Aus der
Medizinischen Universitätsklinik und Poliklinik Tübingen
Abteilung VII, Tropenmedizin
(Schwerpunkt: Institut für Tropenmedizin, Reisemedizin, Humanparasitologie)

Development and implementation of molecular methods to genotype *Plasmodium falciparum* in uncomplicated malaria and severe malarial anemia in Gabon

Thesis submitted as requirement to fulfill the degree „Doctor of Philosophy“ (Ph.D.)

at the
Faculty of Medicine
of the Eberhard Karls Universität
Tübingen

by

Nguyễn, Thế Trọng

from

Ninh Binh, Vietnam

Year of graduation

2020

Dean:	Professor. Dr. I. B. Autenrieth
First reviewer:	Professor. Dr. B. Mordmüller
Second reviewer:	Professor. Dr. S. Wagner

Date of oral examination: 13.12.2019

Dedication

I would like to dedicate this work to my beloved family, especially my daughter, whose beautiful smile I have only been able to see on camera during my PhD time, since she was six months old.

Table of contents

List of abbreviations

ACTs	Artemisinin-based combination therapies
AD	Allelic discrimination
AMA	Apical membrane antigen
BM	Bone marrow
CE	Capillary electrophoresis
CERMEL	Center of Medical Research Lambaréné
CHMI	Controlled human malaria infection
CQ	Chloroquine
CQR	Chloroquine resistance
CSP	Circum-sporozoite protein
Ct-value	Cycle threshold value
DNA	Deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphates
EDTA	Ethylenediaminetetraacetic acid
FACs	Fluorescence-activated cell sorting
FGM	Fougamou
IL-10	Interleukin-10
LA	Lambaréné
LOD	Lower limit of detection
MAF	Minor allele frequency
MGB	Minor groove binding
MM	Mild malaria
MOI	Multiplicity of infection
MSP	Merozoite surface protein
NAT	Nucleic acid testing
NGS	Next generation sequencing
PB	peripheral blood
P. falciparum	*Plasmodium falciparum*
PCR	Polymerase chain reaction
PfCRT	*P. falciparum* chloroquine resistance transporter
RBC	Red blood cell

RDT	Rapid diagnostic test
RNA	Ribonucleic acid
RR	Rural region
RT	Reverse transcriptase
SMA	Severe malarial anemia
SNP	Single-nucleotide polymorphism
SSCP	Single strand conformation polymorphism
TBS	Thick blood smear
TNF	Tumor necrosis factor
WHO	World health organization
WWARN	World-Wide Antimalarial Resistance Network

1 INTRODUCTION

1.1 Malaria burden

Despite the tremendous progress in the fight against malaria over the past decade, the disease remains one of the most important infectious diseases. It was responsible for about 219 million cases and more than 435 000 deaths in 2017 worldwide (1).

Due to the improved access to malaria interventions between 2001 and 2013, a large decline in the overall mortality rate of about 47% has been achieved. Particularly, in African children under five years of age, the mortality rate was reduced by 58%. During the same period, global malaria incidence was reduced by 30%.

Based on this success, in the "Global technical strategy for malaria 2016 – 2030", World Health Organization (WHO) has set goals of at least a 40% reduction in malaria mortality rates and case incidence between 2015 and 2020. However, over the last years, the decline in malaria burden has stalled (**Figure 1**).

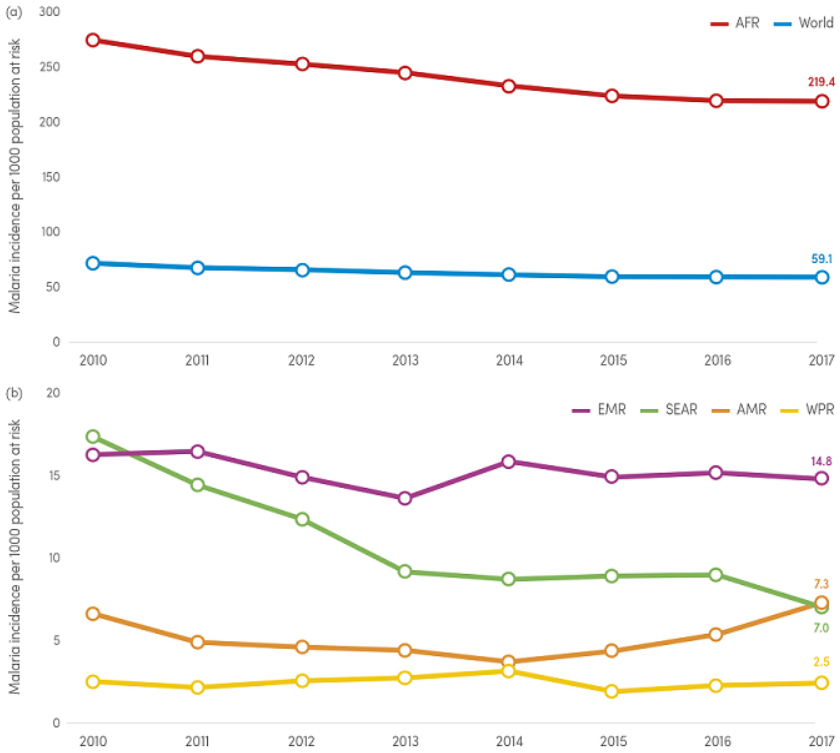

Figure 1. Malaria incidence *(https://www.who.int/gho/malaria/epidemic/cases/en/) (with permission).*

One possible explanation for the halted decline in malaria incidence is climate change (2). As discussed in the Roll Back Malaria's *Action and Investment to Defeat Malaria, 2016 – 2030*, change in temperature and rainfall may create new habitat for mosquitoes in several regions and could result in prolonging transmission season or increase the prevalence of vector, on the other side, drought could shorten transmission season and remove the vector's habitat in some malaria-endemic areas. However, exposing these new regions and populations to malaria may result in higher incidence and death rates due to low acquired-immune level against malaria and lack of treatment.

Another explanation involves the drug resistance of both vector and parasite. Since 2000, a great reduction in malaria mortality and morbidity rate has been linked with an extension on access to the two core vector control interventions: insecticide-treated bed nets and indoor residual spraying, particularly in sub-Saharan Africa. However, these achievements have been threatened by the rise of resistance to insecticides in malaria vector population worldwide. If neglected, insecticide resistance could result in a substantial increase in malaria incidence and death rate.

The emergence of drug and insecticide resistance is additionally constituted by some biological challenges including the high complexity of natural *Plasmodium* infection and diversity of malaria vectors. The former has been a major challenge for the development of new tools for malaria control and eradication e.g. vaccines and new antimalarial drugs.

Gabon is located in Equatorial Africa where malaria is highly endemic. In rural Gabon, as reported in our recent study, there were about 74% of the screened individuals carrying *Plasmodium* parasites, and 66% of these carriers were infected with *P. falciparum.* In addition, the high prevalence of anti-malarial drug resistance especially chloroquine resistance (CQR) is often documented (3).

1.2 Species and life cycle

1.2.1 Human *Plasmodium*

There are up to around 156 identified species of *Plasmodium* (4). Five of those are transmitted between humans and cause malaria: *Plasmodium falciparum*, *P. vivax*, *P. malariae*, *P. ovale curtisi* and *P. ovale walikeri*. *P. falciparum* is responsible for most cases and almost all deaths, especially in children under five years of age in Africa. *P.*

knowlesi is the sixth human malaria parasite. It causes a zoonotic disease that occurs only in Southeast Asia, where *Macaca fascicularis* and *Macaca nemestrina*, the main hosts, live.

1.2.2 Life cycle

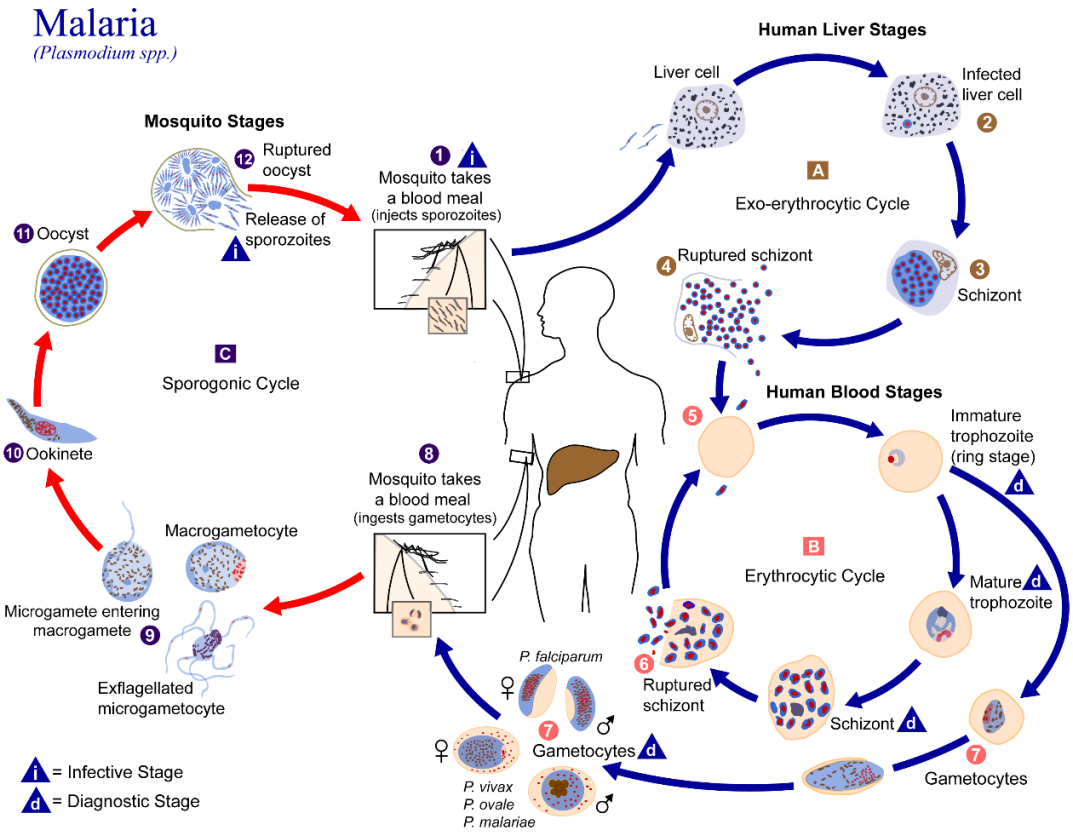

Figure 2. Life cycle of Plasmodium spp. *Source: CDC - DPDx/ Alexander J. da Silva, PhD; Melanie Moser (with permission).*

The unique and complex life cycle of the malaria parasite occurs within two hosts: mosquitoes and humans (**Figure 2**). Genetic recombination and meiosis happen after fertilization when the gametocytes are taken up during the blood meal of a female mosquito, developing into male and female gametes then mate and form a zygote. *Plasmodium* parasites are haploid except for the short diploid period (zygote) in the mosquito midgut. This unique trait allows the molecular approaches which target single-copy nuclear genes for identifying the malaria parasite since each genotype will represent a variant (so-called "strain"). Recombination may generate new variants that help parasite evade host immunity. For every *Plasmodium* species, recombination

events occur only when a mosquito carries multiple parasite strains. As a consequence, recombination rates are directly associated with frequencies of multiple infections and transmission intensity.

1.3 Natural *P. falciparum* infection

1.3.1 Genetic complexity of *P. falciparum* infection

1.3.1.1 Circulating parasite in peripheral blood (PB)

In malaria-endemic regions, individuals are infected repeatedly, and often by multiple parasite strains. The number of circulating parasite strains may vary between different malaria episodes and within the same patient. Most of the reported multiplicity of infection (MOI) was often greater than one. Multiple-strain infection can be a result of multiple infectious bites or a single bite from a mosquito infected with multiple parasite variants. In addition, MOI of the same parasite population could be different due to the sensitivity of genotyping approaches (5). MOI was found to associate with seasonal transmission intensity, the manifestation of the disease in pregnant women (6) and the risk of treatment failure (7,8). There was evidence suggesting the existence of some parasite genotypes that relate to the severity of malaria (9,10).

The multiplicity of malaria infection can complicate the identification of the individual strain. Minor strains can be suppressed by the major ones through resource competition (11), while the density of each parasite strain may change over the course of infection or even after treatment.

A high diversity of parasite populations may intensify difficulties in the development of malaria vaccines. Often, the vaccine protective efficacy is stronger against homologous than heterologous controlled human malaria infections (CHMI) (12) or matched genotype than mismatched genotypes in natural infections (13). In addition, the concept of parasite "strain-specific efficacy" suggests those whole sporozoite vaccines – a new generation of vaccine candidates – may have better efficacy against natural infection if able to cover multiple parasite strains.

1.3.1.2 Sequestering *P. falciparum* in human tissue

Most knowledge on the *in vivo* behavior has been gathered by the investigation of circulating *Plasmodium* in the peripheral blood. In contrast, little is known about this parasite when they are in human tissues including the bone marrow parenchyma.

There is evidence that the human bone marrow (BM) is a preferable place for sequestration and gametocyte maturation of *P. falciparum*.

Mature asexual *P. falciparum* stages (pigmented trophozoites and schizonts) and immature gametocytes are typically not found in peripheral blood (14). Smalley et al. in 1981 have shown that in *P. falciparum*-infected children, the density of immature gametocytes on bone marrow smears was greater than that on peripheral blood smears (15), however, their distribution in each compartment of bone marrow (intra- or extra-vascular) was not reported.

Later, post-mortem histological evidence has revealed the presence of malaria parasites in extravascular compartments of different human tissues including spleen, heart, lung, gut, brain and bone marrow (16,17). It was shown that only a small proportion of mature gametocytes were in the extravascular compartment, the majority being in intravascular spaces (18). Asexual, immature parasites were detected in human autopsies in both intra- and extra-vascular compartments. Extravascular sequestration is suggested to be crucial for gametocytogenesis and transmission of *Plasmodium* to the mosquito (19).

In addition, analysis of sequestering parasite by molecular technologies like polymerase chain reaction (PCR) has rarely been done (20), especially for bone marrow samples; although such investigations may help in understanding the pathogenesis of the severe forms of malaria, particularly anemia and severe anemia.

1.3.2 Diverse disease manifestation

1.3.2.1 Asymptomatic parasitemia

Asymptomatic parasitemia is defined as the presence of *Plasmodium* parasites (asexual forms) in circulation of the infected individual without any associated symptoms (21). In highly endemic areas, asymptomatic parasitemia occur mostly in adults, consist an important proportion of the overall prevalence of infection (3) and is considered as the most important infectious reservoir.

