Moving towards improved malaria control
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Introduction and outline of the thesis
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INTRODUCTION TO MALARIA

Malaria is an infectious disease caused by a parasitic protozoan of the genus *Plasmodium*. In humans, several *Plasmodium* species can cause malaria: *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* (subspecies: *P. ovale wallikeri* and *P. ovale curtisi*) and *P. knowlesi*. Of these species, *P. falciparum* causes the highest morbidity and mortality and was responsible for 99% of cases in sub-Saharan Africa in 2016. Outside Africa, *P. vivax* is more prevalent and represents 64% of the cases in the Americas, over 30% of the cases in Southeast Asia and 40% of the cases in the Eastern Mediterranean regions. In total, the World Health Organization (WHO) estimated 216 million malaria cases and 445,000 deaths in 2016, of which 90% occurred in sub-Saharan Africa.

The vectors responsible for the transmission of *P. falciparum* parasites are mosquitoes of the genus *Anopheles*. When a mosquito is infected with *P. falciparum* and takes a blood meal from the (human) host, it injects sporozoites into the bloodstream. The sporozoites migrate to the liver, where they develop into schizonts. When the schizonts rupture, they release merozoites into the bloodstream. Each merozoite is capable of infecting a red blood cell and is then called trophozoite, which develops into a new schizont. This schizont will rupture about 48 hours later and again, merozoites are released into the bloodstream. The continuous repeat of this asexual cycle can cause rapidly increasing parasite densities. Next to the asexual cycle, some immature trophozoites will develop into female or male gametocytes, the parasite stage responsible for transmission between hosts. When a mosquito takes a blood meal and ingests the gametocytes, these will develop into microgametes and macrogametes and sexually reproduce in the mosquito gut, generating zygotes. The zygotes turn into ookinetes, which invade the midgut wall of the mosquito to develop into oocysts. When the oocysts rupture they release sporozoites, which migrate to the salivary glands of the mosquito. A new bloodmeal and the corresponding injection of sporozoites into a host completes the *P. falciparum* life cycle (Figure 1).
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Figure 1. Life cycle of Plasmodium.

Credits: https://www.cdc.gov/malaria/about/biology/index.html.
CLINICAL FEATURES AND PATHOGENESIS OF PLASMODIUM FALCIPARUM MALARIA

*P. falciparum* malaria is an acute febrile illness associated with high morbidity and mortality, especially in young children. The first symptoms are non-specific and often include irregular fever, headache, abdominal pain, myalgia, nausea and/or vomiting. In uncomplicated malaria, the most important physical findings are fever, mild anaemia and sometimes a palpable spleen. Age is a determinant for the clinical manifestation of severe malaria: while children are more likely to present with severe anaemia and hypoglycaemia, adults have a higher chance to develop acute kidney injury, acute pulmonary oedema and jaundice. Cerebral malaria occurs in all age groups and is an important risk factor for a fatal outcome.

Malarial anaemia is thought to be the result of both haemolysis and inadequate erythropoiesis. Severe anaemia, defined as a haemoglobin (Hb) concentration below 5 g/dL, is an important indicator of life-threatening malaria. Cytoadherence of red blood cells, infected by *P. falciparum*, is a key contributor to parasite sequestration in the microvasculature of organs (like brain, liver, lungs, kidneys and heart), causing reduced microcirculatory flow. This cytoadherence has been associated with severe complications, such as cerebral malaria, and is mediated by the expression of surface antigens on infected erythrocytes. Surface antigens facilitate the binding of infected erythrocytes to endothelial receptors, of which cluster of differentiation 36 (CD36) and intercellular adhesion molecule 1 (ICAM-1) are the most well-known examples.

