Moving towards improved malaria control
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Chapter 7

General discussion
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GENERAL DISCUSSION

The aim of this thesis was to contribute to malaria control efforts by evaluating the accuracy of currently available and newly developed molecular malaria diagnostics and by determining the efficacy and safety of a novel artemisinin-based combination therapy (ACT). Molecular tools were not only evaluated for a potential role in clinical practice, but also for their application in patient follow-up after treatment. Finally, the transmission potential after two different ACTs was addressed by assessing gametocyte dynamics after pyronaridine-artesunate (PA) compared to those after artemether-lumefantrine (AL).

1. Molecular malaria diagnostics
Malaria is traditionally diagnosed by microscopy and since the 1990s also by antigen based rapid diagnostic tests. Upon the development of polymerase chain reaction (PCR) in 1983 (patented in 1987), this technique was soon introduced in many different fields, including malaria research. Since then, numerous PCR-based assays for the detection of Plasmodium species have been developed. PCR was found to be very sensitive, with a detection limit below that of microscopy. Furthermore, it can be used in high throughput formats, can reliably differentiate between species and quantify parasites. Despite its widespread use in research settings, the implementation of PCR in routine diagnostics is not always easy, especially in resource-limited settings. Major obstacles are the need for a stable source of electricity, expensive equipment and highly trained personnel. Several platforms are therefore under development that make implementation easier. A well-known example is loop-mediated isothermal amplification (LAMP), which doesn’t need expensive PCR machines for amplification and has an easy read-out system. An alternative to LAMP is direct-on-blood PCR nucleic acid lateral flow immunoassay (db-PCR-NALFIA), whereby no sample preparation is required and the read-out is done on an easy and fast lateral flow device. The development, laboratory validation and field evaluation of db-PCR-NALFIA with species differentiation capacities was described in chapter 3. To determine which molecular test, if any, would be the most promising to be deployed in a certain setting, both practical aspects and accuracy are of major importance. In chapter 2, an overview was given of the currently available molecular tools for the diagnosis of malaria and a systematic evaluation of their reported
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accuracy was provided, including both traditional PCR-based techniques and newer methods. The systematic review (chapter 2) as well as the evaluation of db-PCR-NALFIA (chapter 3) show high accuracy of molecular tools, and the outcomes of these studies deserve further consideration.

Three traditional and commonly used PCR-based techniques were identified in chapter 2: conventional PCR, nested PCR and real-time PCR. Meta-analysis showed high sensitivity and specificity for all three, and no important differences in accuracy were found. This implies that there are no clear directives, other than practical ones, to choose one technique over the other. However, summary estimates from the meta-analysis should not be interpreted in isolation from the results of the original paper, as considerable heterogeneity exists, especially in the evaluations of conventional PCR. Several other molecular tools for the diagnosis of malaria were identified and included LAMP, (db-)PCR-NALFIA, RNA hybridization assay, PCR–enzyme–linked immunosorbent assay (PCR-ELISA), nucleic acid sequence based amplification (NASBA), ligase detection reaction–fluorescent microsphere assay (LDR-FMA) and photo-induced electron transfer–PCR (PET–PCR)

Sensitivity of these techniques was overall high, while specificity was more variable when microscopy was used as a reference standard. The use of a PCR-based method as a reference standard generally increased the specificity. This was also true for the field evaluation of db-PCR-NALFIA as presented in chapter 3, which showed high sensitivity but disappointing specificity against reference standard microscopy and a clear increase in specificity when qPCR was used as a reference standard. This can be explained by the fact that microscopy is an imperfect reference standard with a higher detection limit than most molecular tools. Thus, when evaluating a molecular test against reference standard microscopy, some false positives may actually represent low-density submicroscopic infections, leading to underestimations of specificity.