1.3.2.2 Uncomplicated malaria

In contrast with asymptomatic parasitemia, in uncomplicated malaria, the presence of parasitemia in circulation of infected individual is accompanied by malaria-associated

symptoms without signs of severity or of any vital organ dysfunction. This is the most common manifestation of malaria in endemic region and is often observed in children.

1.3.2.3 Severe malaria

Most malaria attributable deaths and severe disease are caused by *P. falciparum*. Other non-*falciparum* species were also found to cause severe malaria and the co-infection of different *Plasmodium* species could lead to more severe hematological impairment (22).

1.3.2.3.1 Symptoms defining severe *falciparum* malaria

Severe *falciparum* malaria is defined when a patient is infected with *P. falciparum* with the presence of one of the signs of severity or vital organ dysfunction. Severe *falciparum* occurs mostly in children under 5 years of age and often in some following forms: cerebral malaria (CM), metabolic acidosis, severe anemia, hypoglycemia, pulmonary and renal dysfunction (23).

1.3.2.3.2 Severe malarial anemia

Anemia is defined as a hemoglobin (Hb) concentration of lower than 10.9 g/dL (1). Using WHO's classification guideline, between 2015 and 2017, among children aged under 5 years, the overall prevalence of mild, moderate, severe and any anemia was 25, 33, 3 and 61%, respectively compared to 21, 50, 8 and 79% in group of children who tested positive for malaria by RDT. In tropical regions, not only by malaria, anemia is often caused by other concomitant reasons including other infections (sepsis, intestinal helminthic infection, schistosomiasis), nutritional deficiencies, hemoglobinopathies (thalassemia and sickle cell disease) or other red blood cell abnormalities like glucose 6 phosphate dehydrogenase (G6PD) deficiency (24,25). Anemic children are at a higher risk of developing severe disease including severe malaria.

In other forms of severe malaria e.g. cerebral malaria (CM), metabolic acidosis, renal failure, accumulation and sequestration of parasites in the microvasculature which results in microvascular obstruction and tissue hypoxia is considered the main pathophysiological mechanism. However, the role of malaria parasite in the pathogenesis of anemia is controversial.

Research efforts in understanding the pathogenesis of malarial anemia have suggested the multifactorial relation between SMA and increased red blood cell (RBC) destruction as well as impaired RBC production. The mechanisms of removing RBCs from the

circulation system involves the destruction of RBCs, by a filtration mechanism in the spleen, and phagocytosis. Apparently, parasite-induced destruction of parasitized RBC contributes to the reduction in RBC count, however, parasitemia alone is often not correlated with anemia severity, while the lysis of un-parasitized RBC was demonstrated to be the main contributor to rapid hematological impairment (26).
In peripheral blood, the association between severity of malaria (especially SMA) and structure as well as the diversity of the *P. falciparum* population has been described repeatedly. However, little is known about their local role, e.g. in bone marrow, and it is not clear whether the population structure of *P. falciparum* in bone marrow is associated with the disease pathogenesis.

1.4 Diagnosis

1.4.1 Malaria diagnosis

The outcome of malaria – especially *P. falciparum* malaria – relies crucially on a timely and reliable diagnosis that determines treatment and management. To achieve that, microscopy of thick blood smears and/or malaria rapid diagnostic test (RDT) have been recommended (21,27). Although TBSs are still considered the standard diagnostic tool, malaria RDTs have been used routinely in many field sites as well as in clinical practice, particularly where microscopy is not available (27–29). In Sub-Saharan Africa, RDTs have been increasingly used to test for malaria, from 40% of the tests were performed using RDTs in 2010 to an estimated 75% in 2017 (1).
The expansion in the use of RDT has resulted in an increase in sales of RDTs in Africa from 240 million in 2015 to 276 million in 2017 (1,29). The availability and common use are making RDTs more attractive to research that aims to explore other utilities of this tool e.g. a source of DNA for PCR and other molecular assays.

1.4.2 Sample for diagnosis and studying malaria parasite

In addition to the selection of the adequate method, the rate of success in making diagnosis and genotyping malaria parasites in therapeutic studies might be improved by standardizing blood sampling and storage conditions.
Sample collection in field trials is often done by field workers, therefore, the material should be available in remote regions, collection procedure should be technically easy

and shipment should be at ambient condition. It is also of crucial importance to prevent samples from contamination and degradation of molecular material.

The molecular material for PCR is usually recovered from venous blood, blood spotted on filter paper (FP) (30) or thick blood smears (TBS) (27,31). Venous whole blood sampling is able to serve multiple tasks, including RNA and DNA extraction. It is proven that whole blood samples produce higher quality and quantity of DNA and are easy to handle for DNA extraction. However, venous blood sampling is invasive, requires professional training, special storage and shipping conditions and may result in noticeably lower diagnostic sensitivity compared to capillary blood (27,32). In addition, collection tubes must be treated with anticoagulants which can inhibit PCR reactions. TBS is not commonly used as a source of DNA for molecular assays since the quality of DNA recovered from TBSs is often lower compared to that from filter paper and fresh blood (33).

Capillary blood samples can be collected by finger prick and are used for the preparation of TBSs, filter paper and RDTs. Capillary blood sampling is often the method of choice for withdrawing blood from children and when only a small amount of sample is required. This approach is simple and considerably less invasive.

Since 1995, whole blood spots on filter paper has been most commonly used as a source of DNA for PCR in clinical trials (34). Filter paper can be shipped and stored at room temperature and some materials facilitate the extraction process. The quality of extracted DNA changes according to the filter paper types and extraction procedures. Similar performance was observed between some filter papers commonly used in *Plasmodium* detection by PCR (35,36).

PCR has been successfully performed using DNA recovered from RDTs (37–40) and the amplification success rate was comparable to that of filter paper (41). However, validation of the use of RDTs has been limited to some types of molecular approaches, mostly aimed at detecting parasite and genotyping some common drug-related mutant genes. The use of RDTs as a source of DNA for assessing and monitoring the diversity of parasite population (e.g. *msp* genotyping) is nevertheless uncommon (27). Therefore, validation of the performance of RDTs as a source of DNA for different molecular assays and optimization of the extraction method for the ease of use is of importance (27).

1.5 Current molecular methods for genotyping malaria parasite

1.5.1 Genetic markers

1.5.1.1 Merozoite surface proteins

Research efforts in the 80s and 90s have suggested that the merozoite surface antigens (MSAs) p190 (later known as MSA1), 513 (MSA2) together with the glutamate-rich protein (GLURP) are polymorphic. Analysis of DNA sequences of laboratory strains and field samples has demonstrated that gene coding MSA1 and MSA2 can be divided into blocks that are variable or conserve (42). Due to the polymorphism in length, the variable regions of genes coding MSA1 (block 2, on chromosome 9), MSA2 (block 3, on chromosome 2), and GLURP (R2 region, on chromosome 10) were considered the most useful markers for PCRs aim at genotyping *P. falciparum* (43) as later recommended by WHO (44). This method was considered a standard in many clinical trials conducted between 1995 and 2005 (34). While the assessment of MOI is generally based on the genotyping result of one of the three markers, the distinction of parasite strains may rely on two or even all three markers. In recommended protocols (44), *msp2* was considered the primary marker for genotyping *P. falciparum* followed by *glurp*; *msp1* was only used when these two markers were not able to differentiate parasite strains. The reason for this less common use of *msp1* as the primary marker could be that the identified polymorphic locus was short (often shorter than 500 bp) and the size change is often too small to be analyzed by gel electrophoresis. Later, by using capillary electrophoresis (CE), *msp1* and *msp2* genes were demonstrated to have the same high polymorphism and could be alternatively used as the primary marker for PCR-based genotyping assays and the use of *glurp* could be considered if the distinction is failed with *msp1* and *msp2* (45).

1.5.1.2 Single nucleotide polymorphisms (SNPs)

In humans, single nucleotide polymorphism (SNP) barcoding has been proposed as an alternative to short tandem repeats for kinship testing.

In malaria research, the first molecular SNP "barcode" for genotyping *P. falciparum* has been introduced by Daniels *et al* in 2008. It encompasses 24 bi-allelic chromosomal SNPs distributed across 14 chromosomes of the *P. falciparum* genome and was used as a fingerprint of the parasite genome (46). This methodology in combination with high-

resolution melt analysis requires a small amount of sample, is comparatively inexpensive and was capable of identifying polyclonal infections (47).
The abovementioned nuclear SNP barcode has some limitations including the lack of geographical specificity. In 2014, another molecular barcode has been explored comprising SNPs distributed in extra-nuclear genomes of the mitochondria and apicoplast organelles of *P. falciparum* (48). The barcode was chosen from analyzing the reference genome of *P. falciparum* strain 3D7 together with raw sequences data from 711 *P. falciparum* isolates collected from five geographic regions. A further analysis has resulted in a minimal barcode that is comprised of five mitochondrial and 18 apicoplast SNPs and showed high predictive accuracy in tracing the geographic origin of the parasite. Later, another barcode consisting of 42 nuclear SNPs was explored for genotyping *P. vivax* (49).

1.5.1.3 Microsatellite

Hundreds of microsatellites or simple sequence repeats have been described and used for studying population structure in malaria research. Selected microsatellites that are not under immune selection are often used. Since their individual size is the multiple of the known repeat unit, genotyping results can be better compared across different samples and laboratories. The size of the microsatellites is often measured by capillary electrophoresis on sequencers, and their diversity in length could serve to estimate population structure.
Microsatellite typing in malaria research often involves PCR amplification of several microsatellites. With the limited number of variants at each locus, the discriminatory power of this genotyping approach is low if only one or two microsatellites are used; measuring a high number of microsatellites, on the other hand, complicates the genotyping process.

1.5.1.4 Other length polymorphic markers

Additional markers, such as polymorphisms in *csp, ama* and *trap* genes have been used in malaria research (50–52). However, their use in general strain distinction analyses is uncommon due to low polymorphism in length.

1.5.2 Technical approaches for genotyping malaria parasite

1.5.2.1 Conventional-PCR-based methods

Due to their ease of use, low cost, modest hardware requirements and, genotyping approaches based on conventional PCR are still commonly used in malaria-endemic areas. The target genes of these genotyping approaches shall be polymorphic to maximize the discriminatory power. Often length polymorphisms are chosen and the size of the last PCR product is analyzed directly or after being fragmented by a restriction enzyme on either agarose gel or capillary electrophoresis system.

1.5.2.1.1 Size analysis by Agarose gel electrophoresis

Following the amplification step by PCR, agarose gel electrophoresis is the most popular method for the size analysis of the PCR products, mostly because of its low cost (27). However, this conventional approach is limited by the low resolution and may not provide accurate discrimination of alleles especially in endemic regions where high MOI can lead to *i*) missed detection of minor alleles due to low parasite density, and/or *ii*) misinterpretation since small differences in the length between alleles may be misclassified by gel electrophoresis.

1.5.2.1.2 Size analysis by capillary electrophoresis

a. On a sequencer

An alternative technique for fragment size analysis is CE on DNA sequencers. When used for genotyping a length polymorphic marker, the targeted gene will be amplified by PCR using one pair of fluorescence dye-labeled oligonucleotide primers. Final PCR products (amplicons) then undergo the fragment separation process by CE and the fluorescence signal will be detected by a laser. The fragment size (in base pair) is estimated based on their migration time in relation to the standard calibrator. When used for allelic typing (e.g. microsatellites), different fragments can be detected simultaneously by the use of primers labeled with distinct dyes. CE has provided a more accurate estimation of allele size than gel electrophoresis (53). This can improve the accuracy of discrimination between recrudescence and new infection and thereby refine the study of drug efficacy in clinical trials especially when conducted in high endemic areas (54). However, relying upon sequencer and fluorescent-labelled primers is costly, demanding and difficult to be used in field sites.

b. The QIAxcel Advanced System (Qiagen)

The QIAxcel Advanced System (Qiagen) is one of the commercialized CE systems, manufactured to replace the conventional gel-based sizing analysis of DNA, RNA, and proteins. It consists of 12 independent capillaries. The system is designed for the sizing analysis of a batch of 96 samples on a plate each run with a resolution of 3–5 bp. The PCR product could be analyzed directly in an automated system to avoid hands-in procedures and cross-contamination. In addition, the preparation procedure could be simplified followed by a decreased cost due to the normal PCR condition which does not require florescence-dye labeled primers. In an analysis, the size of the target product is estimated based on their migration time in relation to that of a size marker and an alignment marker (which can be pre-calibrated) using built-in software.

1.5.2.2 SNP barcoding assays

To the time of this thesis, a set of primers and probes for genotyping 24 polymorphic SNPs of *P. falciparum* (see 1.5.2.1) using allelic discrimination (AD) assay is commercially available. This multiplexed, end-point assay has two primer/probe pairs in each reaction, that allows for the detection of the two possible variants at each SNP. In an AD assay, a unique pair of fluorescent dyes (FAM and HEX/VIC) was used to label 24 pairs of TaqMan MGB probes that target 24 SNP positions. One fluorescent dye-labelled probe is a perfect match to one allele and the other is a perfect match for the other allele. During the PCR, the fluorescence dye will emit after being cleaved from the matched probe by the polymerase, the emission will be detected and measured (**Figure 3**).

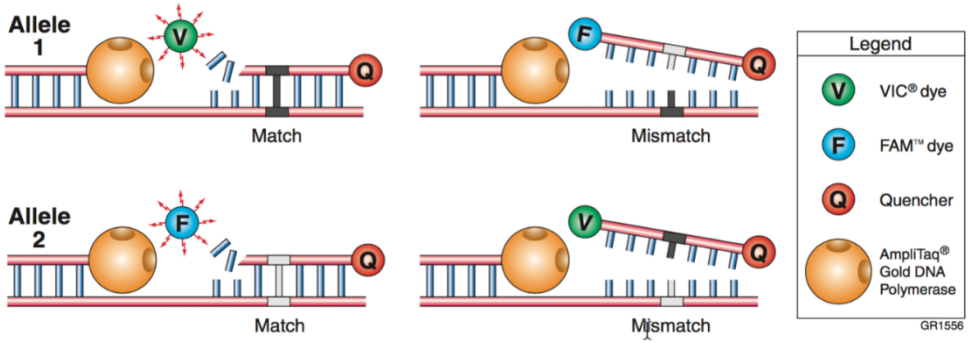

Figure 3. Illustration of AD assay. *Source: Applied Biosystem's Allelic Discrimination Assay Getting Started Guide for the 7900HT System* (55)*(with permission).*

The results are visualized on a scatter plot based on the intensity of the fluorescence signals of the two dyes which were measured at the plateau phase of the amplification. The barcode has been used in some studies and the robustness was compared with conventional (*msp* gene-based) methods (47,56). The overall performance of the entire barcode is not superior to the conventional *msp* genotyping approach, mostly due to the low polymorphism of some SNPs in the studied regions. Minimal but regionally specific barcode with a smaller number of SNPs has been shown to potentially simplify the genotyping procedure (56). Therefore, validation and optimization of a specific barcode for the ease of use in the study area are of particular interest.

