. Analyzing single drug solutions twice a correlation seen was seen.

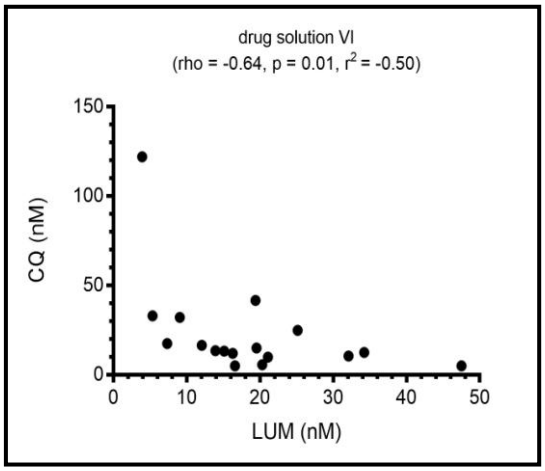




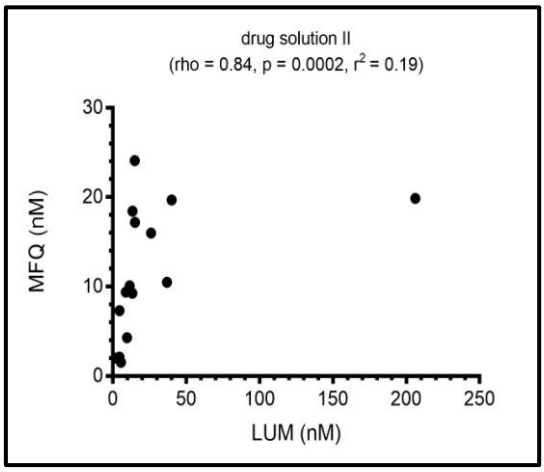
The correlation seen in the analysis including all drug solutions showed a correlation between PPQ and MFQ. This could not be confirmed when analyzing drug solutions individually. The same applied for PPQ and LUM.

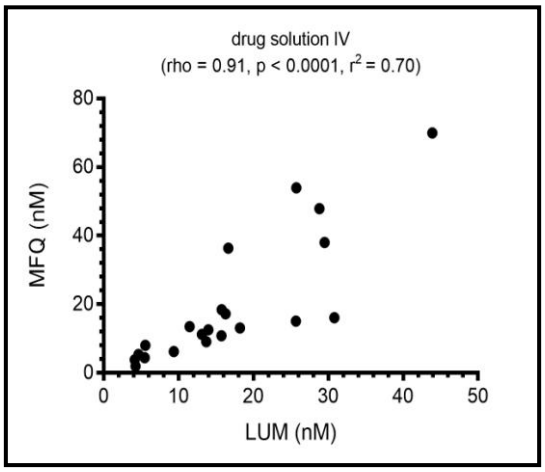
Correlations lumefantrine and chloroquine and mefloquine

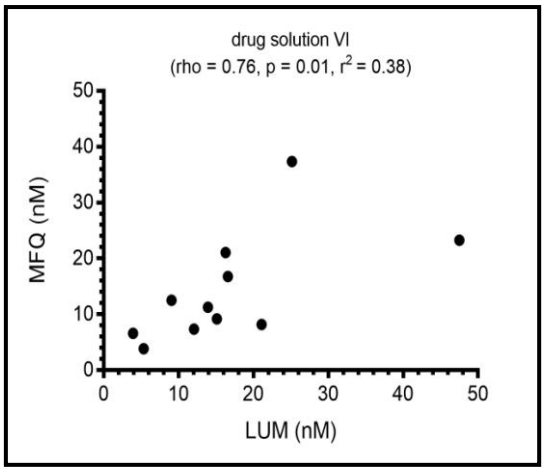
LUM showed a significant negative correlation with CQ in the analysis among all drug solutions. Analyzing single drug solutions, once a significant negative correlation between LUM and CQ was seen.