Some of the above mentioned alternative diagnostics provide clear implementation advantages above the traditional PCRs, of which LAMP and db-PCR-NALFIA are the most extensively evaluated assays, both in the laboratory and in field settings. In terms of input material db-PCR-NALFIA is very convenient (whole blood), whereas LAMP uses several crude and sophisticated isolation techniques and the RNA hybridization assay requires lysed red blood cells. LAMP, NASBA and the RNA-hybridization assay all use
(semi-)isothermal amplification, circumventing the need for PCR-machines. However, the RNA hybridization assay requires an overnight step and is thus less suitable for clinical practice. The read-out is easy and fast for LAMP (UV-light), db-PCR-NALFIA (lateral flow device), PCR-ELISA (ELISA plate reader) and all real-time PCR techniques, although the latter two require specialized equipment. Another promising technique is lateral flow recombinase polymerase amplification (LF-RPA), which combines isothermal amplification with an easy lateral flow-based readout. Laboratory evaluations of LF-RPA reported a limit of detection (LoD) of 0.1–4 parasites/µl, but no results from field evaluations have been described thus far\textsuperscript{18,19}.

The techniques that have recently been developed or are currently under development have their limitations too. Molecular tools still need a stable power source for amplification. Most techniques require a cold chain for storage of reagents, even though LAMP and LF-RPA have the advantage that (part of) their reagents are dried and do not need cold storage\textsuperscript{10,19}. Precise pipetting is essential for the success of molecular techniques, implying that they can only be performed by well-trained technicians. Amplicon contamination is a major concern in many molecular amplification techniques, which can be resolved by closed systems where tubes do not need to be opened after amplification (real-time PCR, NASBA, LAMP). Finally, unlike microscopy, there is very little standardization of molecular malaria diagnostics. Many different assays exist and often in-house assays or published protocols are used, which complicates the comparison between laboratories\textsuperscript{20}. Among laboratories routinely performing molecular diagnostics, proficiency testing and/or external quality assessment programs are essential in monitoring the quality of work\textsuperscript{21}.

The identification of four \textit{P. malariae}, one \textit{P. ovale} and one dual \textit{P. malariae/P. falciparum} infection in the db-PCR-NALFIA field evaluation as described in chapter 3 confirms results from other studies in sub-Saharan Africa that, even though \textit{P. falciparum} is the predominant species, the occurrence of non-\textit{falciparum} infections should remain to be considered\textsuperscript{22,23}. This implies that molecular diagnostics should be able to differentiate between species
or at least use a generic pan-\textit{Plasmodium} target next to a \textit{P. falciparum} specific one. A multiplex format is preferable to multiple tests per patient, and db-PCR-NALFIA has an advantage to LAMP in this respect, as LAMP is difficult to develop into a multiplex format.

The implementation of molecular diagnostics will not have the same impact in every setting. While microscopy and RDTs were shown to underestimate parasite prevalence compared to PCR on a population level\textsuperscript{24,25}, they have sufficient sensitivity to detect most clinically relevant cases and in the near future will remain the most important diagnostics for case management in high transmission settings\textsuperscript{26,27}. However, malaria prevalence is declining in many areas and in (pre-)elimination settings, microscopy and RDTs may not have sufficient sensitivity to reduce transmission in, for example, mass screening and treatment (MSAT) programs\textsuperscript{22,26,27}. Highly sensitive molecular tools are expected to detect more cases and could therefore improve the efficacy of MSAT programs, if implementation issues can be overcome\textsuperscript{14,28}. In this respect, assays like LAMP and db-PCR-NALFIA may be good candidates to be deployed in MSAT programs, where sensitivity, high-throughput and speed are crucial\textsuperscript{11,29}. Recent efforts to develop mobile laboratories that bring (molecular) diagnostics to the field and enable testing on the spot may be helpful to facilitate the implementation of molecular diagnostics\textsuperscript{30,31}.