1.5.2.3 Next generation sequencing

Next-generation sequencing (NGS) has been used for the analysis of the diversity of malaria infection in some trials (57). NGS-based genotyping is able to detect and analyze minor alleles hence often results in higher MOI of *P. falciparum* compared to the conventional method (5). Despite the excellent resolution, NGS is not recommended for use in the field settings due to some limitations: mostly, cost and complicated preparation and analysis procedures.

1.5.2.4 Other genotyping approaches

Some other alternative approaches have been used for genotyping *Plasmodium* in order to distinguish recrudescence from new infection: Southern blotting of paired PCR products (58), single-strand conformation polymorphism (SSCP) (59). However, these methods are not widely used and less standardized (44).

1.6 The implementation of parasite genotyping

1.6.1 Determination of drug efficacy in therapeutic trials

In drug trials which aim to assess the efficacy of antimalarial treatments for uncomplicated malaria, the estimation of efficacy relies crucially on the level of recurrent parasitemia that occurs as result of a recrudescence or a new infection. “New infection” and “recrudescence” are commonly referred to as a difference of parasite strains detected from the pre-treatment sample and recurrent sample. Briefly, a ‘new infection’ is a recurrence of parasitemia after clearance (by treatment), of which, all detected alleles are different from those in the admission sample, on the other side, in

'recrudescence', at least one common allele at each locus is detected from both samples. "Recrudescence" is often considered treatment failure due to drug resistance. However, it is not possible to clinically or morphologically (e.g. by microscopy) distinguish a recrudescence from a new infection. To make this distinction, molecular genotyping techniques are the method of choice, in order to determine whether a sample from admission and recurrent infections carry identical or different parasite strains. Misclassification of recurrent infections ("recrudescence" or "new infection") could possibly lead to imprecise estimation of drug efficacy and delayed curative treatment. Therefore, the World Health Organization has recommended the use of molecular genotyping techniques for all antimalarial drug efficacy trials in highly endemic settings.

1.6.2 Assessment of complexity of infection

Several parasite and host factors have been described to associate with malaria virulence, including parasite species, level of immunity and efficacy of treatment. Within-host diversity of the infecting parasite population and the polymorphism pattern of some genes have been described to associate with the clinical outcome of malaria (9,10,60). In addition, MOI was proposed as a secondary measurement to assess the outcome of intervention trials (61).

Besides the importance of distinguishing parasite strains, diversity of parasite population – often represented by MOI, has also been commonly studied. Assessment of MOI relies upon the sensitivity of the methodology used, may vary between different laboratory settings and is difficult in complex infection, especially in infection with low overall parasite density, or infection where the density of a minor strain is low. In addition, compilation and/or comparison of genotyping outcomes between sites or within a site at different time points is often compromised by the use of non-validated methods.

Therefore, it is of importance to establish, validate and standardize the genotyping approaches that are reproducible, cost-effective, field deployable, simple to perform and able to provide genotyping results in order to detect parasite strains with low density and help to assess MOI with high accuracy.

1.6.3 Documentation of drug resistance

Antimalarial drug resistance has been described mostly for *P. falciparum* and *P. vivax*. *P. falciparum* has been reported to develop resistance to nearly all current antimalarials drugs especially chloroquine.

Resistance to chloroquine (CQ) of *Plasmodium* was first documented in the Greater Mekong region after World War II and quickly spread to Africa. The spread of CQ resistance was followed by a deplorable treatment failure rate worldwide and has contributed to a frightening increase in malaria mortality in the second half of the 20th century. At their intra-erythrocytic stage, *P. falciparum* parasites take up hemoglobin and host cytoplasm to grow and replicate. Hemoglobin digestion happens in an acidic digestive vacuole and releases toxic hematin that – if accumulated – may cause parasite death. CQ is proposed to kill the parasite by interfering with the excretion of hematin which results in the accumulation of this toxic product inside the parasite (62). Examination of CQ-sensitive and resistant clones have localized the CQ resistance determinant to the *P. falciparum* chloroquine resistance transporter (*Pfcrt*) gene on chromosome 7. Four out of 10 described point mutations happen from locus 72 to 76 of *Pfcrt* gene, namely C72S, M74I, N75E, K76T are common in *P. falciparum* populations in Africa (63). Some point mutations are often found to happen together and the most frequently observed response haplotypes in Africa are CVMNK (the wild type or CQ sensitive), SVIET (CQR) and SVMNT (CQR). The haplotype SVMNT and other point mutations are very rarely observed in this region (3).

Artemisinin-based combination therapies (ACTs) has become the replacement for CQ as the first-line treatment for uncomplicated malaria. Although ACTs remain the most effective treatment, resistance against artemisinin derivatives and partner drugs have been documented. Artemisinin resistance is associate with delayed parasite clearance and usually does not lead to complete treatment failure. Resistance to the partner drug is more frequent and may require a change in regimen. However, in most of the cases, patients can still be cured with ACTs (64).

The mechanism that maintains the high prevalence of CQ-resistant haplotypes and other drugs is not well understood. Therefore, regularly monitoring drug resistance is of importance (65).

2 OBJECTIVE

Against this background, my thesis was aimed to assess the performance on a large scale of archived RDTs as a source of DNA for molecular assays that aim at detection, estimation of parasite diversity, and characterization of chloroquine resistance of *P. falciparum* in asymptomatic parasitemia and uncomplicated malaria. The current prevalence of CQ-resistant haplotypes in Gabon was correspondingly reported.

In order to enhance the resolution and robustness of parasite genotyping, this thesis was focused on establishing molecular tools including *i*) *msp*1-gene-targeted PCRs followed by the sizing analysis on an automated and affordable CE system, and *ii*) a minimal SNP barcode for discrimination of parasite strains for the use in drug trials in Gabon.

As an exploratory objective, in this thesis, I utilized the *msp1*-gene-targeted genotyping method to assess the diversity of *P. falciparum* parasite populations in peripheral blood and bone marrow of anemic malaria patients and the association between parasite diversity and severity of malarial anemia.

3 METHODS

3.1 Declaration of ethical approval

The study entitled "Characterization of population genetic diversity and molecular genotypes of drug-resistant *Plasmodium falciparum* using samples from routine Rapid diagnostic tests in Gabon" was approved by the scientific review committee and the Institutional Ethics Committee (CEI) of CERMEL.

The archived RDTs were mainly collected from a clinical trial (NCT03201770) named "Phase IIIb/IV Cohort Event Monitoring Study To Evaluate, In Real Life Setting, The Safety And Tolerability In Malaria Patients Of The Fixed-Dose Artemisinin-Based Combination Therapy Pyramax®" which was approved by the Institutional Ethics Committee of CERMEL and the National Ethics Committee for research of Gabon – Comité National d'Ethique pour la Recherche (CNER).

The ethics committees of the International Foundation for the Albert Schweitzer Hospital in Lambaréné and the ethics committees of the University of Münster and the University of Tübingen, Germany have approved the study entitled "A longitudinal study comparing age-matched triplets to investigate the etiology of severe anemia in children with *Plasmodium falciparum* malaria in Lambaréné, Gabon". Bone marrow and peripheral blood samples collected for the study were used for this thesis.

3.2 Study sites and sample collection

3.2.1 Study site

Clinical samples were collected in Lambaréné – Gabon. *In vitro* cultured *P. falciparum* parasites were reared at the Institute of Tropical Medicine – University of Tübingen – Germany. DNA extraction for RDT samples and filter paper, *Pfcrt* genotyping PCR was done in Lambaréné – Gabon. All other laboratory procedures were performed at the Institute for Tropical Medicine in Tübingen. An aliquot of the extracted DNA from RDTs was transported to Tübingen, another half was kept at -20^{0}C at CERMEL.

3.2.2 Standard calibrator

The preparation of the standard calibrator was described in detail else where (27). In brief, a field sample (with TBS parasitemia of *P. falciparum* of 6 840 000 p/mL) obtained from a malaria patient was ten-fold serially diluted with malaria-free group O+

blood (lowest parasitemia 68 p/ml). Five µl of each dilution (6 dilutions) was spotted on new RDTs (SD BIOLINE Malaria Ag *P.f*/Pan) and 10µl was spotted on each circle of filter papers (Whatman ™ 903 Protein Saver Card). All 6 RDTs and filter papers (6 circles) were kept at ambient temperature overnight before DNA extraction (27).

3.2.3 Archived RDT collection

As a part of the screening process of an ongoing drug trial (NCT03201770) and routine clinical activities, RDTs were collected and stored from June 2017 to July 2018. The full panel of inclusion and exclusion criteria could be retrieved from the trial registry NCT03201770 (27).

Three different types of RDTs – all WHO prequalified – were used: VIKIA® Malaria Ag Pf/Pan (IMAccess, Lyon, France), Paracheck Pf® (Orchid Biomedical Systems, Goa, India) and SD BIOLINE Malaria Ag *P.f*/Pan (Standard Diagnostics Inc, Hagal-Dong, Korea). The general lower limit of detection of these three types of RDT is ≤200 parasites/µl, according to manufacturers' specifications. Archived positive RDT cassettes were allowed to dry then stored at ambient temperature in a sealed pouch, until further use (27).

During the study time from June 2017 to July 2018, a total of 1008 used RDT cassettes have been collected, RDTs that had both demographic data and positive readable test lines (with *P. falciparum*) were used for further investigations (27).

3.2.4 Sample preparation for barcoding assay

Samples for validation of the SNP barcoding assays were taken from the *P. falciparum in vitro* culture. Briefly, six *P. falciparum* laboratory strains were cultured: 3D7, HB3, Dd2, 7G8, D10, and W2. Parasites were gathered at the ring stage (parasitemia of the culture was approximately 3.5% and consists of >95% ring form, quantified by microscopy and fluorescence-activated cell sorting – FACS) and used for the assays. For the lower limit of detection assays (LLODs), two *P. falciparum* strains 3D7 and Dd2 were used. The templates' DNA concentrations were measured using the Nanodrop spectrophotometer, each sample was measured five times and the mean concentration was used to generate the sample's serial dilution. A serial dilution of a DNA mix (of 3D7 and Dd2) was prepared (material extracted from culture is devoid from human DNA) as shown in **Table 13**.

To assess the utility of the SNP barcoding assays in distinguishing recrudescence and new infections: seven pairs of samples were taken from a drug efficacy trial conducted in Lambaréné – Gabon (NCT02198807), each pair had one sample collected on admission (visit Day 0, before treatment) and the other was collected at day of recurrence (Day X).

3.2.5 Bone marrow and blood collection – SMA study

A total of 91 children were recruited into this study after all inclusion and exclusion criteria had been full-filled, briefly:

Inclusion criteria:

- Written or witnessed informed consent by parent/guardian
- Infection with *P. falciparum*.
- Parasitemia > 1,000 parasites/µL
- Age 1 year to 6 years
- Plasma iron >10µmol/L
- Leukocytes <12,000/µL
  - MCV ≥ 70fL in children aged 1-2 years
  - MCV ≥ 73fL in children aged 2-4 years
  - MCV ≥ 75fL in children aged 5-6 years
- MCH between 28 and 34pg
- HbAA or HbAS
- Residency within 100km

Additional entry criteria for group SMA:

- Severe normochromic normocytic anemia with Hb ≤ 5g/dL and Hct ≤ 15%

Additional entry criteria for group MMA:

- Age matched (± 6 months) to a child from SMA group
- Hb > 5g/dL and < 10g/dL
- Thrombocytes > 30,000/µL
- Lactate < 3mM
- Blood glucose > 50mg/dL
- No signs of complicated malaria

Additional entry criteria for group MMW:

- Age matched (± 6 months) to a child from SMA group
- Hb ≥ 10g/dL
- Thrombocytes > 50,000/µµL
- Lactate < 3mM
- Blood glucose > 50mg/dL
- Hemozoin-containing neutrophils < 2%
- Parasitemia of between 1,000 and 100,000 parasites/µL, no schizonts
- No hospitalization due to malaria in patient history
- No malaria treatment as measured by Wilson&Edeson test (for chloroquine and quinine) and Lignin-Test (for sulfonamides)

Exclusion Criteria:

- Sickle cell anemia
- Malnutrition (body size and body mass index below 80th percentile)
- Blood transfusion within the past month
- Signs of complicated malaria (other than anemia or hyperparasitemia): cerebral malaria, renal or pulmonary dysfunction.
- Known filarial (*Loa loa*, *Mansonella perstans*), bacterial or viral infection (HIV, HBV, HCV, Parvovirus B19)
- Other severe diseases, including malignant disease, renal, hepatic or pulmonary dysfunction not due to malaria
- External or internal bleeding.

Patients were enrolled and classified into three groups based on the peripheral Hemoglobin (Hb) concentrations at the admission visits. All patients were hospitalized and were treated with quinine and clindamycin (standard regimen at the time at the Albert Schweitzer Hospital) (66).

Blood transfusion and other supportive treatment were upon study clinician's judgment. The active follow-up period was two months (treatment period day 0 to day 4, control visits on day 14, day 28 and day 56) and was prolonged for 2 more months (from the last infection) in case of recurrent parasitemia.

Blood sampling: From each patient, 5 ml of heparinized blood and 1 ml of EDTA treated blood was obtained directly before antimalarial treatment and blood transfusion.

500 µL of blood was stored at -80°C in glycerolyte (1.3 mL of glycerolyte was added to 1000 µL blood).

Bone marrow aspirations used for this work were taken on admission (Day 0) and on day 56. Bone marrow was aspired from the posterior iliac spine either under local anesthesia or under midazolam/pethidine anesthesia. Informed consent for the procedure was taken separately and study participation was independent of the willingness to undergo bone marrow puncture. 2mL of bone marrow was aspired (1mL to EDTA-coated tube, 1mL to the heparinized tube) and frozen at -80ºC together with glycerolyte (500 µL blood + 700 µL glycerolyte).

Positive controls (*P. falciparum* strain 3D7 for 18S RT-PCR, strain 3D7 for *msp1*/K1, strain Dd2 for *msp1*/MAD20 and strain 7G8 for *msp1*/RO33) were prepared using cultured parasite. We used malaria parasite-free blood as negative controls for molecular assays.