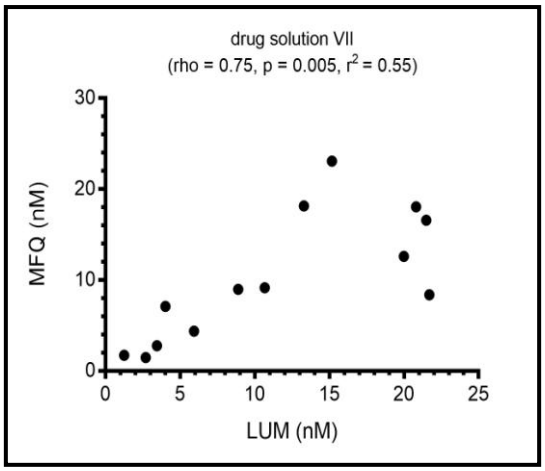


Four drug solutions did show a significant positive correlation between LUM and MFQ.

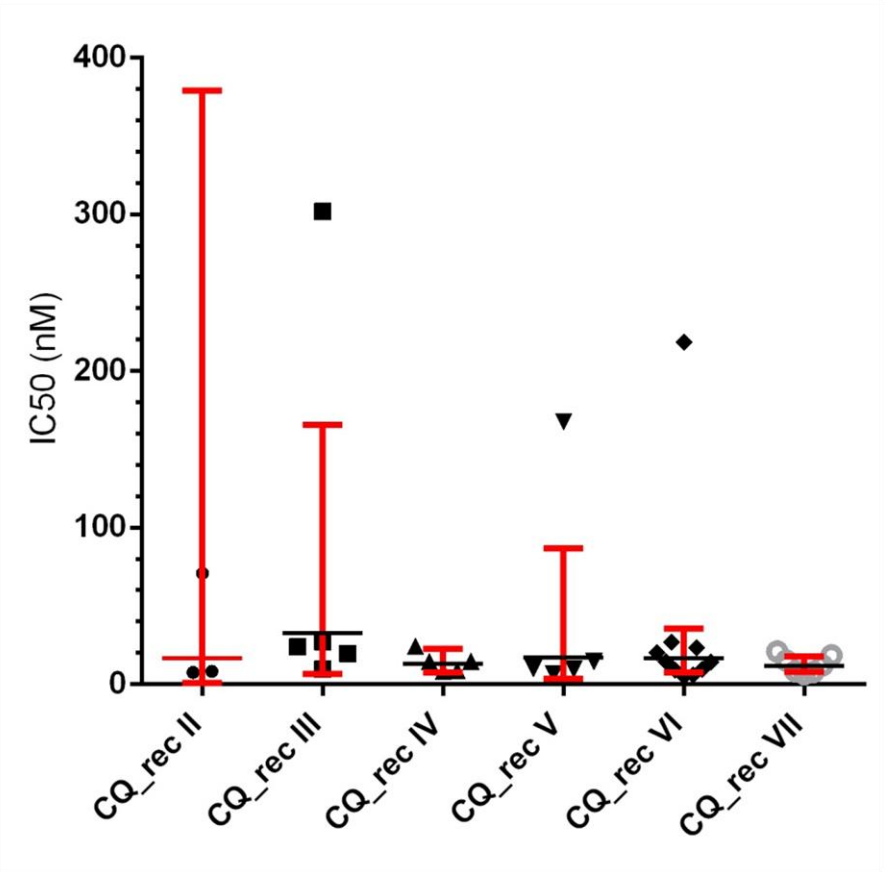




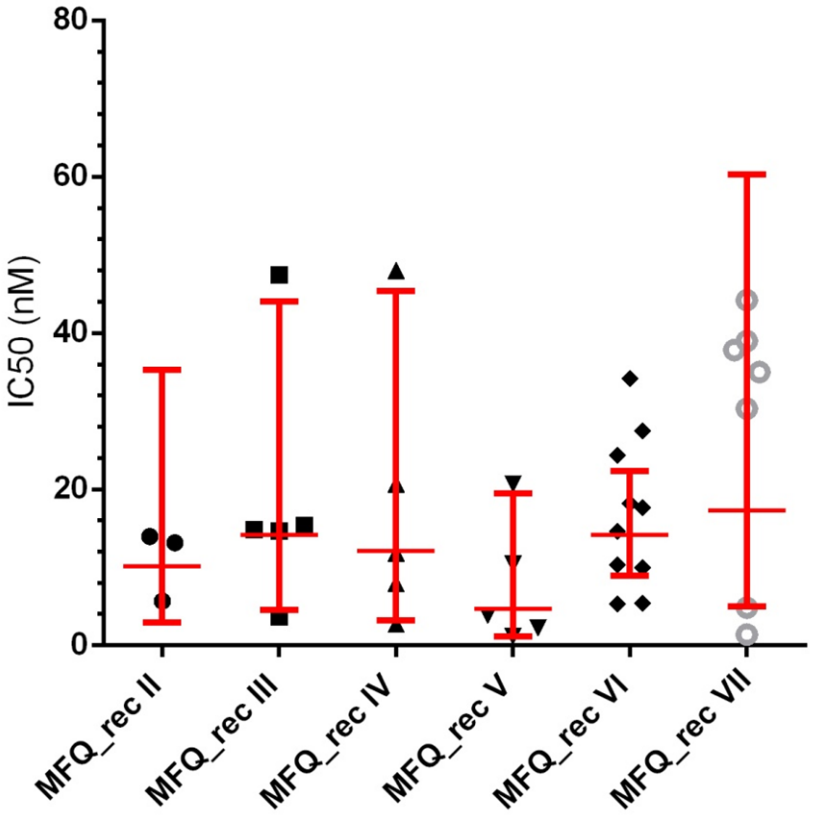




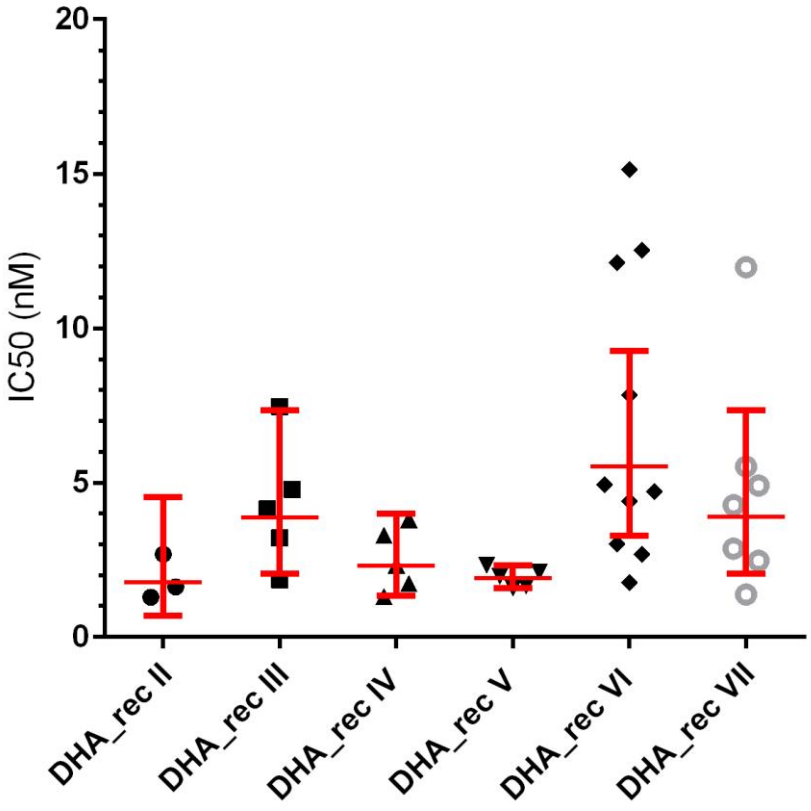