Molecular diagnostics can also be applied for monitoring parasite clearance after treatment. Patient outcomes may benefit from a sensitive and reliable method that detects possible resistance early and enables timely adjustment of 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. In \textit{Chapter 6}, it was found that residual submicroscopic parasitemia detected by db-PCR-NALFIA at day 7 after the start of artemisinin-based combination therapy (ACT) treatment was associated with recurrent parasitemia (odds ratio: 3.410, 95% CI: 1.513 - 7.689, \(P=0.003\)). Remarkably, the association between residual parasitemia and parasite recurrence was not found when using qPCR as a detection method (odds ratio: 0.701, 95% CI: 0.312 - 1.578, \(P=0.391\)). This difference between qPCR and db-PCR-NALFIA may be explained by a difference in LoD (~0.2 and 1 p/μl, respectively), whereby qPCR detects very low parasite densities that may not be of relevance for later treatment failures. While the odds ratio is valuable for
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research purposes, predictive values (which compose the odds ratio) are more useful in clinical practice. Here, it became clear that a positive db-PCR-NAL-FIA at day 7 was actually not predictive for treatment failure. The factor that contributed most to the positive odds ratio was the negative predictive value (NPV): a negative db-PCR-NAL-FIA test at day 7 was in most cases predictive for an adequate clinical and parasitological response (ACPR) during follow-up (NPV: 82.4% (95% CI: 75.8 – 87.4)).

2. Pyronaridine-artesunate for the treatment of malaria

To reinforce malaria control efforts, there is not only need for highly accurate diagnostics, but also for new, effective and safe antimalarial drugs. Pyronaridine-artesunate (PA) is a novel artemisinin-based combination therapy (ACT) that was found to be well tolerated and effective for the treatment of uncomplicated \textit{P. falciparum} malaria and the blood stage of \textit{P. vivax} malaria in previous phase III studies\textsuperscript{32–36}. However, further confirmatory data for PA are warranted, especially for young children. In chapter 4, the results of a randomized controlled non-inferiority study were described, comparing the efficacy and safety of PA to that of artemether-lumefantrine (AL) for the treatment of uncomplicated \textit{P. falciparum} malaria in Kenyan children aged 6 months to 12 years. Despite the fact that the planned sample size was not reached, PA was found to be well tolerated and non-inferiority of PA to AL was demonstrated. This finding is in line with a previous study in children\textsuperscript{35} and PA may be a good candidate for inclusion in pediatric malaria treatment programs.

In the study described in chapter 4, children <20 kg received the pediatric formulation of PA, consisting of soluble granules instead of tablets. Pediatric ACT formulations have been developed to overcome difficulties with the treatment of young children using standard tablet formulations. Problems include inability to swallow entire tablets, inappropriate dosing when using crushed tablets and vomiting due to unpalatable drugs\textsuperscript{37}. As a consequence, compliance may not be optimal in young children\textsuperscript{37}. Besides for PA, pediatric formulations also exist for AL, artesunate-mefloquine (AS-MQ) and artesunate-amodiaquine (AS-AQ), consisting of dispersible or soluble tablets, granules or powder for suspension\textsuperscript{37–40}. A systematic review and meta-analysis showed that pediatric ACT formulations had similar efficacy and safety profiles compared to the standard tablet formulation, but fewer patients
AL is the most widely used first-line treatment for uncomplicated *P. falciparum* malaria in Africa, with good safety and efficacy records\(^41\). It needs to be taken twice a day for three days with fatty food for optimal absorption of lumefantrine. PA, on the other hand, needs to be taken only once per day for three days and does not need to be taken with food. The same holds true for dihydroartemisinin–piperaquine (DHA–PPQ), AS–AQ and AS–MQ\(^42\). The advantage of the six-dose regimen of AL is that it allows for optimally effective drug concentrations over the complete treatment period. However, a regimen with extra doses may lead to poorer adherence in unsupervised treatment settings\(^43\). Furthermore, patients with acute malaria often have a poor appetite, which may compromise the efficacy of lumefantrine\(^44\). The half-life of lumefantrine is relatively short (approximately 3.2 days) and exposes patients to a risk of early reinfection because it provides little prophylactic effect shortly after treatment\(^44,45\). The partner drugs of other ACTs have longer half-life estimates: 13.2 days for pyronaridine, 28 days for piperaquine, 9 days for amodiaquine and 21 days for mefloquine\(^42,46–48\). Indeed, when comparing PA to AL, a previous study found that PA had a lower reinfection rate than AL on day 28 and 42 after treatment initiation\(^32\). This finding could not be confirmed in the study described in chapter 4, although a slightly higher proportion of reinfections occurred in the AL group compared to the PA group. DHA–PPQ is the ACT with the longest half-life and several studies have shown fewer reinfections after this drug compared to AL\(^49–51\). Even though longer half-lifes can provide a prophylactic effect after treatment, which is beneficial for the individual patient, this comes at a cost of subtherapeutic drug levels in the weeks after treatment that may provide a selective force for the emergence of drug resistance\(^52\). With the existing and expanding range of ACTs to choose from as first-line treatment, the above mentioned drug characteristics are important to take into consideration. For example, in high transmission areas where