3.3 Nucleic acids extraction

3.3.1 RDT and filter paper preparation

3.3.1.1 Archived RDTs

RDT cassettes were opened individually using scissors and forceps. After each sample, scissors and forceps were sterilized in alcohol 100% and DNA AWAY™ solution (Molecular BioProducts, San Diego, USA.) (27) then allowed to dry at room temperature before working with other RDTs.

The test strip (nitrocellulose part) was taken out from the case and any plastic cover on the strip was stripped off (27). The part of the test strip (see **Figure 4**) which contains DNA was dissected and used for DNA extraction (67). DNA was eluted in 50 µl.

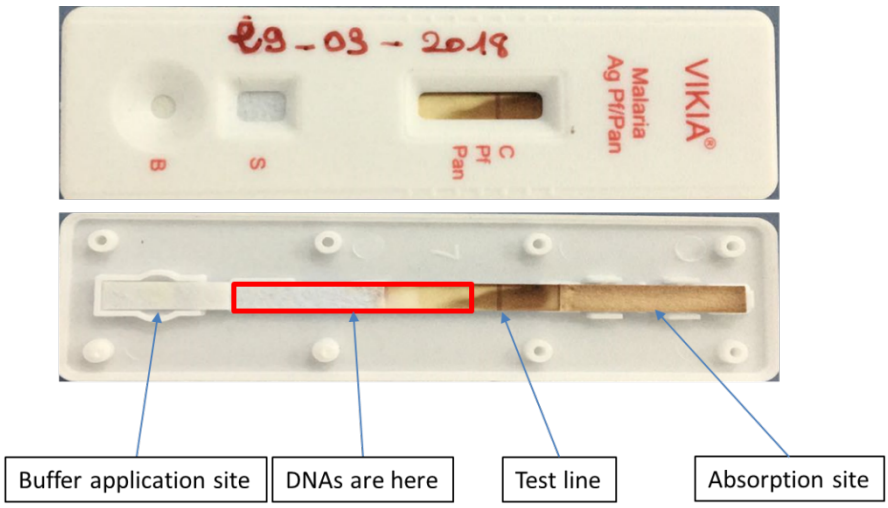

Figure 4. Illustration of RDT preparation for DNA extraction.

3.3.1.2 Filter paper

The DNA extraction was done using a half of each circle (containing approximately 5µl of the sample) of filter paper (27). Elution volume was 50 µl.

3.3.2 Peripheral blood and bone marrow samples

For both peripheral blood and bone marrow samples, DNA extraction was done using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instruction for blood (27). Fifty µL of PBS 1X was added to each sample before adding proteinase K and starting extraction. The elution volume was 100 µl.

3.3.3 Nucleic acids extraction protocol

Nucleic acid extraction from cultured parasites, RDTs, and dried blood spots was performed using QIAamp DNA Blood Mini Kit (Cat No: 51106) (27) following manufacturer's instruction.

The common extraction procedure was as follow:

- Add protease from the QIAamp DNA Blood Mini Kit (the amount depends on the type of sample, mentioned below)
- Transfer sample (amount depends on the type of sample) to a 2 mL Eppendorf tube
- Add lysis buffer AL (amount depends on the type of sample) and mix by vortexing for 15 seconds

- Spin tubes shortly to remove drops from the inside of the cap
- Incubate at 56°C for 10 minutes while shaking
- Spin tubes shortly to remove drops from inside of the cap
- Add 100% Ethanol (similar to the volume of buffer AL) and mix by vortexing for 15 seconds
- Spin tubes shortly to remove drops from inside of the cap
- Load lysate to a QIAamp Mini spin column without wetting the rim and centrifuge at 6000x g (8000 rpm) for 1 min. (Repeat this step until all lysate are applied to column)
- Place the column in a clean 2 mL collection tube
- Add 500 µL wash buffer AW1, without wetting the rim and centrifuge at 6000xg (8000 rpm) for 1 minute
- Place the column in a clean 2 ml collection tube
- Add 500µL wash buffer AW2, without wetting the rim and centrifuge at full speed (17000xg) for 3 mins.
- Place the spin column in a clean collection tube and centrifuge at full speed (17000xg) for 1 min to remove all wash buffer residues from the column.
- Place the spin column in a sterile, DNase/RNase-free certified 1.5 mL Tube
- Add 100µL (50µL for RDT and filter paper) pre-warm DNAase-free water directly to the filter of the spin column.
- Incubate for 3-5 min at room temperature and centrifuge for 2 min at 6000xg (8000rpm) to elute total NAs.

Table 1. Volume of samples and reagents used for extraction according to the type of sample.

	Sample volume (µL)	**Protease (µL)**	**Buffer Al (µL)**
Parasite culture	200	20	200
RDT	5	20	200
Filter paper	5	20	200
Blood in glycerolyte	150 (+ 50 µL of 1x PBS)	20	200
Bone marrow in glycerolyte	150 (+ 50 µL of 1x PBS)	20	200

3.4 Real-time PCR for quantification of malaria parasite (18S RT-PCR)

Malaria parasite detection and quantification were done using a Taqman probe-based Pan-*Plasmodium* Real-time PCR that targets a highly conserved region of 18S rRNA as described elsewhere (27,68) with a modification: reverse transcriptase was not used. Primers and probes sequences used for the assay are described in **Table 4**.
Reaction mixes was made using TaqMan™ RNA-to-CT™ 1-Step Kit (Thermo Fisher Scientific, Foster City, CA, USA) following the manufacturer's instructions for non-RT PCR.

Table 2. Master mix preparation for 18S Pan-Plasmodium quantitative real-time PCR.

Reagent component	**Volume**	**Final concentration**
TaqMan® RT-PCR Mix (2X)	5 µl	1x
PLU3 TaqMan Probe (10µM)	0.15 µl	150nM
PLU3 Forward Primer (10µM)	0.4 µl	400nM
PLU3 Reverse Primer (10µM)	0.4 µl	400nM
Total Nucleic acids	2.5 µl	
Nuclease-free H_2O	1.55 µl	
Final volume per reaction	10 µl	

After the preparation, on a robotic pipetting system (QIAgility, Qiagen, Germany, Cat No./ID: 9001532), master mix was loaded into each well of a 384-well PCR plate together with DNA extracted from samples (in triplicate), one positive control (*P. falciparum* NF54) and one non-template control (in duplicate for both negative and positive controls). PCR was done on a LightCycler 480 Instrument II (Roche, Basel, Switzerland) with the following thermal conditions:

Table 3. Cycling conditions for 18S real-time PCR.

Cycles	Temp.	Time	Step
1	96°C	10 min	Polymerase activation
45	95°C	15 sec	Denaturation
	62°C	1 min	Annealing/Extension/Acquiring
1	40°C	10 sec	Cooling

Quantification analysis was done with LightCycler® 480 SW v1.5.1 software. Successful amplification was defined as a Ct-value of lower than 40.

3.5 Nested PCR for identification of *Pfcrt* mutations

Nested PCR was used to genotype codons 72 – 76 of the *Pfcrt* gene, primers, and probes sequences are given in **Table 4**.

To improve the sensitivity, *Pfcrt* gene was pre-amplified by conventional PCR as follows (27): 20µl of total reaction volume was made by adding 2.5µl of the template to 17.5µl of a master mix containing: 1x PCR buffer, 0.4µM of each primer, 0.25 mM dNTPs, 1U of Taq polymerase (Qiagen, Hilden, Germany). The PCR was done on a MyCycler (BioRad, Germany) with the following thermal conditions: 94ºC for 5 minutes followed by 25 cycles of 94ºC for 30 seconds, 55ºC for 30 seconds and 72ºC for 30 seconds, final annealing at 72ºC for 10 minutes.

The product of the pre-amplification PCR was used as the template for the nested multiplex Real-time PCR on a LightCycler 480 Instrument II using SensiMix™ II Probe Kit (Bioline GmbH., Germany) (27) following the manufacturer's instructions. Sequences of primers and *Pfcrt* haplotypes specific probes are given in **Table 4**. Each sample was analyzed in duplicate. DNA of *P. falciparum* parasites of strains NF54, Dd2, and 7G8 were prepared from culture and used as positive controls for genotyping three haplotypes CVMNK, CVVIET, and SVNMT, respectively.

3.6 *msp1* genotyping

3.6.1 Amplification of *msp1* gene by conventional PCR

Msp1 genes were genotyped by nested PCR using previously published pairs of primers (45).

The conserved region of the *msp1* gene was amplified in the primary PCR with a total volume of the reaction of 20µl consisting of 5µl of template, 15µl of master mix (which

contains 1x AmpliTaq Gold buffer, 1.5mM $MgCl_2$, 0.25mM dNTPs, 1 U of AmpliTaq Polymerase and 250nM each primer). The PCR was performed using a MyCycler (BioRad, Germany) with the following thermal condition (27): 10 minutes at 94°C followed by 35 cycles of 94°C in 15 seconds, 58°C for 30 seconds, 72°C for 1 minute and 72°C for 10 minutes for the final extension.

Nested PCR (27) amplified block 2 of the *msp1* gene using 3 pairs of primers specific for 3 families of the *msp1* gene: K1, MAD20, and RO33 (sequences of primers are in **Table 4**). Three nested PCR reaction mixes were made with the total volume of 20µl containing 2.5µl of template, other reagents were added at the same concentration as used in the primary PCR. Except for the annealing temperature was at 61°C, thermal conditions of the nested PCRs were similar to that of the primary PCR. *P. falciparum* strains NF54, Dd2, and 7G8 were used as positive controls for K1, MAD20, and RO33 families of the *msp1* gene, respectively.

3.6.2 Amplicon sizing using capillary electrophoresis.

Amplicon sizing by capillary gel electrophoresis was done on a QIAxcel Advance system (QIAGEN, Hilden, Germany) (27) according to the manufacturer's instructions for the OM400 protocol. Each batch has 88 samples and 8 controls (7 positive controls and 1 non-template control).

Materials:

- QIAxcel DNA High Resolution Kits (catalog number: 929002).
- QX DNA Size Marker 50–800 bp (50µl) v2.0 (Qiagen, cat.no. 929561) and,
- QX Alignment Marker 15bp/1kb (Qiagen, cat. No. 929521).

Amplicon size was analyzed by software QIAxcel ScreenGel v1.5.0 as described elsewhere (27). Data were analyzed and reported when positive controls were positive with a single peak (NF54: 241±3 bp, Dd2: 205±3 bp and 7G8: 153±3 bp). A peak was defined as a countable allele when it was greater than 100 bp and contributed more than 10% of total peak height. Within one sample, multiple peaks with less than 6 bp differences were considered as one allele and the average size was calculated and used for further analyses.

Table 4. Primer and probe sequences (27).

msp1	Primary PCR		A	AAGCTTTAGAAGATGCAGTATTGAC
			B	ATTCATTAATTTCTTCATATCCATC
	Nested PCR	K1	Fwd	AAATGAAGAAGAAATTACTACAAAAGGTGC
			Rev	GCTTGCATCAGCTGGAGGGCTTGCACCAGA
		MAD20	Fwd	AAATGAAGGAACAAGTGGAACAGCTGTTAC
			Rev	ATCTGAAGGATTTGTACGTCTTGAATTACC
		RO33	Fwd	TAAAGGATGGAGCAAATACTCAAGTTGTTG
			Rev	CATCTGAAGGATTTGCAGCACCTGGAGATC
Pfcrt	Pre-amplification		Fwd	TGGTAAATGTGCTCATGTGTTT
			Rev	AGTTTCGGATGTTACAAAACTATAGT
	RT-PCR		Fwd	TGGCTCACGTTTAGGTGGAGGTTCTTG
			Rev	ACTGAACAGGCATCTAACATGGATATAGC
	Probes		CVMNK	TGTGTAATGAATAAAATTTTTGCTAA
			CVIET	TGTGTAATTGAAACAATTTTTGCTAA
			SVMNT	AGTGTAATGAATACAATTTTTGCTAA
18S PCR	PLU3 Forward			GCTCTTTCTTGATTTCTTGGATG
	PLU3 Reverse			AGCAGGTTAAGATCTCGTTCG
	PLU3 Probe			**VIC**-ATGGCCGTTTTTAGTTCGTG-**NFQ-MGB**

Red-highlighted letters represent the mutation points

Fwd: forward primer

Rev: reverse primer

3.7 Dual-probe endpoint genotyping (SNP barcoding assays)

Primers and probe sequences used for this barcoding assays can be retrieved elsewhere (46).

Table 5. Barcoding assays*. Minor and major alleles were determined in the original work* (46).

Assay number	Assay name	R/S number	Possible alleles	Major allele	Minor allele	3D7
1	Pf_01_000130573		C/T	C	T	C
2	Pf_01_000539044		A/G	A	G	G
3	Pf_02_000842803		T/C	T	C	C
4	Pf_04_000282592		T/C	T	C	T
5	Pf_05_000931601		G/C	G	C	C
6	Pf_06_000145472	rs45343635	C/G	C	G	C
7	Pf_06_000937750		A/G	A	G	G
8	Pf_07_000277104	rs45339742	A/G	A	G	G
9	Pf_07_000490877	rs45343970	A/T	A	T	A
10	Pf_07_000545046	rs45338103	C/T	C	T	C
11	Pf_07_000657939	rs45403113	T/C	T	C	T
12	Pf_07_000671839		A/G	A	G	G
13	Pf_07_000683772	rs45339189	C/T	C	T	C
14	Pf_07_000792356	rs45341962	A/C	A	C	A
15	Pf_07_001415182	rs45403212	C/A	C	A	C
16	Pf_08_000613716	rs45405749	C/A	C	A	C
17	Pf_09_000634010	rs45408065	C/T	C	T	C
18	Pf_10_000082376	rs45409692	A/T	A	T	A
19	Pf_10_001403751	rs45410415	A/C	A	C	A
20	Pf_11_000117114	rs45415497	G/A	G	A	G
21	Pf_11_000406215		A/C	A	C	A
22	Pf_13_000158614	rs45422484	C/T	C	T	T
23	Pf_13_001429265		G/T	G	T	T
24	Pf_14_000755729		G/T	G	T	G

Master mixes was prepared following the instruction of the manufacturer for the TaqMan Universal PCR Master Mix (Applied Biosystems Catalog number 4364343).

Table 6. Master mix preparation for barcoding PCR.