Appendix 4



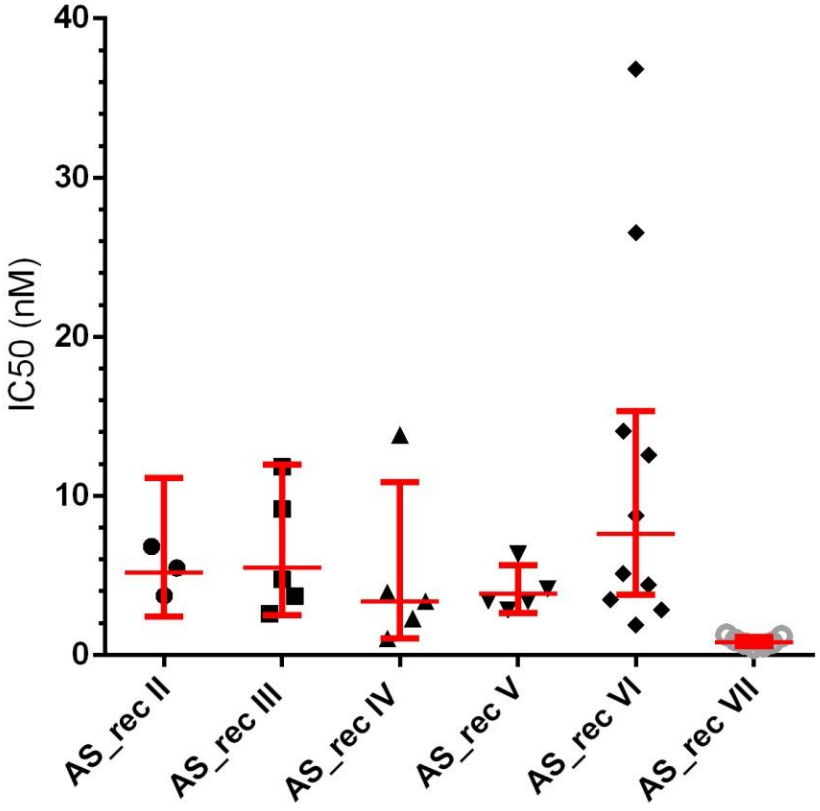
CQ_rec	II	III	IV	V	VI	VII
Number of values	3	5	5	5	10	7
Minimum	7.70	9.63	8.41	6.56	4.93	6.67
25 % Percentile	7.70	14.68	8..63	8.08	8.42	7.93
Median	8.35	23.99	14.55	10.22	14.34	12.12
75% Percentile	70.94	164.40	19.56	91.07	24.35	18.62
Maximum	70.94	301.60	24.31	167.60	218.30	21.02
Geometric mean	16.59	32.71	13.13	17.35	16.56	11.83
Lower 95 % CI of geometric mean	0.73	6.47	7.65	3.46	7.73	7.80
Upper 95 % CI of geometric mean	378.70	165.40	22.54	87.01	35.49	17.92