experienced drug-related vomiting and gastrointestinal disorders\(^37\). Thus, the availability of convenient, safe and effective pediatric ACTs, including PA, may benefit the management of malaria in children. However, very young children are not always well represented in (pediatric) efficacy studies\(^35,36\). For example, in the present study, 40.6% (41/101) of participants in the PA group received the granule formulation, but none were <1 year old. Both the present study and previous reports conclude that there is a need for further PA efficacy and safety data in infants\(^35,36\).
people experience frequent re-exposure, a long-lasting prophylactic effect may prevent a large number of reinfections and thereby protect the individual as well as avoid further transmission\textsuperscript{53}. In low transmission areas, on the other hand, a long-acting drug regimen may be less advantageous and other characteristics, such as gametocytocidal properties, may be more important (see part 3 of this discussion)\textsuperscript{54}.

Containment of artemisinin-resistance in western Cambodia and the Thailand–Cambodia border area is urgently needed for local and global malaria control efforts and new effective antimalarial treatment options are therefore required\textsuperscript{55}. Selecting another ACT for this purpose is not necessarily excluded, at least before a new generation of non-artemisinin antimalarials becomes available, because thus far ACTs retain efficacy in the absence of resistance to the partner-drug\textsuperscript{56}. When partner-drug resistance is present, treatment failure rates increase and resistance can spread. Because pyronaridine showed high in vitro activity against multidrug resistant \textit{P. falciparum} and had not regularly been used as a monotherapy, it was hoped that pyronaridine partner-drug resistance was uncommon\textsuperscript{57,58}. However, in western Cambodia, a recrudescence rate of 10.2\% after PA was reported, versus 0\% for AS–MQ\textsuperscript{34}. This is indicative of reduced \textit{P. falciparum} susceptibility to the pyronaridine, although further confirmatory studies are warranted. Furthermore, in the same study, parasite clearance times after PA and AS–MQ were found to be significantly increased in Cambodian patients compared to patients from other countries, which is suggestive of artemisinin resistance\textsuperscript{34}. Given the urgent need for effective antimalarial treatments in Cambodia and for confirmatory efficacy data of PA in this area, a recent study assessed the efficacy of PA for the treatment of uncomplicated \textit{P. falciparum} malaria in an area of artemisinin resistance in western Cambodia\textsuperscript{58}. The overall PCR-corrected day-42 ACPR was found to be 87.9\% (95\% CI: 80.6 – 93.2), which is below the WHO-recommended threshold of 90\% ACPR for first-line treatment of \textit{P. falciparum} malaria\textsuperscript{59}. The exact reason why the efficacy of PA in western Cambodia is not as high as in other areas, remains unknown at present. Cross-resistance of pyronaridine with piperaquine was suggested, but \textit{in vitro} data indicate that this is not the case\textsuperscript{56}. To date, AS–MQ is still effective in western Cambodia, but the WHO expert group on drug efficacy and response recommends efficacy and safety evaluations of PA + atovaquone–proguanil (AP) to be conducted in case the alarming scenario becomes true that there are no other treatment
options in this region. These findings indicate that even a novel drug with very little exposure, may not be equally effective in every area. However, despite the issues in Western Cambodia, PA has important clinical utility in African (chapter 4) and other Asian countries, and possibly in other parts of Cambodia or in combination with other antimalarials.