Component	Volume for 1 reaction
2x TaqMan Master Mix	2.5 µL
20x Assay Working Stock	0.25 µL
Template	2.25 µL
Total volume per well	**5 µL**

3.7.1 Preparation of the reaction plate.

The DNA concentration of each sample and positive control were measured by NanoDrop Spectrophotometer. DNA was diluted to a similar concentration (1 ng/µl) across all samples before mixing with the prepared master mix on a 96-well PCR plate (LightCycler® 480 Multi-well Plate 96, white, Cat # 04729692001).

3.7.2 Perform a PCR plate read

Table 7. Cycling conditions for barcoding PCR.

Step	Temp	Time	No of Cycles
Polymerase activation	95°C	10 minutes	1
Denaturation	95°C	15 seconds	40
Annealing/extension	60°C	1 minute	
Cooling	40°C	30 seconds	Hold

Acquisition format as dual probe: VIC/HEX (excitation – detection: 533 – 580nm) and FAM (excitation – detection: 465 – 510nm).

All of the PCR assays was done on a Light Cycler 480 Instrument II PCR system. The End-point genotyping result was analyzed and visualized using the built-in software LightCycler® 480 SW, version 1.5.1.

3.8 Statistical analysis

R software (version 3.5.1) was used to analyze all the data mentioned in this thesis and 95% confidence intervals were given where possible. A two-sided p-value of <0.05 was considered statistically significant.

3.8.1 For work on RDTs (27)

Cycle threshold (Ct) values were used in absolute quantification analyses to extrapolate parasitemia of the samples. The extrapolation was made in relation to Ct-values of a set of standard calibrators using linear regression analysis (with an assumption that equal amounts of blood (5 µl) were spotted on each RDT and calibrators).
Positive PCR results were considered as negative (0 parasites/ml) if Ct values >40 and excluded from the analysis. Comparison of Ct-values of two serial dilutions: RDT and filter paper was done using a paired T-test.
Individuals were stratified into three groups according to their age: (i) children <5 years (ii) children between 5 to 18 years and (iii) adults >18 years old and three groups based on their residence: Lambaréné (semi-urban), Fougamou (semi-urban) and surrounding rural areas (radius approximately 10km).
The MOI was calculated for individuals and the population. For an individual, MOI is the sum of the number of alleles detected by *msp1* genotyping (including 3 gene families):

MOI = number of K1 allele + number of MAD20 allele + number of RO33 allele

Population MOI was the mean MOI of all individuals and calculated by dividing the total of individual MOI by and the total number of PCR positivity. Sample with MOI = 1 was considered as a monoclonal infection the other positive samples were considered as polyclonal infections.
MOI parameters were displayed as mean numbers, but while comparing MOI between age or location groups, the non-parametric Kruskal-Wallis H and Wilcoxon rank-sum tests were used. The decadic logarithm (Log10) of parasite densities were compared between age groups using these tests.
Linear and logistic regression models were used as shown in **Table 8**. In all models, location as an independent variable was defined based on different population density: rural areas, Fougamou and Lambaréné. The relation between age and Log10 transformed parasite density was analyzed by a linear regression model.

Table 8. Regression models used.

Test	Dependent variable	Independent variables
Poisson regression model	MOI	age, location
Binomial regression model	CQ resistance	age, location
	PCR success	storage duration
Linear regression model	Log10 parasitemia	age

3.8.2 For SNP barcoding assays

Minor allele frequency was calculated for each SNP as previously described (49). In brief, each allele found in a polymorphic genotype was considered to contribute a half of an allele found in a monomorphic genotype. Allele frequency was calculated for each SNP using allele counts.

A new infection is defined when the parasite genotype at day X (day of recurrence) has at least 1 distinct SNP compared to the genotype of day 0 (admission). Therefore, only monomorphic SNP result was used to distinguish parasite genotypes.

3.8.3 For work on SMA samples

The recruitment process allocated patients into three groups based on Hb concentration, but in data analysis, patients were classified as severe malarial anemia (SMA) if RBC < 2.8 Mio/ml, and moderate malarial anemia (MM) if RBC >2.8 Mio/ml regardless of Hb concentration.

4 RESULTS

4.1 Performance of RDTs as source of DNA for molecular assays

4.1.1 Demographic details of uncomplicated malaria and asymptomatic *P. falciparum* parasitemia

A total of 669 positive RDTs were collected from uncomplicated malaria and asymptomatic *P. falciparum* parasitemia including 332 (50%) female. The median age was 8 years (Interquartile Range - IQR: 4-15). 185/669 (28%) were children aged under 5 years, 346 (52%) between 5 and 18 years and 138 (21%) aged >18 years. 125 (18.7%) samples were collected from Lambaréné and from rural areas, 122 (18%) were from Fougamou and 422 (63%) from other rural areas **Table 11**. The mean storage duration was 113.2 days (range: 9 – 231 days).

4.1.2 Success rates of different PCRs using DNA extracted from RDTs

Using positive RDTs as a source of DNA for 18S quantification real-time PCR, *Pfcrt* PCR, and *msp1* genotyping PCR, RDTs produced 96.6%, 87.4%, and 88.5% amplification success rates, respectively (**Table 9**). Longer storage duration of RDTs did not change the amplification success rate of 18S PCR notably.

Table 9**. **Proportion of positivity of molecular assays using DNA extracted from RDTs (27)**.**

	Time of sample storage				
	<1 month (N = 138)	1-3 months (N = 57)	3-6 months (N = 361)	>6 months (N = 113)	Total (N = 669)
Assay					
18S PCR	135(97.8%)	52(91.2%)	353(97.8%)	106(93.8%)	646(96.6%)
Pfcrt PCR	120(87%)	49(86%)	314(87%)	102(90.3%)	585(87.4%)
msp1 PCR	112(81.2%)	52(91.2%)	322(89.2%)	106(93.8%)	592(88.5%)

N: sample size

Table 10. Lower limit of detection of PCR protocols using DNA extracted from RDT.

Parasitemia (p/mL)	18S RT-PCR	*Pfcrt* PCR	*msp1* PCR	RDT
6840000	+	+	+	+
684000	+	+	+	+
68400	+	+	+	+
6840	+	-	+	-
684	+	-	-	-
68	+	-	-	-

4.1.3 For quantification of parasite density by 18S PCR

4.1.3.1 RDTs versus FP

Two serial DNA dilutions were made from 6 RDTs and 6 circles of filter paper. Absolute quantitative real-time PCR targeting 18S genes were run in triplicates. Ct-values were used to analyze the performance of these two DNA sources. All samples of these series including the lowest parasitemia (68 parasites/ml) were positive. Ct-values of two groups of samples RDT and filter paper were similar (**Figure 5**).

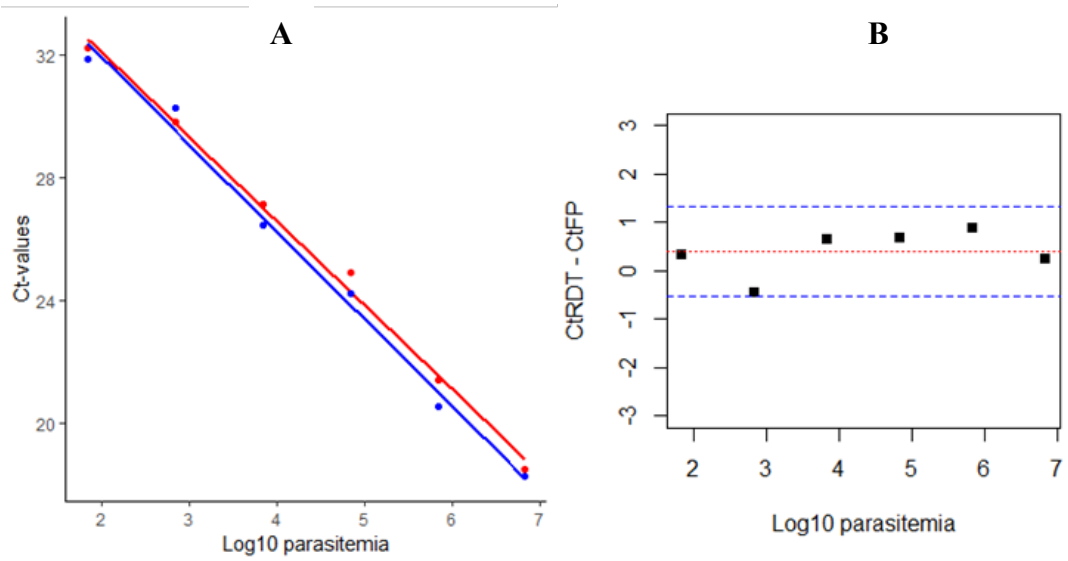

Figure 5. Comparison of the performance of RDTs and FPs as source of DNA for quantitative real-time PCR. ***A****: The correlation between Ct-values and peripheral parasitemia: RDT (red) and filter paper (blue). The PCRs were performed for each sample in triplicate and the means of Ct-values were represented by these dots. The straight lines visualized the linearity between the mean Ct-values and decadic logarithm transformation of parasitemia of the RDT group (red, adjusted* R^2*: 0.9944) and FP group (blue, adjusted* R^2*: 0.9892)* (27). ***B****: The Bland Altman plot visualized the agreement between the Ct-values of PCRs which were performed using two types of samples, red line: mean of differences, blue lines: 95% confidence intervals of the differences. The positive difference between the Ct values of the RDT group and the FP group implied that the DNA load extracted from RDT was lower than that from the FP, but the difference was not significant.*

4.1.3.2 Parasitemia in uncomplicated malaria and asymptomatic *P. falciparum* parasitemia

Extrapolated parasitemia from Ct values of PCRs (using the linear model mentioned in 4.1.3.1) was excluded from analysis if lower than 1 p/mL. Parasitemia in the group of children aged <5 years (geometric mean: 3548 parasites/mL (95% CI: 2188 – 4677)) was significantly higher than that in the other two age groups: 5 to 18 years and >18 years, with geometric mean of parasitemia of 1259 (95% CI: 891 – 1698) and 776 (95% CI: 457 – 1318) parasites/mL, respectively (**Figure 6**).

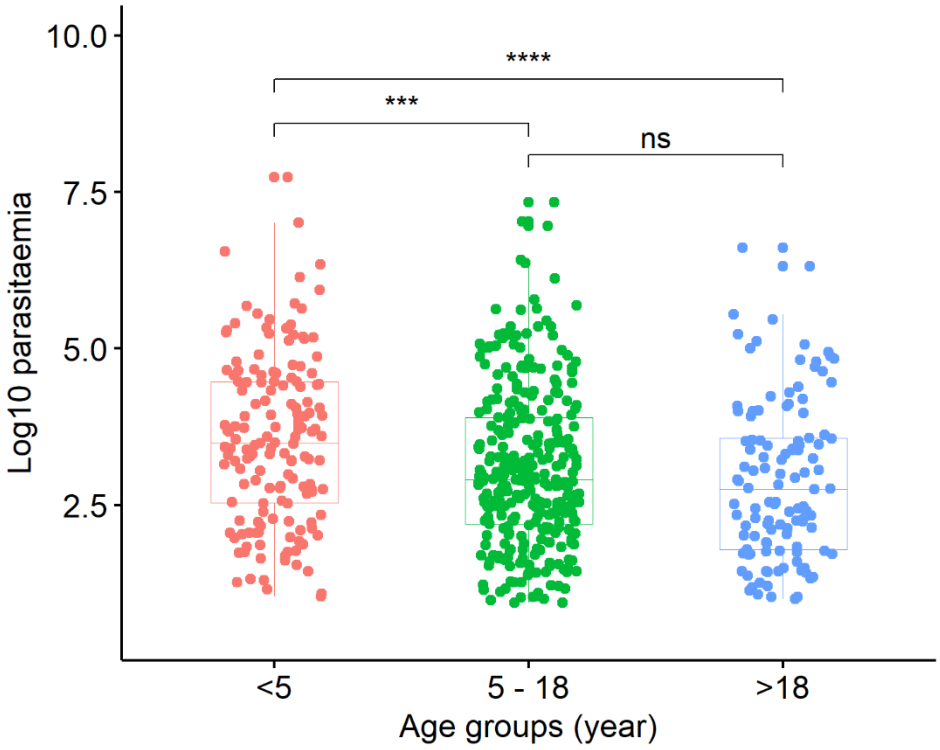

Figure 6. Parasitemia in different age groups of uncomplicated malaria and asymptomatic P. falciparum parasitemia. *The significance levels: significant (***: <0.001, ****: <0.0001) and nonsignificant (ns)* (27).

4.1.4 For assessment of diversity of *P. falciparum*

4.1.4.1 Capillary electrophoresis and visualization of allele size by QIAxcel

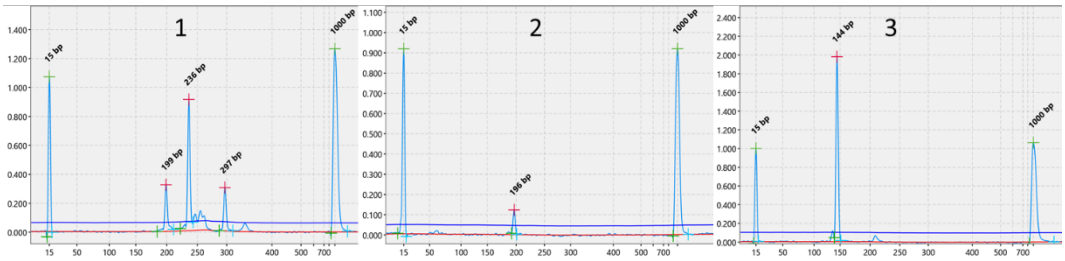

Figure 7. An example of the msp1 genotyping result. *Graphs 1,2 and 3 represent CE results of msp1 families RO33, MAD20, and K1, respectively. Each peak (except the ones with the sizes of 15bp and 1000bp – which are size markers) shown in this graph represents one single allele (strain). MOI of the sample was 5.*

4.1.4.2 MOI in association with age and region

The highest individual MOI was 11. The MOI of the population was: 2.6 (95% CI: 2.5 - 2.8). The MOI was higher in groups of ages <5 years (2.8 (95% CI: 2.6-3.1)) and 5 to 18 years (2.7 (95% CI: 2.5-2.9)) than in group of ages >18 years (2.1 (95% CI: 1.9-2.4)). MOI for samples from Lambaréné was 2.0 (95% CI: 1.8-2.3), significantly lower than Fougamou 2.8 (95% CI: 2.5- 3.1) and rural areas 2.8 (95% CI: 2.6- 2.9) (**Figure 8**). Poisson regression model confirmed the significant adverse association of both age and location and MOI (age: β = -0.0046, p-values = 0.019 and location: β =-0.314, p = 0.0004) .