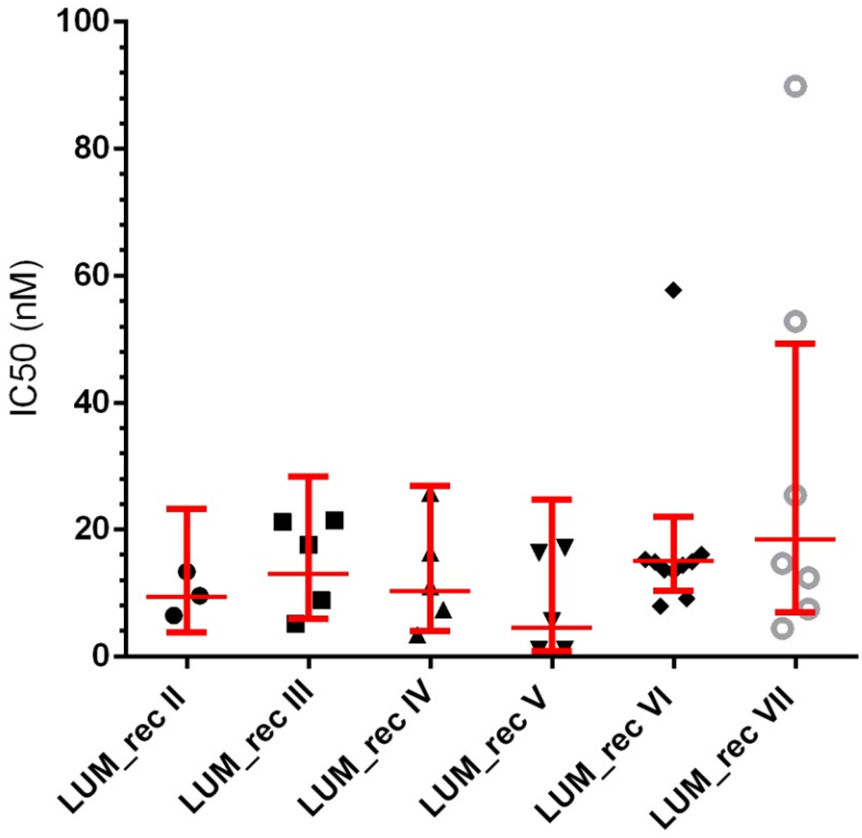
MFQ_rec	II	III	IV	V	VI	VII
Number of values	3	5	5	5	10	7
Minimum	5.68	3.63	2.75	1.19	5.35	1.40
25 % Percentile	5.68	9.15	5.37	1.73	8.79	4.83
Median	13.15	14.87	11.79	3.86	16.09	34.99
75 % Percentile	13.94	31.40	34.31	15.54	25.15	38.99
Maximum	13.94	47.47	47.99	20.64	34.15	44.22
Geometric mean	10.13	14.19	12.07	4.68	14.12	17.32
Lower 95 % CI of geometric mean	2.91	4.57	3.21	1.13	8.91	4.98
Upper 95 % CI of geometric mean	35.31	44.03	45.38	19.48	22.37	60.28