The most important safety concern about PA in previous studies was the transient increase of the hepatic enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST). A systematic review and meta-analysis showed that ALT and AST elevations were four times more frequent after PA than after other ACTs. Elevations were present at day 3 and 7 after treatment initiation and levels were normalized or decreasing by day 28. Grade 3/4 toxicities were defined as ALT or AST levels >5 times the upper limit of normal (ULN) and occurred at day 7 for ALT in 0.9% (24/2709) of patients and for AST in 0.3% (9/2711) of patients. These rises in ALT and/or AST were rarely associated with increases in total bilirubin (0.2%, 7/2815), in which case drug-induced liver injury is a serious concern (Hy’s law). One of the 7 patients also had elevated alkaline phosphatase (ALP), indicative of an alternative underlying cause and precluding the patient as a Hy’s law case. Importantly, none of the patients had clinical sequelae as a result of the liver enzyme changes and all elevations were transient. An independent data and safety monitoring committee, including hepatotoxicity experts, concluded that even though PA treatment is associated with transient transaminase elevations, the early onset and rapid resolution are consistent with direct low-level toxicity. Because the treatment regimen of PA is only 3 days, the risk of progressive liver injury was estimated to be very small. Reassuringly, a study assessing safety after re-treatment with PA confirmed that hepatotoxicity events did not increase after treating multiple malaria episodes. Surprisingly, and in contrast to previous studies, the trial as described in chapter 4 did not find any grade 3 or 4 toxicities. Although this is an encouraging safety finding, it should be noted that the sample size was smaller compared to other studies and not for all participants (enough) serum was available to perform all ALT/AST measurements. Other safety findings were in line with previous reports. Mean hemoglobin concentrations decreased by ~0.8 g/dL at day 3, but recovered by day 28, similar to other ACTs. No serious adverse events occurred and most
common adverse events after PA treatment included headache, vomiting and coughing. Overall, the present study confirms the results from earlier reports that PA was well tolerated and had a similar adverse event profile compared to other ACTs.

While microscopy based parasite clearance after PA or AL treatment was very good in both the present and earlier studies, residual submicroscopic parasitemia as detected by molecular techniques, was found to be common in the study reported in chapter 6. For example, of the study participants who received PA or AL, respectively 96.0% (97/101) and 94.8% (91/96) cleared all parasites by day 3 as determined by microscopy. However, the qPCR-based parasite clearance at day 3 was only 21.6% (16/74) after PA and 37.7% (26/69) after AL. This finding is not exceptional: high levels of submicroscopic residual parasitemia after ACT treatment have been described previously. In the present study, qPCR based parasite clearance at day 2 appeared to be faster after AL compared to PA, but this difference was less clear at day 3 and absent at day 7 after treatment initiation. Importantly, while the qPCR-based prevalence remained high during the first days after treatment, the parasite density rapidly decreased for both PA and AL, in agreement with previous reports. Day 1 densities were <2% of the enrollment value and day 7 densities were around 0.02% of baseline. The persistence of low parasite densities after apparently successful ACT treatment may shed new light on parasite clearance dynamics after ACT. The clinical relevance of this persistence remains to be further investigated, as there appears to be conflicting evidence. While two studies found an association between residual parasitemia shortly after treatment and parasite recurrence during follow-up, this association was not found by Chang et al. The present study found an association between recurrent parasitemia and db-PCR-NALFIA but not qPCR positivity at day 7. To further establish the clinical relevance of residual submicroscopic parasitemia after ACT treatment, estimated by molecular techniques, it is important to assess the viability of these low-density infections, for example by attempting to culture the residual parasites. Viable residual submicroscopic parasites may clear slowly, remain as low-density chronic infections or cause a recrudescence. In either case, these parasites might be an important contributing factor to ongoing transmission.
3. Monitoring gametocyte dynamics after treatment