The prevalence of monoclonal infection observed in LA was lower than that in the regions (df = 2, p-value = 0.004).

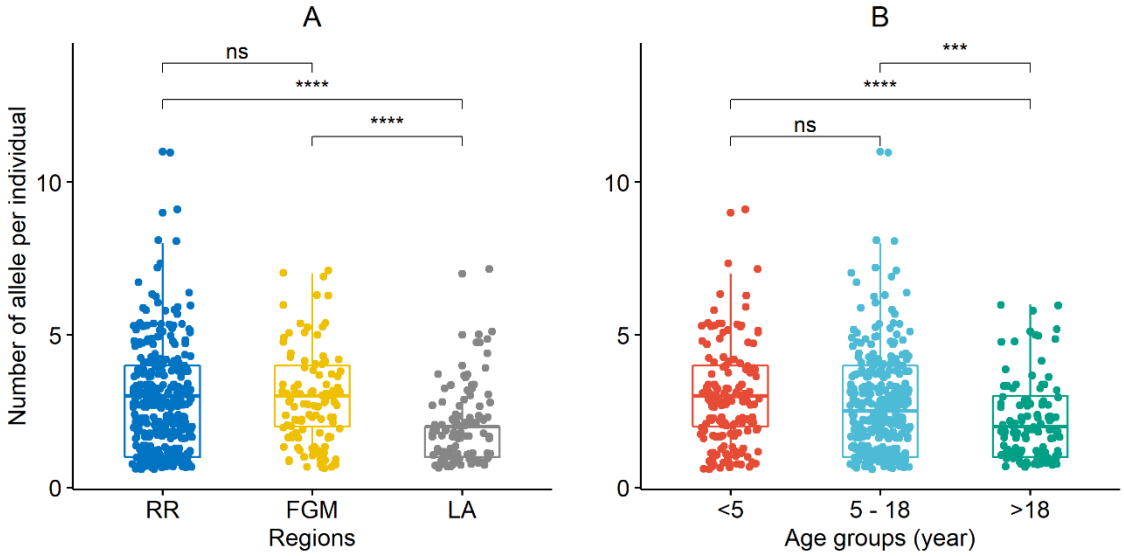

Figure 8. MOI across studied regions (A) and age groups (B)(27)***.*** *MOI was displayed as the number of alleles per individual in y-axis. Box plots were shown with medians and interquartile ranges (IQR). In order to avoid overlapping, each number of alleles per individual (countable number) was visualized by a jitter point. The significance levels were: significant (***: <0.001, ****: <0.0001) and nonsignificant (ns). RR: rural areas, FGM: Fougamou, LA: Lambaréné.*

4.1.4.3 Prevalence of *msp1* alleles in uncomplicated malaria and asymptomatic *P. falciparum* parasitemia

Five hundred ninety five samples were positive with *msp1* genotyping PCR, the prevalence of three *msp1* gene families were K1: 75% (n=446); MAD20: 47% (n=275); and RO33 51% (n=305).

A total of 27.5% (n=163) of the *msp1* PCR positive isolates were monoclonal infections (**Table 11**). *msp1*/K1 alleles were found in the major proportion of monoclonal infections, whereas *msp1*/ K1 + RO33 and K1 + Ro33 + MAD20 alleles were more commonly observed in polyclonal infections (**Table 11**). The allele sizes for each of the families of the *msp1* gene were 133 - 374 bp (K1), 133 - 311 bp (MAD20) and 111 - 258 bp (RO33) (**Figure 15**). There was no specific allele for any age group or region.

Table 11. msp1 PCR positivity and the distribution of msp1 genotypes in uncomplicated and P. falciparum asymptomatic infection across geographic regions (27)**.**

	FGM	**LA**	**RR**	**Total**
N (%)	122(18.2)	125 (18.7)	422 (61.3)	669
msp1 PCR positivity (%)	111 (18.7)	107 (18.1)	374 (63.2)	592
Monoclonal infection	24 (21.6)	43 (40.2)	96 (25.7)	163 (27.5)
Polyclonal infection	87(78.4)	64 (59.8)	278 (74.3)	429 (72.5)
K1 only	18	40	88	146
MAD20 only	10	15	31	56
RO33 only	11	14	39	64
K1 + MAD20	14	10	61	85
K1 + RO33	24	17	66	107
MAD20 + RO33	8	4	14	26
K1 + MAD20 + RO33	26	7	75	108

4.1.5 For genotyping CQ-resistant *P. falciparum*

The overall prevalence of CQR genotypes in the study population was 78.5%.

Prevalence of CQ sensitive genotypes in the group of children aged <5 years was 26%,

followed by 48% of CVIET genotype (CQR) and 27% of mixed infection with sensitive and CQR genotypes **Table 12**. The prevalence of these genotypes (sensitive, CVIET and mixed strains) in other two age groups was: 18.8%, 44.2%, and 37%, for the group aged from 5 to 18 years, and 22.6%, 47.0%, and 30.4% for adults, respectively (**Figure 9**).

The prevalence of CQ resistant genotypes was not significantly different between three age groups (p-value = 0.2). The prevalence of CQR genotypes in Lambaréné (65.7%) was lower compared to that of the rural areas (Fougamou and other rural areas – 81.3%) (p-value = 0.0017) **Table 12**. Logistic regression analysis showed an association between living in Lambaréné and lower prevalence of CQR genotypes of *P falciparum* (β = - 0.809, p-value = 0.011 after correction for age).

Table 12. Prevalence of Pfcrt haplotypes, age distribution and msp1 genotyping result across regions (27)**.**

	FGM	**LA**	**RR**	**Total**
N (%)	122(18.2)	125 (18.7)	422 (61.3)	669
Age				
≤ 5 years (%)	23 (18.8)	24 (19.2)	138 (32.7)	185 (27.7)
≥ 5 to 18 years (%)	80 (65.6)	58 (46.4)	208 (49.3)	346 (51.7)
Adults (>18 years) (%)	19 (15.6)	43 (34.4)	76 (18.0)	138 (20.6)
Pfcrt-PCR positivity (%)	109 (18.6)	108 (18.5)	368 (62.9)	585
CQ sensitive (CVMNK) only (%)	21(19.3)	37(34.3)	68(18.5)	126 (21.6)
CQ resistance 1 (CVIET) only (%)	43 (39.4)	50(46.3)	174(47.3)	267(45.6)
CQ resistance 2 (SVMNT) (%)	0	0	0	0
Mix (R and S) (%)	45(41.3)	21(19.4)	126(34.2)	192(32.8)

Regional prevalence of CQ resistance (by summing up the prevalence of resistance and mix infection) was 65.7% in LA and 81.3% in rural areas (including FGM and other rural areas). R: chloroquine resistance; S: chloroquine-sensitive; FGM: Fougamou; LA: Lambaréné; RR: rural regions, CQ: chloroquine

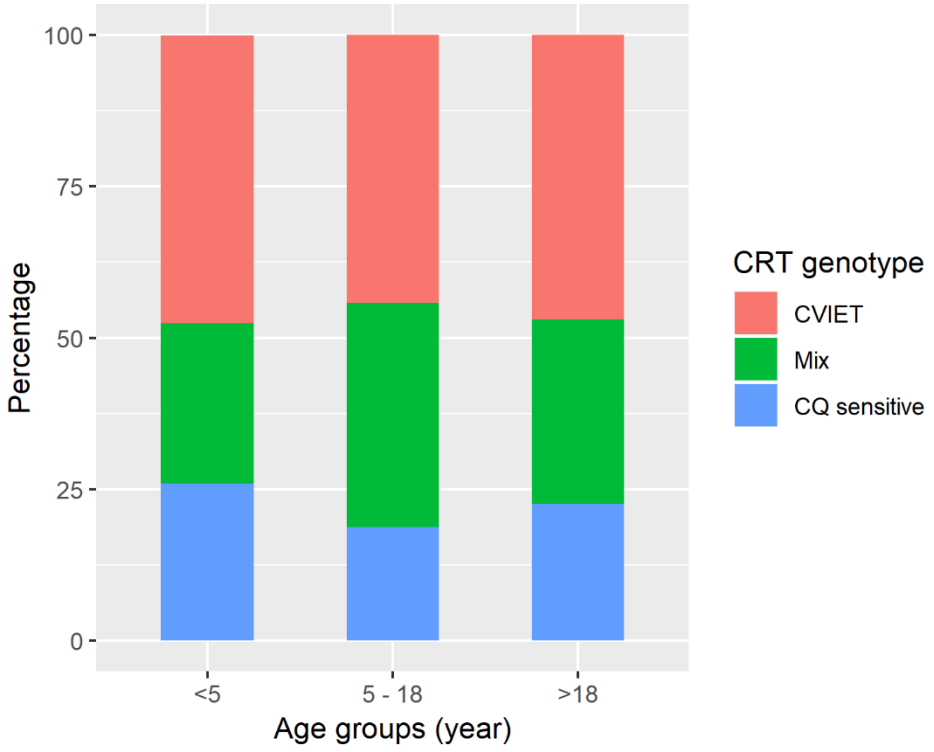

Figure 9. The prevalence of Pfcrt haplotypes was similar between age groups. *Mix infections: infected by both sensitive and resistant genotypes of P. falciparum. SVMNT haplotype was absent in this population* (27).

4.2 Performance of the barcoding assays

4.2.1 Validation of the SNP barcoding assays

The 24-SNPs barcoding assays successfully genotyped 6 *in vitro* cultured well-defined laboratory *P. falciparum* strains: 3D7, Dd2, HB3, 7G8, W2, D10. The fluorescence signal was automatically analyzed by the software and visualized in a scatter plot, followed by manual allele calling.

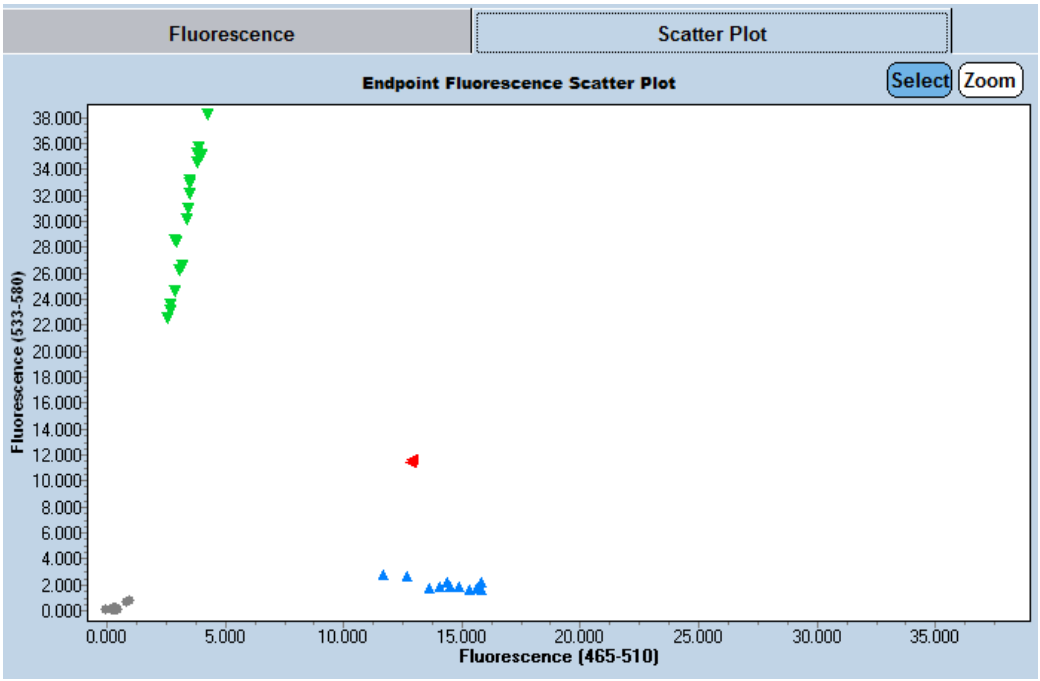

Figure 10. An example of the genotyping result of P. falciparum strain 3D7 together with a non-template control and a field sample. *A scatter plot was generated by the built-in software using the protocol of end-point analysis. The signal intensity of two florescent dyes was measured and plotted; green dots represent major alleles only which were labeled with VIC dye (in y-axis, with florescence excitation 533nm and detection 580nm), blue dots for minor alleles only which were labelled with FAM florescence (x-axis, excitation: 465nm and detection: 510nm) and red dots represent both alleles.*

For monoclonal samples (e.g. cultured lab strains), distinct clusters were shown in the scatter plot (as green and blue dots only), and allele calling is determined visually from the plot.

A total of 8 field isolates collected from Gabon were genotyped to estimate the complexity of infection. Three out of eight (37.5%) samples were monoclonal whereas 5 out of 8 (62.5%) samples were polyclonal. Sample J12 appeared to be the most complex infection since 10 out of 24 SNPs were polymorphic.

4.2.2 Limit of detection of minor strain in mixed infection

The assay for SNP C/T on chromosome 13 at position 000158614 was done for the serial dilutions of a mix of two parasite strains: 3D7 and Dd2. All 7 dilutions were genotyped.