These surface antigens have extensive antigenic variation, which equips the parasite with tools to escape the host immune system, as antibody-mediated immunity is largely directed against *P. falciparum* surface antigens. Although cross-reactivity against different surface antigens exists, and the level of cross-reactivity might increase in high transmission settings, antibody responses are often surface antigen specific. To acquire immunity, it is therefore necessary to develop a range of different antibodies, which implies frequent exposure. This is the reason why children under five years of age living in endemic areas are more vulnerable to clinical malaria and severe disease, compared to older children and adults who have acquired an antibody response that suppresses parasitaemia and clinical symptoms. However, asymptomatic infections are still frequently observed in adults and older...
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children in endemic areas, providing evidence that sterile immunity is not reached\textsuperscript{20,21}. Apart from young children, pregnant women represent another risk group for malaria. Even though adult women in endemic areas often have acquired immunity, \textit{P. falciparum} expresses a different surface antigen during pregnancy (variant surface antigen 2–chondroitin sulphate A (VAR2CSA)), to which especially primigravidae have a very limited antibody response\textsuperscript{22}. 
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MALARIA CONTROL

Even though malaria still has a huge impact on human health, wide-scale interventions have led to important reductions in overall malaria morbidity and mortality. The estimated number of cases dropped from 262 million in 2000, to 237 million in 2010 and 216 million in 2016\(^2\). Since 2010, the incidence rate is estimated to have decreased globally from 76 to 63 cases per 1000 people at risk. The largest decline in incidence rate was reported in Southeast Asia (48%), followed by the Americas (22%) and sub-Saharan Africa (20%)\(^2\). The WHO Global Technical Strategy for Malaria (GTS), endorsed by the World Health Assembly, set clear targets of reducing global malaria case incidence and mortality by at least 90% by 2030, compared to 2015\(^{23,24}\). It should be noted however, that from 2014 to 2016 the reduction in incidence has stalled and that the reductions were lowest in countries with the highest burden\(^2,25\). In these countries, the incidence rate reduction needs to be substantially accelerated to meet future goals\(^25\).

In high or moderate transmission areas, control programs aim to reduce the number of cases mainly through improving case management and prevention\(^26\). Case management includes diagnosis of suspected cases and subsequent treatment of those who test positive with effective antimalarials. Prevention includes the use of prophylactic medication in high risk groups and vector control, for example through indoor residual spraying (IRS) and the use of long-lasting insecticide-treated bed nets (LLITNs)\(^27,28\). The availability of effective and safe vaccines could also play a major role in malaria control and elimination. Several vaccine candidates are under development, of which RTS,S/AS01, a pre-erythrocytic stage vaccine, is the most advanced. RTS,S is a vaccine for young children that provides partial protection against *P. falciparum* malaria\(^29–31\). It received a positive opinion from the European Medicines Agency (EMA) and pilot programs will start to implement the vaccine in selected areas of Kenya, Ghana and Malawi in 2018\(^32\).

In countries with low transmission that approach elimination, strong surveillance systems are recommended besides prevention and case management, striving to actively or passively detect and treat every infection. These surveillance systems are advised to focus on the detection of at risk populations or -regions\(^24,26\). An additional measure could be the implementation of Mass Drug Administration (MDA) campaigns, which aim to administer antimalarial treatment to entire defined populations at the same time. This
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could reduce the risk of parasitaemia and, especially when a gametocytocidal drug like primaquine is given, interrupt transmission\textsuperscript{33}. However, there are still issues with optimizing MDA coverage, defining the target population and primaquine safety\textsuperscript{34–36}. In contrast to MDA, Mass Screening and Treatment (MSAT) aims to screen an entire defined population and treat those who are positive. MSAT based on rapid diagnostic tests (RDTs) is currently not recommended, because it was shown to be ineffective at reducing malaria incidence, most likely because large proportions of (low parasite density) infections remained undetected and thus untreated\textsuperscript{37}. A highly sensitive point-of-care test that rapidly detects low parasite densities missed by RDTs, thereby identifying all infected individuals, would be very helpful for both surveillance systems and MSAT programs\textsuperscript{24}.

From the above can be concluded that malaria control consists of at least three main components: prevention, diagnosis and treatment. All three need to be represented in different ways in different transmission settings. In this thesis, the focus lies on the latter two, which are discussed in the sections below.
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DIAGNOSIS

The signs and symptoms of malaria are non-specific and clinical suspicion of malaria is usually mainly based on the presence or a history of fever. Thus, malaria cannot reliably be distinguished from other causes of fever based on signs and symptoms only and, as a consequence, a clinical diagnosis has low specificity and easily leads to overtreatment. Additionally, the true cause of a non-malarial fever requiring alternative treatment may be missed when relying on clinical symptoms only. Therefore, the WHO recommends that patients suspected of malaria should all be tested by microscopy or RDT for parasitological confirmation of the diagnosis.