With the increasing efforts to control and eliminate malaria, it becomes highly important to evaluate not only the efficacy of antimalarial drugs against asexual *Plasmodium* stages, but also their effect on gametocytes. Any effective antimalarial reduces the duration of infectiousness compared to untreated or partially treated individuals by killing asexual parasites, the source of gametocytes. Gametocytocidal activity can further reduce the transmission potential, but differs between drugs and stages of development. While immature *P. falciparum* gametocytes are susceptible to most commonly used antimalarials to a certain extent, this is not the case for mature gametocytes. Thus, especially mature gametocytes can persist and cause onward transmission when asexual parasites have been cleared. Gametocyte carriage might even be increased after treatment with certain antimalarial drugs, like chloroquine and SP, either by continued gametocyte production (possibly enhanced by drug-induced stress caused by slow-acting antimalarials) or by efflux of sequestered gametocytes. Treatment with ACTs generally results in lower levels of gametocyte carriage and posttreatment transmission potential compared to non–ACTs. Nevertheless, gametocytocidal differences exist between ACTs. A systematic review and meta-analysis evaluating the gametocyte dynamics after four different ACTs found that the appearance of gametocytemia among patients without gametocytes at enrollment, was higher after DHA-PPQ and AS-AQ than after AL and AS-MQ. Additionally, among patients with gametocytes at baseline, gametocyte clearance is faster after AS-MQ and slower after DHA-PPQ, compared to AL. The difference between AS-AQ and AS-MQ is remarkable and cannot be explained by artemisinin dosing or treatment outcome, but may be related to the non-artemisinin partner-drug. Indeed, *in vitro* drug screening assays indicated that developing gametocytes appear to be more susceptible to mefloquine and lumefantrine than to amodiaquine.

It is essential to determine the gametocytocidal activity of not only the most commonly used ACTs, as described above, but also that of novel antimalarial treatments. In the case of PA, previous studies found no significant difference in gametocyte clearance rate compared to AL or AS-MQ. However, these studies were all based on microscopically detectable gametocytes. Already in the 1930s it was observed that mosquitoes can become infected after taking a blood meal that did not contain microscopically detectable gametocytes.
Molecular methods later confirmed the presence of submicroscopic gametocytes capable of transmission\textsuperscript{71}. Thus, because gametocytes often circulate at low densities, microscopy is not sensitive enough to detect all gametocyte carriers relevant for transmission. Molecular methods provide more accurate estimates of post-treatment gametocytemia\textsuperscript{66}. In chapter 5, the gametocyte dynamics after PA and AL treatment were evaluated using QT–NASBA and quantitative reverse transcriptase PCR (qRT–PCR). The duration of gametocyte carriage and gametocyte circulation time were found to be slightly longer after PA. This suggests that the transmission potential may be higher after PA than after AL, which needs to be confirmed using mosquito feeding assays. Future studies should also confirm the position of PA relative to other ACTs in terms of gametocyte clearance time.

While the above mentioned drugs lack activity against mature gametocytes, highly effective gametocytocidal treatments do exist. Primaquine is the most well-known example: it actively clears mature \textit{P. falciparum} gametocytes, which results in a shorter duration of posttreatment (submicroscopic) gametocyte carriage\textsuperscript{72–75}. However, primaquine does not effectively clear asexual parasites and immature gametocytes, and thus needs to be combined with an effective ACT for the treatment of \textit{P. falciparum} malaria\textsuperscript{75}. Despite the advantage in terms of reducing transmission potential, the use of primaquine is limited due to its hematological toxicity in people with glucose–6–phosphate dehydrogenase (G6PD) deficiency\textsuperscript{76,77}. Hemolysis induced by primaquine in G6PD deficient individuals can occur after a single dose and is dose dependent. In low transmission settings, the WHO initially recommended a dose of 0.75 mg base/kg ‘when the risk of G6PD deficiency is considered low or when G6PD testing is available’\textsuperscript{78}. With this recommendation the WHO targeted two situations: malaria elimination programs and to prevent the spread of artemisinin resistance\textsuperscript{79}. Unfortunately, G6PD testing is not widely available in all malaria endemic areas. Thus, the WHO recommended that single–dose primaquine used as a gametocytocide for \textit{P. falciparum} should be reduced to a dose of 0.25 mg base/kg, as initial evidence indicated that this was safer and equally effective compared to higher doses\textsuperscript{80}. However, at the time of this recommendation, additional data were still required to determine the safety and efficacy of low–dose primaquine. Reassuring results were obtained in non–G6PD individuals\textsuperscript{81–83}. Future safety studies of low–dose primaquine need to be conducted in individuals with G6PD deficiency to determine whether primaquine
community interventions without testing for G6PD deficiency can be done safely. The above mentioned studies used primaquine in combination with DHA-PPQ, AL and SP-AQ. A potential future combination with PA would require safety evaluations as well. Finally, the challenge of finding safer alternatives to primaquine is ongoing. Methylene blue was recently evaluated and found to be well tolerated and efficacious for preventing transmission.