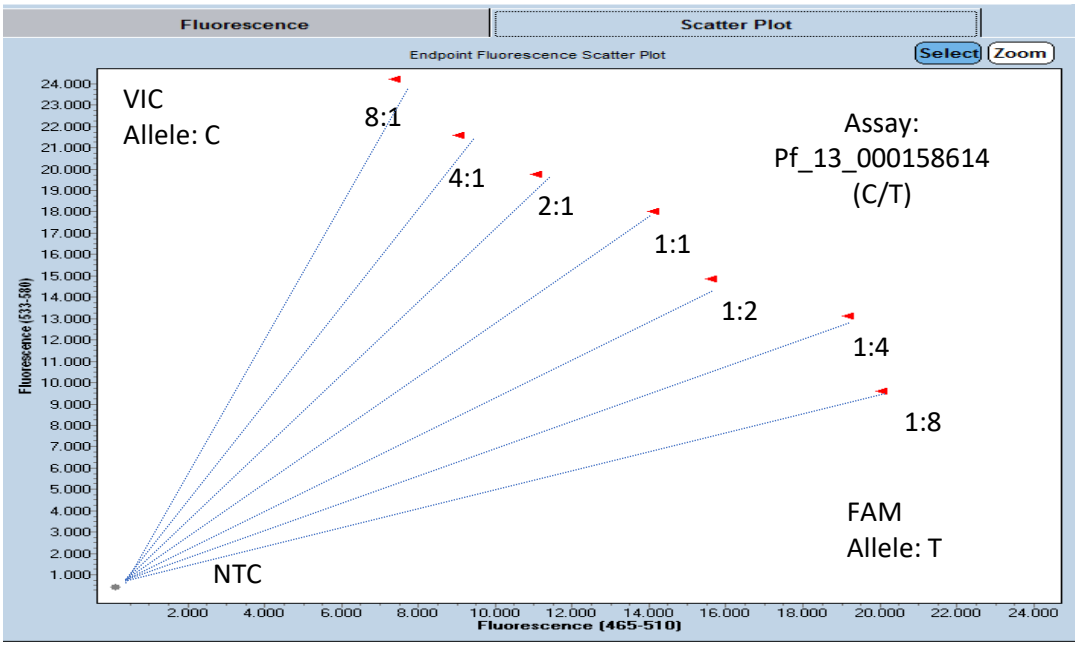

Figure 11. The plot of 1 SNP genotyping of serial samples created by mixing DNA of 2 P. falciparum lab strains (3D7 and Dd2) with different ratios. *The location of red dots on the scatter plot reflex the mixing ratio of two parasite strains 3D7 (allele C) and Dd2 (allele T). NTC: non-template control.*

Table 13. Concentration of parasite DNA in the serial dilution and sensitivity of assay Pf_13_000158614*. SNP gen: SNP genotyping result.*

Name of sample		**A-2**	**A-1**	**A1**	**A2**	**A3**	**A4**	**A5**	**A6**	**A7**	**A8**	**A9**
3D7/Dd2 DNA ratio		16:1	10:1	8:1	4:1	2:1	1:1	1:2	1:4	1:8	1:10	1:16
Concentration (ng/µL)	3D7	9.4	9.1	8.9	8.0	6.7	5.0	3.3	2.0	1.1	0.9	0.6
	Dd2	0.6	0.9	1.1	2.0	3.3	5.0	6.7	8.0	8.9	9.1	9.4
SNP gen for 3D7 (C)		+	+	+	+	+	+	+	+	+	NA	NA
SNP gen for Dd2 (T)		NA	NA	+	+	+	+	+	+	+	+	+

Genotyping results of the samples with mixing ratios greater than 8:1 and smaller than 1:8 showed that minor alleles cannot be reliably discriminated when they contribute less than ~12.5% of the major alleles density in the same sample.

4.2.3 Discrimination between recrudescence and new infection

Seven pairs of samples collected from a drug efficacy trial in Gabon were successfully genotyped. There were 6 out of 7 (85.7%) samples classified as new infections, 1 out of 7 (14.3%) was not classifiable due to the too high complexity of both samples (Day 0

and Day X). SNP 15 was excluded from our analysis since all of the PCR results were negative.

4.2.4 Establishment of a minimal SNP barcode

Table 14. Genotyping result of 21 samples at 9 SNP loci. *Minor allele freq: Minor allele frequency.*

SNP	**5**	**6**	**7**	**9**	**10**	**11**	**13**	**14**	**22**	
Allele variants	**G/C**	**C/G**	**A/G**	**A/T**	**C/T**	**T/C**	**C/T**	**A/C**	**C/T**	
F078_SCR	C	C	A	A	U	T	C	A	C	
F079_D42	C	C	G	A	C	T	C	A	C	
F079_SCR	C	G	U	A	C	T	C	C	T	
F080_D35	U	C	A	A	C	U		C	C	
F080_SCR	G	C	G	T	T	T		A	T	
F081_D35	C	C	G	A	C	T	T	C	C	
F081_SCR	U	G	G	T	C	U		C	T	
F090_D63	U	U	G	A	C	C		C	C	
F090_SCR	C	C	A	U	C	T	C	A	T	
F095_D42	G	C	A	U	T	T	T	A	C	
F095_SCR	G	C	A	T	C	T	C	C	C	
F098_D63	C	G	A	A	T	T	C	A	T	
F098_SCR	C	C	U	T	C	T	C	C	U	
J11	G	G	A	T	T	C	C	C	C	
J12*	U	C	U	T	U	C	C	A	T	
J13	C	G	G	T	U	U		C	C	
J14	G	C	A	T	T	C	T	A	C	
J20	G	C	A	T	C	C	C	A	T	
J23	G	C	G	T	T	C	T	A	T	
J25*	U	U	U	A	C	T	C	C	T	
J3S	G	G	A	T	U	T	C	A	C	
Minor allele freq	0.5	0.341	0.432	0.432	0.386	0.364	0.263	0.477	0.455	**24-SNPs barcode**
Amp success rate (%)	100	100	100	100	100	100	86.4	100	100	
Uniqueness calling	90.50%									90.50%

Amp success rate: amplification success rate. *: samples with non-unique SNP barcode. A: Adenine; C: Cytosine; G: Guanine; T: Thymine; U: mix of two alleles; blanks: amplification failed. Sample F078_D42 was excluded from this analysis due to the negative result at 5/9 SNPs.

A compiled analysis using the sample from all experiments showed the minor allele frequency (MAF) of the 24 SNPs ranged from 0.02 to 0.5.

Based on MAF (>0.25) and amplification success rate (>80%), 9 assays number 5, 6, 7, 9, 10, 11, 13, 14, 22 were selected for a minimal barcode. The minimal barcode showed similar discriminatory power to the 24-SNPs barcode (**Table 14**).

4.3 *P. falciparum* in peripheral blood and bone marrow of anemic malaria

4.3.1 Parasitemia

4.3.1.1 Between BM and PB

A total of 91 thick blood smears (TBS) made from peripheral blood samples were read. The mean of log10 transformed parasitemia was: 4.35 (95% CI: 4.20, 4.50).
Ct values from PCR using bone marrow samples are significantly lower than those using peripheral blood samples ([Paired t.test] p-value < 0.0001).

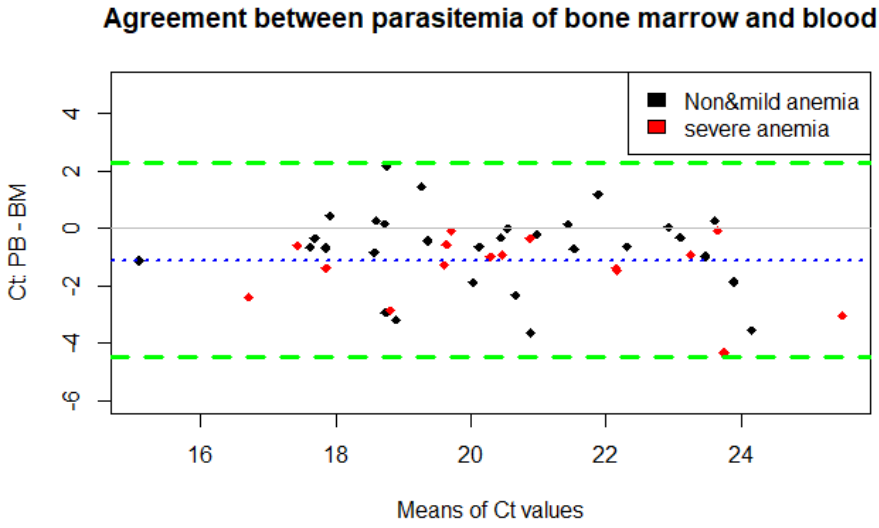

Figure 12. Bland Altman plot visualizes the agreement of the Ct-values of PCR using peripheral blood (PB) and bone marrow (BM). *The green lines represent 95% confidence intervals. Y-axis: the difference between Ct-values of PCR using PB samples and BM samples from the same patient. The blue line represents the mean of the differences. The difference in Ct values between PB and BM samples was negative in 100% severe malarial anemia cases whereas in the group of non- and mild anemia, the positive differences were observed in some samples.*

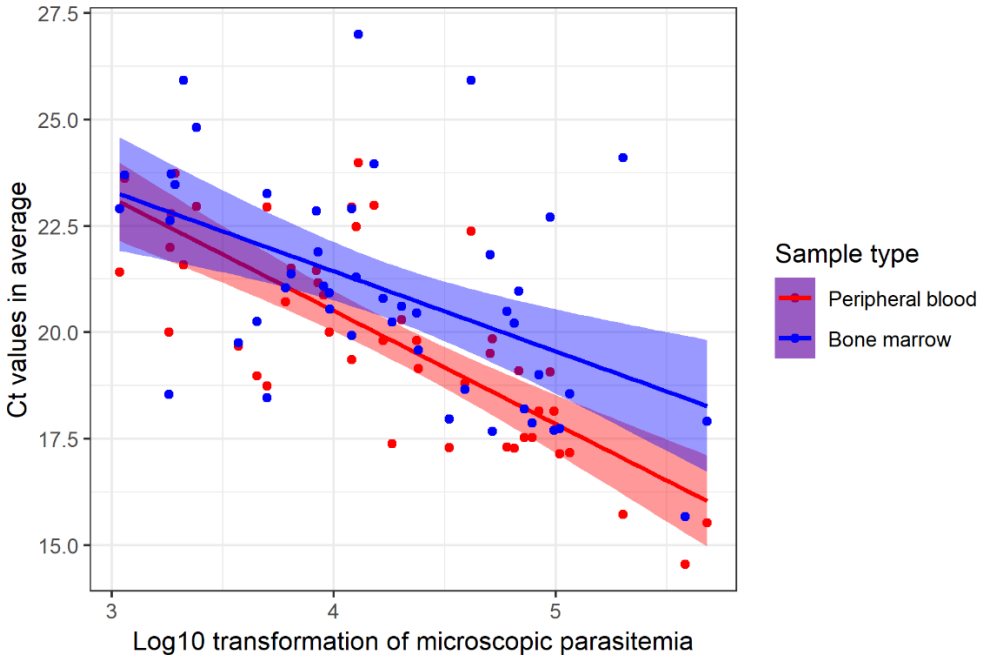

Figure 13. The correlation between the Ct-values of qPCR and TBS results. *Y-axis represents the average Ct-values of triplicate PCR.*

There are significant correlations of Ct values and microscopic parasitemia. With bone marrow samples: p-value = 0.00032, Adjusted R2: 0.2355 and peripheral blood samples: p-value < 0.0001, adjusted R2: 0.5759 (**Figure 13**).

The results from a binomial regression model showed that anemia was independent of parasitemia assessed by PCR using bone marrow samples as well as peripheral microscopic TBS results.

4.3.1.2 Between SMA and MM

Peripheral parasitemia between SMA group (Log10 parasitemia = 4.34 (95% CI: 4.07, 4.61)) and MM group were similar (Log10 parasitemia = 4.35 (95% CI: 4.19, 4.52)), p-value = 0.93.

4.3.2 Multiplicity of infection

4.3.2.1 Different MOI between BM and PB of the same patient

MOI of the parasite populations in bone marrow was 3.06 (95% CL: 2.69, 3.44) and in peripheral blood was 3.11 (95% CL: 2.70, 3.51). There was no difference observed between MOI of the parasite population in these two compartments, the result was confirmed by a binomial regression model.

The difference in the number of alleles was observed in 8/47 pairs (17%) of the sample (from 08 patients).

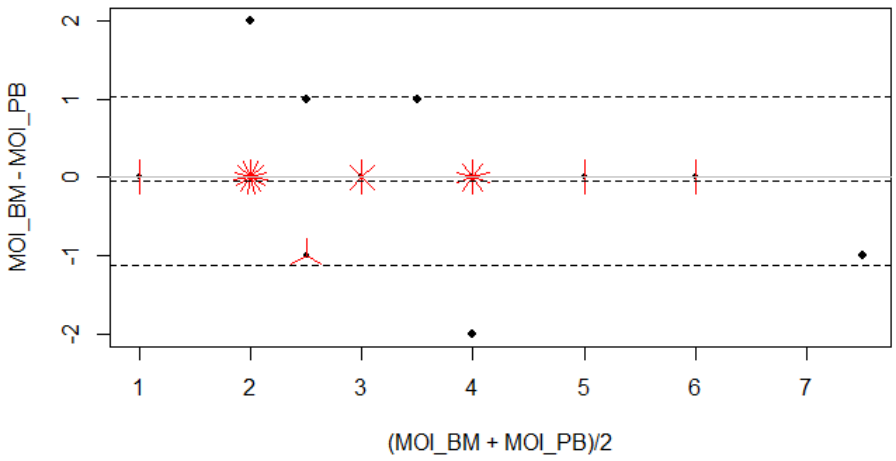

Figure 14. Agreement of genotyping result using bone marrow samples (MOI_BM) and peripheral blood samples (MOI_PB). *X-axis: average MOI of 2 types of samples from the same patients, y-axis: the difference between MOI of 2 types of samples. Flower patterns: represent the number of identical results.*

In 8/8 pairs of samples, the discordant alleles (the one detected in 1 type of sample but was not detected in the other type sample) were minor alleles.

4.3.2.2 MOI was not a predictor of anemia and blood transfusion

MOI of the group of SMA was 3.12 (95%CI: 2.54, 3.70), and was not different from that of the MM group 3.10 (95%CI: 2.55, 3.65), p-value = 0.57 (Wilcoxon test)

MOI of the group of patients with blood transfusion was 2.88 (95% CI: 2.33, 3.44) and was not different from that of the group without blood transfusion (3.17 (95% CI: 2.67, 3.66)), p-value = 0.72. The result was also confirmed by a binomial regression model.

4.3.3 *msp1* allele prevalence in MM and SMA

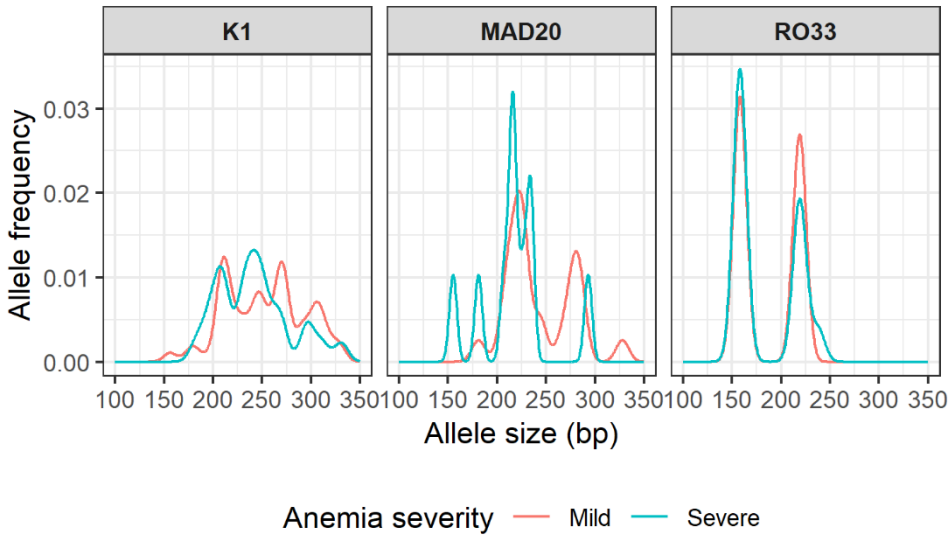

Figure 15. The distribution of allele size among the groups of patients with SMA and MM. *There was no msp1 allele that is specific for any of the level of severity of anemia.*

Table 15. Combination of msp1 genotypes in anemic malaria cases.