Both endemic and non-endemic countries require good diagnostic facilities, in the latter case to detect imported malaria in travellers returning from endemic areas. However, the resources required for proper diagnosis are not equally distributed. While developed countries can equip their laboratories with all the required diagnostic facilities, they are often non-endemic for malaria and hospitals only see a limited number of imported cases, which may lead to lack of expertise. Developing countries, on the other hand, have the highest burden in terms of malaria cases, but resources are often lacking to properly equip (all) laboratories and educate laboratory staff. This difference is important in the debate about advantages and drawbacks of diagnostics, because the applicability of a tool in clinical practice or surveillance programs largely depends on the resources available at the (intended) site of implementation.

The most common methods for the diagnosis of malaria are described below, from a perspective that mostly fits endemic developing countries and whereby, next to accuracy, user friendliness, required resources and costs are of major importance.

Microscopy

Microscopy is based on morphological detection and identification of Plasmodium parasites in stained thick and thin blood films. A major advantage of microscopy is the ability to differentiate between Plasmodium species, which can be important for treatment decisions. Additionally, it is possible to quantify the number of parasites. This is especially useful in the case of P. falciparum malaria, where parasite densities may become very high and parasitaemia is an indicator for treatment outcome. Microscopy is
also inexpensive (around 0.5 USD)\textsuperscript{42} and it is possible to distinguish between sexual and asexual stages of the parasite, which provides information on the transmission potential of patients\textsuperscript{49,50}. Finally, microscopy provides a method to identify blood abnormalities while screening for malaria\textsuperscript{51,52}.

A disadvantage of microscopy, on the other hand, is that the sensitivity may not be good enough in every situation. Even though expert microscopists can reach a limit of detection (LoD) of 4–20 parasites/µl, under field conditions a LoD of 50–100 parasites/µl is more realistic\textsuperscript{53–55}. Other issues are the training level required to reliably detect and quantify parasites, the maintenance of equipment, the need for electricity and a relatively long throughput time from sample collection to test result (30 to over 60 minutes)\textsuperscript{47,56}. The most commonly used stain is Giemsa, with staining times from 10 minutes (fast method) to 60 minutes (slower method which provides optimal quality with less stain)\textsuperscript{51,57}. To shorten the staining time, methods like Field stain have been developed, which provide good quality slides in less time, although Giemsa staining is reported to have a more consistent quality\textsuperscript{46,58}. Microscopes have been adjusted to fluorescence based battery operated systems, making the read-out easier and providing possibilities to perform microscopy in settings where no electricity is available\textsuperscript{59–61}. Quantitative Buffy Coat (QBC) is also a fluorescence based method using acridine orange staining. Parasites can easily be seen in a microcentrifuge tube under ultraviolet light and QBC has similar sensitivity and specificity compared to conventional light microscopy\textsuperscript{62–64}. However, although QBC is easy to perform, species identification and quantification are not possible and additional equipment is required compared to traditional microscopy\textsuperscript{62}. Thus, Giemsa stained slides in combination with light microscopy are still the optimal method of choice for malaria microscopy.

**Rapid Diagnostic Tests**

Rapid diagnostic tests (RDTs) for malaria are antigen detection assays with a lateral flow based read-out. RDTs were designed and developed to overcome some major limitations of microscopy: they are fast (time from sample collection to test result: approximately 20 minutes), easy to perform and do not require electricity or specific equipment\textsuperscript{56}. The cost per test is generally higher than for microscopy: around 2 USD, depending on type, manufacturer and supplier\textsuperscript{42}. However, in contrast to microscopy, no other equipment is needed to perform RDTs.
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Commercialized RDTs use histidine rich protein-2 (HRP-2), *Plasmodium* lactate dehydrogenase (pLDH) and/or *Plasmodium* aldolase as targets, of which the first two are the most common\(^65\). HRP-2 is produced by asexual stages and young gametocytes of *P. falciparum*. It is a very specific antigen, but may persist for more than four weeks after treatment\(^56,66,67\). HRP-2 based tests are therefore not suitable to monitor parasite clearance after treatment\(^66,67\).