Not only the type of drug and drug dosing influence the gametocyte response and transmission potential after treatment, but also the gametocyte sex ratio may play an important role. In vitro studies found that male gametocytes are more susceptible to most commonly used antimalarial drugs compared to females. The ratio of male to female P. falciparum gametocytes is usually unequal, with a clear female bias, presumably because each male gamocyte can produce eight microgametes and thus fertilize up to eight females. Most commonly found sex ratios are 1 male to 3–5 female gametocytes. The hypothesis was raised that if male gametocytes are indeed cleared faster from the circulation, the infection might actually be sterilized before a clear drop in the female dominated gametocyte density occurs. Thus, differentiation between male and female gametocytes may be important. QT-NASBA is commonly used for the detection of posttreatment gametocytemia, but is female specific. In chapter 5, a recently developed qRT-PCR was used to evaluate the male and female gametocyte response after PA and AL. No indications were found that PA or AL preferentially cleared male gametocytes. In fact, while the prevalence of male gametocytes was lower at baseline, they appeared to clear slower than females. Even though this seems to contrast with the previous in vitro findings, it is important to note that the in vitro assays measured the ability of gametocytes to activate rather than the presence of mRNA, which may be detectable in nonfunctional gametocytes still present in the circulation. Thus, despite the clear added value of molecular techniques, functional assays that determine gametocyte fitness or infectivity remain crucial in assessing transmission-blocking properties of antimalarial drugs.

Notwithstanding the importance of identifying the gametocytocidal properties of antimalarial drugs, many gametocyte positive individuals are asymptomatic and, while they contribute considerably to onward transmission, they do not seek treatment. Thus, even though the gametocytocidal activity of
the first-line ACT can influence transmission, this effect may be small on a population basis when transmission is mainly driven by the asymptomatic reservoir. The success of efforts to reduce transmission through treatment regimens therefore depends on the ability to reach both the symptomatic and asymptomatic population. For example, including asymptomatic carriers in treatment campaigns, where possible using transmission blocking drugs like primaquine, may have more impact on transmission than the choice of ACT.

Finally, there evidently is a relationship between sexual and asexual parasite clearance. A longer duration of asexual clearance is inevitably associated with a longer duration of gametocyte carriage, because the time at which asexual parasites are cleared determines when the generation of new gametocytes stops. Indeed, both microscopy-based and submicroscopic data from previous studies indicate that longer clearance times were related to increased gametocyte carriage. Chapter 6 confirmed this and found that residual submicroscopic parasitemia at day 7 was associated with higher prevalence and density of gametocytes at baseline, day 3 and day 7. The study by Beshir et al. showed that residual submicroscopic parasitemia was not only associated with increased gametocyte carriage but also translated into a higher transmission potential to mosquitoes. Besides prolonged clearance time, antimalarial treatment failure also has previously been related to increased gametocyte carriage. Mutations in pfcr and pfmdr1 genes conferring chloroquine resistance, as well as dhfr genes conferring SP resistance were associated with increased gametocyte carriage and transmission. The relation between resistance associated mutations, gametocyte carriage and transmission potential indicates that increased gametocyte carriage or transmission after treatment may serve as an early indicator of drug resistance. Currently, this may particularly be relevant for the early detection of artemisinin resistance, which is characterized by slow clearance and may also be associated with increased gametocyte carriage.