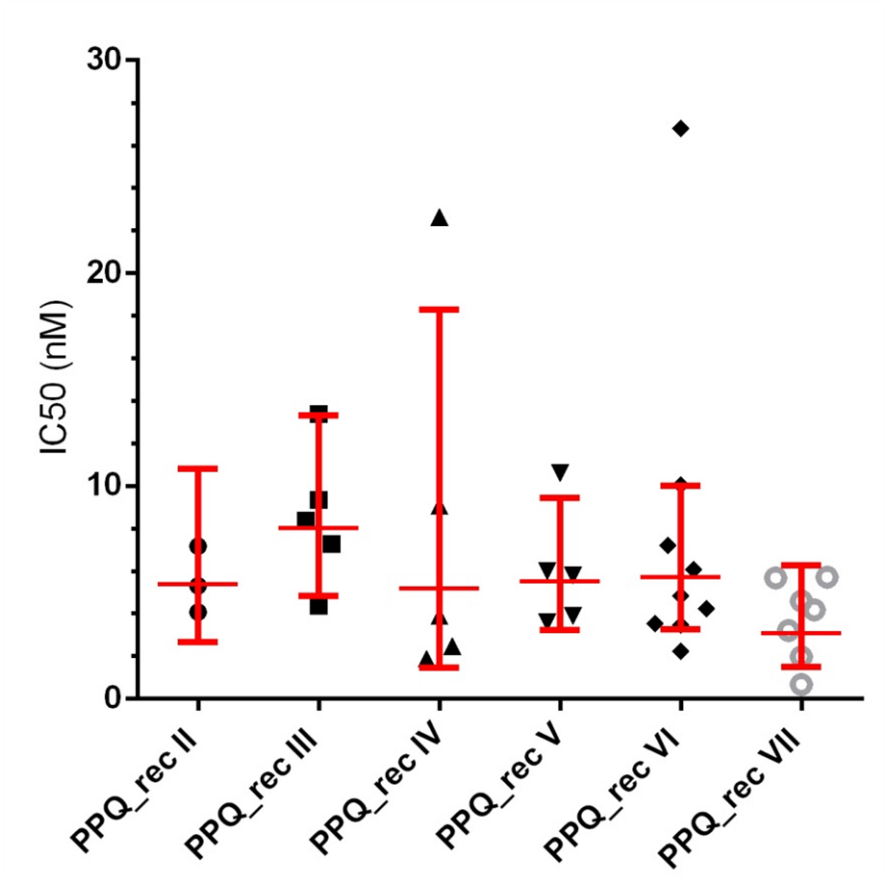
DHA_rec	II	III	IV	V	VI	VII
Number of values	3	5	5	5	10	7
Minimum	1.29	1.86	1.31	1.60	1.77	1.38
25 % Percentile	1.29	2.54	1.52	1.63	2.94	2.47
Median	1.62	4.15	2.32	1.97	4.82	4.28
75 % Percentile	1.29	2.54	1.52	1.63	2.94	2.470
Maximum	2.69	7.46	3.81	2.32	15.14	11.98
Lower 95 % CI of geometric mean	0.69	2.05	1.33	1.58	3.29	2.06
Upper 95% CI of geometric mean	4.54	7.35	4.00	2.32	9.27	7.34



AS_rec	II	III	IV	V	VI	VII
Number of values	3	5	5	5	10	7
Minimum	3.72	2.60	1.04	2.83	1.89	0.47
25 % Percentile	3.72	3.15	1.66	3.11	3.33	0.57
Median	5.47	4.74	3.38	3.39	6.94	0.79
75 % Percentile	6.83	10.49	8.88	5.24	17.20	1.19
Maximum	6.83	11.81	13.84	6.33	36.83	1.26
Geometric mean	5.18	5.48	3.37	3.86	7.60	0.80
Lower 95 % CI of geometric mean	2.41	2.51	1.04	2.63	3.76	0.57
Upper 95 % CI of geometric mean	11.12	11.97	10.87	5.66	15.33	1.13



LUM_rec	II	III	IV	V	VI	VII
Number of values	3	5	5	5	10	7
Minimum	6.47	5.17	3.44	1.07	7.94	4.43
25 % Percentile	6.47	7.02	5.42	1.09	12.38	7.49
Median	9.59	17.64	11.02	5.57	14.66	14.67
75 % Percentile	13.38	21.34	21.05	16.72	15.50	52.81
Maximum	13.38	21.47	25.76	17.11	57.75	89.92
Geometric mean	9.40	12.98	10.34	4.50	15.08	18.46
Lower 95 % CI of geometric mean	3.80	5.95	3.98	0.82	10.35	6.91
Upper 95 % CI of geometric mean	23.21	28.32	26.88	24.69	21.98	49.33



PPQ_rec	II	III	IV	V	VI	VII
Number of values	3	5	5	5	9	7
Minimum	4.08	4.36	1.90	3.59	2.25	0.67
25 % Percentile	4.08	4.36	1.90	3.59	2.25	0.67
Median	5.31	8.38	3.90	5.81	4.84	4.18
75 % Percentile	7.17	11.37	15.86	8.31	8.64	5.69
Maximum	7.17	13.38	22.65	10.61	26.81	5.73
Geometric mean	5.38	8.03	5.19	5.53	5.74	3.09
Lower 95 % CI of geometric mean	2.67	4.84	1.47	3.24	3.28	1.52
Upper 95 % CI of geometric mean	10.84	13.33	18.30	9.44	10.03	6.28