pLDH can be species-specific for *P. falciparum* or *P. vivax*, although there is also ‘pan-malarial’ pLDH, detecting *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*\(^68\). In contrast to HRP-2, pLDH is cleared rapidly from the circulation after treatment, but sensitivity against reference standard microscopy is lower compared to HRP-2 based assays\(^69,70\). On the other hand, false positive HRP-2 tests might occur in patients who recently cleared a *P. falciparum* infection and in patients with rheumatoid factors, resulting in lower specificity compared to pLDH based tests\(^69,71,72\). Generally, both HRP-2 and pLDH based RDTs can reliably detect up to around 100 parasites/µl, similar to microscopy under field conditions\(^42\).

Sensitivity is low at parasitaemias <100/µl and RDTs are therefore unsuitable to detect these low-density infections. Another issue with RDTs is that storage and use in tropical climates can affect their stability\(^73,74\). Finally, the presence of *P. falciparum* parasites with deletions in the *pfhrp* gene may be a concern for case management and control\(^75\). In Amazon River basin regions in South America, parasites lacking the *pfhrp2* gene are common, hence the use of HRP-2 based RDTs is not recommended in these areas since several years\(^76\). More recently, a number of studies described *pfhrp2* deletions and mutations leading to false negative RDTs in African countries\(^77–80\). Further studies are required to establish the potential diagnostic impact of *pfhrp2* deletions in Africa.

In recent years, new targets have been explored that might improve accuracy and stability of currently used RDTs. Amongst others, these targets include *Plasmodium falciparum* heat shock protein 70 (*PfHsp70*), dihydrofolate reductase–thymidylate synthase (DHFR–TS), heme detoxification protein (HDP), glutamate rich protein (glurp), merozoite surface protein 1 (msp1) and topoisomerase 1\(^81–85\). To date, neither of these targets is used outside research settings.

Despite their clear shortcomings, the user-friendliness combined with adequate sensitivity for most clinical cases make that RDTs can be very valuable in situations where microscopy is unavailable or difficult to perform\(^38\).
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Molecular Diagnostics

Molecular diagnostics may overcome limitations of both microscopy and RDTs, with improved sensitivity being the main advantage. Polymerase Chain Reaction (PCR) is the most well-known molecular technique with an assay specific detection limit that ranges from 0.004 to 30 parasites/µl\(^86\). PCR based tests can be designed in such a way that they distinguish between *Plasmodium* species. A nested PCR developed by Snounou et al. is a commonly used method that can accurately differentiate between *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*\(^87\). Quantification is possible with the use of molecular probes or a SYBR green dye in real-time assays\(^88,89\). Especially real-time techniques are suitable for high throughput formats because of their integrated read-out, reducing the time required per sample\(^90\). Finally, molecular methods can be used to detect mutations and thereby differentiate between drug resistant and drug sensitive parasite strains\(^91,92\).

However, most molecular tools are difficult to implement in low-resource settings because they are complex and require specialized equipment. PCR-machines have high purchase and maintenance costs and need a constant supply of electricity. In most cases, nucleic acid extraction is required before amplification. The storage of reagents involves a cold chain and time from sample collection to test result varies between assays but generally takes 1 to 6 hours\(^90\). The cost per test highly depends on the assay used and the number of samples tested, but can be estimated at 0.4 to 5 USD for the reagents only\(^90\). The read-out either requires real-time machines or gel electrophoresis systems that produce toxic waste.

Despite these implementation difficulties, the value of molecular tools has been acknowledged and efforts are being made to overcome the limitations\(^93,94\). The platforms that are most well developed for malaria diagnostics with one or more advantages compared to traditional PCR-based systems are described below.

Loop mediated isothermal amplification (LAMP), for example, is an isothermal platform. The amplification of DNA runs at a single temperature, thereby circumventing the need for expensive and maintenance dependent thermocyclers used for PCR\(^95,96\). LAMP is relatively fast (<1 hour) and has an easy fluorescence or turbidity-based read-out system. The reported LoD is
1–5 parasites/µl, sensitivity is >89.5% and specificity >85.0%, compared to a PCR-based reference standard\textsuperscript{96–101}. Disadvantages of LAMP include the need for DNA extraction, the lack of an internal amplification control and difficulties to develop multiplex assays.