4. Future perspectives
Despite the clear challenges associated with the implementation of molecular diagnostics in resource-restricted areas, molecular tools potentially play an important role in especially malaria elimination settings. Obviously, strengthening of local laboratory and health care facilities is crucial to enable
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the implementation of molecular tools in a growing number of laboratories in the future\textsuperscript{101,102}. However, the development of new methods and/or adjustments to currently existing techniques may have substantial impact as well. Several tests with clear implementation advantages over traditional PCR-based methods have been described in this thesis, but there are still issues remaining that complicate their use and require attention. These include the dependence of molecular methods on electricity, the necessity of a cold-chain for most assays and, in the case of MSAT programs, reachability of large populations with short time to test result. Possible solutions that are currently explored include the development and use of mobile laboratories with on-board PCR-facilities, heat-stable (lyophilized) reagents and solar- or battery powered portal PCR-machines\textsuperscript{30,31,103}.

A major threat to malaria control and elimination is drug resistance, hence the search for new drugs, drug combinations and treatment regimens is of key importance. Nowadays, all new drugs (including non-ACTs) are developed as combination therapies to improve efficacy and reduce the risk of drug resistance development. Highly favorable are antimalarial drugs that provide ‘Single Encounter Radical Cure and Prophylaxis’ (SERCaP), which may have a high impact on malaria control\textsuperscript{104}. So far, this has only been achieved with SP and focusing just on SERCap potentially excludes valuable drugs that require longer treatment courses\textsuperscript{55}. Examples of new drugs that are in phase 2 of the development pipeline include ferroquine, cipargamin, artefenomel, MMV048, DSM 265 and KAF 156\textsuperscript{105}. As it will take at least several years before these candidate drugs will become generally available, the clinical development of alternative regimens of existing drugs to encounter the spread of artemisinin and partner drug resistance is essential\textsuperscript{55}. A relatively simple possibility is to extend the treatment course from 3 to 5–7 days, which has proven to be effective\textsuperscript{99}. However, with the use of fixed dose combination therapies, longer exposure to the artemisinin component implies that partner drug exposure is increased as well, which may lead to higher levels of toxicity or reduced tolerability\textsuperscript{55}. Alternatively, it has been proposed to give two courses of different ACTs sequentially, providing cross protection between the two partner drugs\textsuperscript{106}. Finally, triple therapies are successfully used for tuberculosis and HIV and studies are underway that evaluate the safety and efficacy of triple ACTs, containing two slowly eliminated partner drugs and one
artemisinin component\textsuperscript{55,107}. The advantage of both sequential and triple ACTs is that partner drugs can be chosen such that high resistance to one partner drug is associated with lower resistance to the other, for example: lumefantrine and amodiaquine or piperaquine and mefloquine\textsuperscript{55}.

Finally, the availability of effective and safe vaccines play a major role in malaria control and elimination. Several vaccine candidates are under development, of which RTS,S, a pre-erythrocytic stage vaccine, is the most advanced. A phase III multicenter study involving 15,459 children from 7 African countries estimated that 829 clinical malaria episodes per 1000 children were averted by RTS,S over an 18 month follow-up period\textsuperscript{108}. An overall vaccine efficacy of 46\% was obtained, which is low in comparison to vaccines for other diseases, but efficacies of 30–50\% are justified by the WHO based on the magnitude of the worldwide malaria problem\textsuperscript{109,110}. Pilot implementation of RTS,S is planned to start in Ghana, Kenya and Malawi in 2018\textsuperscript{111}. However, a recent study found that the vaccine efficacy of RTS,S drastically decreased over a 7-year period after vaccination\textsuperscript{112}, which emphasizes the need for alternative vaccines. The vaccine development pipeline consists of pre-erythrocytic stage vaccines (PfSPZ, GAP, CVac), blood stage vaccines (chemically attenuated parasites, AMA1-RON2, PfRH5) and transmission blocking vaccines (Pfs25, Pfs230, Pfs47)\textsuperscript{111}. All are in the preclinical phase, phase 1 or phase 2 of development (in contrast to RTS,S, which is in stage IV). It is aimed to combine the most promising vaccine candidates targeting different stages of parasite development in the future to achieve the highest efficacy\textsuperscript{111}. 
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