Nucleic acid sequence based amplification (NASBA) is also an isothermal technique, based on the amplification of RNA\textsuperscript{102}. The reaction runs at 41 °C, does not include a DNA denaturing step and a T7 polymerase sequence on one of the primers ensures amplification of single stranded RNA only. A major advantage of NASBA is that it can be used to detect stage specific genes, for example \textit{Pfs25} in \textit{Plasmodium falciparum} gametocytes\textsuperscript{103}. NASBA runs for <1 hour, can be used in a quantitative format (QT-NASBA), is highly accurate and can detect very low parasite densities (0.02 parasites/µl)\textsuperscript{104,105}. However, the detection requires a molecular beacon and therefore specialized real-time machines, which limits the applicability of NASBA in low-resource settings.

PCR Nucleic Acid Lateral Flow Immunoassay (PCR-NALFIA) is using a different way to overcome the limitations of conventional molecular tools. This assay is based on a 1 hour PCR protocol, with a lateral flow based read-out. Lateral flow devices are commonly used for serological applications (like RDTs), but detect labelled PCR-amplified products in the case of PCR-NALFIA\textsuperscript{106}. The nitrocellulose of the lateral flow device is coated with a line of antibodies against a label on the forward primer (eg digoxigenin (DIG)). The reverse primer has a biotin label and detection takes place through neutravidin labelled carbon. Neutravidin has a very high affinity to biotin. When a PCR-amplicon has been formed, it will bind to the antibodies on the nitrocellulose through the label on the forward primer and will be visualized by the binding of neutravidin labelled carbon to the biotin label on the reverse primer (Figure 2).

Main advantages of this technique include the high sensitivity (LoD: 1 parasite/µl), the possibility to differentiate between species in a multiplex assay and the use of an internal amplification control\textsuperscript{107}. Additionally, PCR-NALFIA has been transferred to a time and reagents saving direct-on-blood format, whereby no nucleic acid extraction is required\textsuperscript{108}. 
Figure 2. Principle of NALFIA detection.
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TREATMENT

Following the diagnosis of malaria, timely and effective treatment with antimalarial drugs is essential. However, the development of drug resistance is a common threat to malaria control, both historically and in current practice. Antimalarial drug resistance is defined by the WHO as: “the ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within tolerance of the subject”\(^{109}\). Extensive monitoring of treatment efficacy is thus needed for the timely detection of drug resistance. Additionally, the development of new drugs is a continuous challenge to ensure the availability of effective treatment options\(^{110,111}\).

Quinine, extracted and isolated from the bark of the Cinchona officinalis tree, was the main drug for the treatment of malaria up to the 1920s, when more effective synthetic antimalarials became available. Of these synthetic drugs, chloroquine was the most frequently used\(^{112}\). However, \textit{P. falciparum} resistance to chloroquine emerged in Southeast Asia and South America by the late 1950s and in Africa by the late 1970s. Chloroquine resistance became widespread in many \textit{P. falciparum} endemic areas by the 1980s. Polymorphisms in the \textit{P. falciparum} chloroquine resistance transporter (\textit{pfcrt}) or multidrug resistance transporter 1 (\textit{pfmdr1}) genes were found to be responsible for chloroquine resistance\(^{113–115}\). To date, chloroquine remains effective against \textit{P. vivax} in many areas, but it is only in a number of South American countries still recommended for the treatment of \textit{P. falciparum} malaria \(^2\). Due to the decreased effectiveness of chloroquine, quinine started to play an important role again. Despite sporadic observations of quinine resistance, it remains an important drug until today, particularly as an intravenous treatment for severe malaria\(^2,116\).

Another important antimalarial is sulfadoxine–pyrimethamine (SP). Upon the development of pyrimethamine as an antimalarial, it was combined with sulfadoxine to increase efficacy and prevent or delay the development of resistance. However, resistance developed soon after the introduction of SP in Thailand in 1967 and rapidly spread throughout Southeast Asia, causing up to 90% treatment failures to SP in the Thai–Cambodian border area in the early 1980s\(^{117}\). In Africa, SP–resistance was not common until the late 1990s but has quickly spread since then\(^{118,119}\). Mutations associated with SP–resistance are found in the dihydrofolate reductase (\textit{pf dhfr}) and dihydropteroate
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synthase (pfdhps) genes. SP is currently no longer used as first-line treatment. However, it is still in use as intermittent preventive treatment during pregnancy (IPTp) due to its adequate safety profile and prophylactic effect. Indeed, it was found that even in high-resistance areas, IPTp is still an effective protective measure against low birth weight and maternal anaemia, which are common clinical consequences of malaria in pregnancy.

Artemisinin was isolated in 1971 from *Artemisia annua*, known as qinghao to Chinese herbalists for over 2000 years, and proved to be a very potent antimalarial treatment. When used as a monotherapy, the short half-life of artemisinin or artemisinin derivatives implies that a treatment course of at least 7 days is required. Not completing the full course can lead to treatment failure, even in the absence of drug resistance. Combining artemisinin derivatives with a longer half-life partner drug enabled a reduction in treatment duration and decreased the chance of drug resistance development.

Examples of such partner drugs include amodiaquine, lumefantrine and mefloquine. Mainly to prevent the development of artemisinin resistance, the use of artemisinins as monotherapy is not recommended since 2006. Instead, the WHO recommends the use of artemisinin based combination therapies (ACTs) for the treatment of uncomplicated *P. falciparum* malaria. ACTs were implemented in most *P. falciparum* endemic countries during the first decade of the 21st century, replacing failing therapies like chloroquine and SP. To date, the most commonly used ACTs include artemether–lumefantrine (AL), artesunate–amodiaquine (AS–AQ), artesunate–mefloquine (AS–MQ) and dihydroartemisinin–piperaquine (DHA–PPQ). Pyronaridine–artesunate (PA) is a novel ACT that received a positive opinion from the European Medicines Agency (EMA) in 2015, is approved in a growing number of African and Asian countries and is on the WHO list of essential medicines. It is currently not (yet) included in national treatment guidelines.

With the increasing efforts to reduce malaria transmission, it became highly important to evaluate not only the potential of ACTs to cure the asexual stage of the parasite, but also their effect on the transmission stage (gametocytes). ACTs are generally effective against asexual stages and immature gametocytes, while their activity against mature gametocytes is incomplete (in contrast to primaquine, see Discussion). However, differences between ACTs in the gametocyte response after treatment exist. A recent meta-analysis showed that the appearance of gametocytaemia in patients without gametocytes at baseline was lower after AL and AS–MQ compared to DHA–PPQ and
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AS-AQ. Among patients with gametocytes at baseline, clearance was faster after AS-MQ and slower after DP, compared to AL. This meta-analysis by the Worldwide Antimalarial Resistance Network (WWARN) hypothesized that the non-artemisinin partner drug is a relevant determinant for differences in the post-treatment gametocyte response\textsuperscript{127}. To accurately evaluate the gametocyte response after ACT treatment, molecular tools are particularly suitable since posttreatment gametocyte densities often fall below the detection threshold of microscopy\textsuperscript{128}. As described above, QT-NASBA is a sensitive and reliable technique for the detection of submicroscopic gametocytes, targeting the female-specific \textit{Pfs25}\textsuperscript{103,129}. Recently, a sex-specific quantitative reverse transcriptase PCR (qRT-PCR) has been developed and evaluated, differentiating between female (\textit{Pfs25}) and male (\textit{PfMGET}) gametocytes\textsuperscript{130}. This differentiation may be important, because the minority male population (normally 3–5 females to 1 male) was shown \textit{in vitro} to be more sensitive than females to a range of antimalarial drugs\textsuperscript{131}. Thus, faster clearance of the male gametocyte population during or after treatment might sterilize the infection, while the female-dominated gametocyte density may not be reduced to the same extent\textsuperscript{132}.

Finally, ACTs were initially believed to play an important role in containing resistance. However, in 2009, artemisinin resistance was reported in the Thai-Cambodian border, characterized by delayed parasite clearance\textsuperscript{133}. Artemisinin resistance was found to be associated with point mutations in the \textit{P. falciparum} kelch protein gene on chromosome 13 (K13) and it is currently spreading through Southeast Asia, threatening malaria control and elimination measures\textsuperscript{134–136}. There are major concerns that artemisinin resistance will spread to the African continent, where cure rates after ACT are currently still excellent\textsuperscript{134}. Extensive monitoring of drug efficacy, testing new combinations of existing drugs and the identification and evaluation of new antimalarial compounds are important measures against the further spread of artemisinin resistance\textsuperscript{135}.
AIMS AND OUTLINE OF THIS THESIS

This thesis is divided in two sections. In section A, an overview is given of the currently available molecular diagnostics, their reported accuracy and possible ways to overcome implementation difficulties. Additionally, the development, laboratory validation and field evaluation of a simplified molecular tool is described: the direct-on-blood PCR Nucleic Acid Lateral Flow Immunoassay (db-PCR-NALFIA). Section B describes the results of a clinical trial investigating the efficacy and safety of the novel ACT pyronaridine-artesunate (PA) compared to the commonly used artemether-lumefantrine (AL) for the treatment of uncomplicated *P. falciparum* malaria in children. This study was conducted in Mbita, Western Kenya, a rural area with moderate transmission intensity and peaks around the long and short raining seasons (March–May and October–November). In addition to treatment efficacy, the effect of PA and AL on gametocytes was evaluated and the persistence of residual submicroscopic parasitaemia after treatment was assessed by molecular methods.

A. Molecular tools for the diagnosis of malaria

Both practical aspects and accuracy data are essential in determining which molecular test, if any, would be the most promising to be deployed in a certain setting. Chapter 2 provides an overview of the currently available molecular tools for the diagnosis of malaria and a systematic evaluation of their reported accuracy, including both traditional PCR-based techniques and newer methods. In chapter 3, the development and validation of two multiplex db-PCR-NALFIA assays is described, one detecting pan-*Plasmodium* and *Plasmodium falciparum* (pan/*P. falciparum*), and the other detecting pan-*Plasmodium* and *Plasmodium vivax* (pan/*P. vivax*). It also presents the results of a prospective evaluation in Western Kenya, where index test pan/*P. falciparum* db-PCR-NALFIA is compared to an RDT and evaluated against microscopy and real-time PCR as reference standards.

B. Evaluating drug efficacy, gametocyte dynamics and parasite clearance

Chapter 4 describes the results of a randomized controlled phase III non-inferiority trial, where the efficacy and safety of PA was compared with AL for
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the treatment of uncomplicated *P. falciparum* malaria in Kenyan children aged ≤12 years. While there are multiple reports providing reassuring efficacy and safety data of PA in adults and adolescents, this study provides much needed confirmatory data for PA in children.

In *chapter 5*, the QT–NASBA based gametocyte response after PA-treatment was compared to that after AL. Furthermore, a quantitative reverse transcriptase PCR (qRT-PCR) was used to differentiate male and female gametocyte dynamics. This is the first report to describe the kinetics of submicroscopic gametocytes after PA treatment in comparison to AL.

Molecular diagnostics can also be applied for monitoring (asexual) parasite clearance dynamics. Submicroscopic residual parasitaemia was previously found to be common after treatment and associated with a higher transmission potential and increased risk of treatment failure\textsuperscript{137}. The availability of a sensitive and reliable method to monitor treatment efficacy would help clinicians and researchers to detect possible resistance early and adjust treatment regimens where necessary, especially when such test is able to predict a failure shortly after treatment initiation and can be performed in field settings. *Chapter 6* describes (submicroscopic) parasite clearance after PA and AL as determined by real-time PCR and db–PCR–NALFIA. Furthermore, the association between the molecular test result on day 7 after treatment initiation and subsequent treatment failure or success was established.

Finally, in *chapter 7* the findings of this thesis are discussed.
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REFERENCES

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57. WHO. Basic malaria microscopy – part I: Learner’s guide. 2nd Edn. (World Health Organization, 2010).


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119. WHO. Guidelines for the treatment of malaria. in (World Health Organization, 2006).


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