	Anemia			
	Mild (N = 30)		Severe (N = 17)	
	BM (%)	PB(%)	BM(%)	PB(%)
K1 only	13 (44)	14 (47)	2 (12)	3 (18)
MAD20 only	3 (10)	3 (10)	0	0
RO33 only	1 (3)	1 (3)	2 (12)	2 (12)
K1 + MAD20	7 (23)	7 (23)	5 (29)	3 (18)
K1 + RO33	3 (10)	3 (10)	5 (29)	5 (29)
MAD + RO33	0	0	0	0
K1 + MAD20 + RO33	3 (10)	2 (7)	3 (18)	4 (23)
Total	30 (100)	30 (100)	17 (100)	17 (100)

5 DISCUSSION

5.1 RDTs are excellent source of DNA for large scale investigational studies

Archived RDTs are a reliable source of DNA for Realtime PCR and the amplification success rate is comparable to that achieved from filter paper (27,41). PCR using DNA extracted from both sources could detect *Plasmodium* parasites at as low density as 68 parasites/mL. The yield of DNA recovered may vary among different designs of RDT. Lower DNA yield was reported for RDT types that have a plastic cover on top of the nitrocellulose strip (40). The limit of detection and success rate of molecular assays using DNA extracted from RDT also rely on the extraction methodology (41) and, better result was achieved by using commercialized column-based kits. Besides, absolute quantification of malaria parasites using archived RDT samples is possible, which expands the contribution of RDT to the success of field studies. However, the accuracy of the quantification process can be influenced by the ununiformed blood volume spotted on RDT (69). The extrapolated parasitemia in this work, therefore, served only the analyses with the assumption that blood volumes on every RDT were identical (both on the field RDT and controls). The storage duration (within 6 months at room ambient) of archived RDTs did not interfere with either DNA quality or the amplification success rate (27).

The sensitivity and specificity of quantitative real-time PCR which targets 18S genes (especially multiple copy genes as the approach used in this thesis) were demonstrated to be higher compared to that of other conventional methods. Additionally, parasite density in uncomplicated and asymptomatic *P. falciparum* parasitemia is usually low and is often undetectable by microscopy. Therefore, the amplification success rates of *msp1* genotyping PCR and *Pfcrt* (single-copy gene) genotyping PCR are often relatively lower than that of our 18S-targeted real-time PCR.

RDTs are used to detect parasite-specific antigens (hrp2, lactate dehydrogenase and aldolase) which were demonstrated to circulate in human blood even after parasites are removed, therefore, RDT may still show a positive result in some transient infections for days after parasite clearance. That reason, together with the absence of negative RTD in the analyses may explain the amplification failure rates observed in this work.

In line with results from previously published work, we demonstrated that being able to provide a diagnosis in minutes and serve as a source of DNA for molecular testing, malaria RDTs could potentially represent the key of large scale retrospective analyses of parasite populations (27).

5.2 The pattern of parasitemia in study populations in Gabon

Age and exposure to malaria parasites are described as two major factors that define the risk of developing parasitemia and illness. Both age and exposure to malaria were found to induce and regulate the host immunity to malaria. When analyzing the effect of each factor independently on the immune response to the first malaria episode between children and naïve adults, the result surprisingly showed that the effect of age alone was small, and the main determinant was malaria exposure (70). Often, these two factors correlate, especially in malaria-endemic regions, therefore, higher parasitemia is observed in children more often than in adults (71).

In children aged from 9 months to 5 years, results from TBS has revealed that, except for immature gametocytes density and prevalence, asexual and matured parasite density in bone marrow samples and peripheral blood was similar (15). Since TBS from bone marrow samples were not made in this study, we analyzed Ct-values from the qPCR results to compare the DNA load between samples. The extrapolation from Ct-values to parasitemia in bone marrow might result in an imprecise estimation because most of the mature parasites e.g. trophozoites and schizonts are accumulated in bone marrow (15), each of them contains often more than one genome. By PCR, the parasite DNA concentration (regardless of the stage of the parasite, represented by Ct-values) in peripheral blood was revealed to be slightly higher than that in bone marrow samples (**Figure 12**).

The comparison might be additionally biased due to the fluctuation of peripheral parasitemia. In fact, parasites at mature stages and gametocytes were found in bone marrow while absent in the periphery as described in a case report (72). In our study, the number of asexual parasite count in TBS partly but significantly correlates with the Ct-values from PCR using peripheral blood as well as bone marrow samples.

In line with results from the previous study which emphasized the role of lysis of non-parasitized RBC in the pathogenesis of malarial anemia (26), in this study population, peripheral parasitemia (from TBS reading) and Ct-values of PCR using bone marrow

samples are not predictors of severity of anemia. That implies that admission parasitemia is a poor predictor of malarial anemia and, suggests to further look into the other characteristics of the infection e.g. parasite diversity.

5.3 *msp1* genotyping revealed high diversity of *P. falciparum* parasite in Gabon

5.3.1 Automated CE enhances the resolution of conventional-PCR-based genotyping.

Approaches used to estimate the diversity of the malaria parasite populations are still basing on conventional PCR, and the ones targeting the length-polymorphic genes have been commonly used. The performance of these approaches relies mainly on the resolution of the sizing system and could be enhanced by using capillary electrophoresis (27).

Our study was the first in Lambaréné – a field site of numbers of malaria-related trials – to use QIAxcel Advanced System – the high resolution, automated capillary gel electrophoresis to analyze the population genetic diversity of *P. falciparum* (27). This system provides a high discriminatory power (three base-pairs difference) and is able to detect up to 11 alleles in one infection. Such an automated system allows for sizing analysis of batches of 96 samples in one run. The outcome could be used for different analyses in order to characterize the malaria parasite, including the length of the amplicons, identification of major and minor alleles, and the relative proportion of each allele. Despite a lower resolution of this system compared to that of sequencers when genotyping short tandem repeats (microsatellites) (73,74), this system could be a part of a field-deployable approach for genotyping other marker genes (like *msp* genes) with higher accuracy compared to conventional gel electrophoresis.

5.3.2 Parasite diversity in uncomplicated malaria and asymptomatic *P. falciparum* parasitemia

P. falciparum merozoite surface protein 1 (MSP1) involves the invasion process of these parasites into the human erythrocytes. Studying the polymorphism and the fluctuation in the diversity of this important gene in malaria-endemic areas may help understanding selection pressure. Indeed, patterns of the multiplicity of malaria infections were shown across age groups and geographic regions, thus, a high polymorphism of the *msp1* gene in the studied population has been recorded (27).

The population MOI was 2.6 (2.0 for Lambaréné and 2.8 for Fougamou and rural areas), which is in line with results from recent reports (75). That implies the decrease in the complexity of malaria infection as compared to what was observed in 2000 (76) – before ACTs became the first-line therapy.

Immunity against malaria is acquired after exposure to *P. falciparum* parasites, therefore, it relates to transmission intensity and age of individuals. As a result, parasite diversity is often lower in adults than in children. After correction for the effect of age, the result showed a significant correlation of difference in the geographic location on MOI. In regions with higher economic status, it is possible that drug pressures can cause fluctuation of parasite clones in patients, results in disappearance or reappearance of some parasite strains (76).

The MSP1, particularly transcription of *msp1*/K1 and MAD20 gene families have been highlighted as potential targets for *P. falciparum* vaccine development (77). In this study, the prevalence of *msp1*/K1 was predominant, followed by *msp1*/RO33 and *msp1*/MAD20 families. However, there were no specific alleles for any region or age. This result is in line with that from other studies conducted in different lab settings and geographic regions (76,78). *P. falciparum* allele size of around 240 bp was the most prevalent (26.7%), that size is similar to the length of the *msp1* gene of strain NF54 (241 bp) – which is the parasite strain used in some clinical trials in Lambaréné (27).

5.3.3 *P. falciparum* diversity in BM and PB of patients with malarial anemia

At the population level, similar to what is observed in the placenta and umbilical cord blood (20), MOI of the parasite in bone marrow is similar to that in peripheral blood. In severe malaria patients, a higher prevalence of *msp1*/MAD20 alleles (10) or *msp1*/K1 alleles (9) was observed. In our study, the prevalence of *P. falciparum msp1*/K1 and *msp1*/K20 alleles in monoclonal infections was higher in mild and non-anemic malaria group. There was no association between the prevalence of other alleles and anemia. In addition, there is no specific allele size for severe malarial anemia. Prevalence of *msp1* alleles in the population and their contribution to the severe form of malaria may change over time due to selection pressure. Causing mild and asymptomatic malaria may be a useful strategy for the malaria parasite to improve fitness costs since mild and asymptomatic malaria cases are demonstrated to be the main source of transmission. The difference in the prevalence of some alleles of *msp1* genes in this study population

over time (our result compared with the result of Kun et al 1998) may reflect the ongoing change within the parasite population which is probably due to selection pressure. The finding highlighted the need for the frequent monitoring of malaria parasite in endemic regions.

In a significant proportion of patients (17%), the number of parasite strains identified in the bone marrow differs from that in peripheral blood. The difference in the observed MOI between peripheral blood and bone marrow might be the result of the within-host competition (11) – in which, resource competition may lead to the suppression of some minor parasite strains – or the interaction between parasite and host's immunity.

The limitations of this analysis were: first, the small sample size since the initial study design was not for this purpose and second, the effect of the geographic factor was not corrected as in the work with uncomplicated malaria and asymptomatic *P. falciparum* parasitemia we have demonstrated that the difference in MOI could also be observed in different regions and age groups.

5.4 A minimal SNP barcode for genotyping *P. falciparum* in Gabon

The primary goal of this work was to validate the established SNP "barcode" for genotyping *P. falciparum* in our lab setting and optimize the barcode to a number of SNPs that are specific for the malaria parasite in our studied area and allow high throughput.

Regardless of the genotyping method, the distinction between new infections and recrudescence remains difficult, especially in highly complex infections. Although minor strain which consists of a negligible proportion of total parasite load should not affect the treatment outcome, the definition or the cut-off values by which the minor strains are defined is still not well characterized. It depends on the techniques used and the author's preference. The most general cut-off is around 20% for conventional methods (79) (parasite strain consists lower than 20% of the overall parasite load should be considered negligible) or could be 10% for more advanced methods (80). With SNP barcoding assay, the genotyping result of samples that contain 1 or 2 parasites strains was displayed in colors in a scatter plot, therefore the interpretation can be done with bare eyes. However, in poly-genomic infections, where the difference in parasite density between major and minor alleles is large (minor alleles density are less than ~12.5% of the overall parasite load), the identification of alleles becomes more complicated. One

drawback of this genotyping approach is the genotype calling process for complex infections (when both minor and major alleles are detected in the greater number of SNP) since the rearrangement and estimation of all the SNP result relies (if exists) on analyses that use many assumptions (81).

The entire barcode was developed using sequence data from 18 isolates collected from different endemic regions, 5 out of these 18 were from Africa (82). The barcode is robust when using 24 most polymorphic SNPs that are able to distinguish parasites with high predictive accuracy. However, using all 24 SNPs for the work in one geographic region is not necessary. To this point, a smaller but powerful barcode is of our interest. Here, we described a minimal barcode consisting of 9 SNPs that showed a similar discriminatory power to that of the entire 24-SNPs barcode. The amplification success rate was shown to be different between the SNP assays, even when applied to the same sample, therefore the sensitivity of the whole barcode assays should not be represented by the lower limit of detection of any of the 24 SNP assays. In addition, besides the ability to give results after a one-step-PCR and without using any sizing or imaging analysis, the SNP barcoding assays were shown to have comparable performance in distinguishing parasite strains to *msp* method (56). More important, minimizing the number of SNP assays can reduce the cost of this method (56).

Another possible application of this method could be to differentiate parasite clones used to test the efficacy of vaccine candidates against heterozygous challenges since identified strains could be distinguished by using one or two SNPs.

This work had some limitations. First, the number of samples used to validate and analyze the discriminatory power of the barcode was small. Second, the number of SNP of the minimal barcode remains unsatisfactorily high (9 SNPs). Therefore, validating the barcode assay using a bigger number of samples will potentially result in a better selection of SNP assays.

5.5 Prevalence of CQ-resistant haplotypes in Gabon remains high

In Gabon, chloroquine has been replaced by artemisinin-based combination therapies (ACTs) in 2005, these drug combinations became the first-line regimens for the treatment of uncomplicated malaria since then. Before this change, the prevalence of the CQ resistance-related genotypes was close to 100%. A few years after the change in national guidelines, several studies have documented a regional reduction in the

prevalence of CQ-resistant alleles (83,84). Another study has documented a low prevalence of the CQ-susceptible wild-type haplotype CVMNK in samples collected during 2011 – 2014 (85). A recent study conducted in rural Gabon has concluded a high (89%) overall prevalence of CQR genotypes (3). Although the use of amodiaquine was connected with the persistence of a high prevalence of CQR genotypes, strong evidence is scarce.

The prevalence of CQ-resistant haplotypes in Lambaréné was found to be lower than that in Fougamou and rural areas. The prevalence of CQ-resistant haplotypes is independent of age. All of the isolates carry CQ-resistant haplotype CVIET (triple mutation at codons 74, 75 and 76), haplotype SVMNT (double mutation at codons 72 and 76) was not found (27). These findings highlight the necessity of further investigations to better understand the mechanism behind the persistence of CQ resistance.

5.6 Conclusion

Rapid diagnostic tests are the multifunctional tool for field studies on *P. falciparum*. Besides the great contribution to the success of treatment and management of malaria by giving the prompt diagnosis, RDTs are also a good source of DNA for molecular assays that aim at genotyping malaria parasites and possibly parasite quantification. The resolution of the conventional *msp1*-gene-targeted PCR-based genotyping method was enhanced by CE on an affordable and automated system. The minimal but specific SNP "barcoding" assays for discrimination of *P. falciparum* strains from Gabon has been established. Using these tools, high diversity and prevalence of CQ-resistant haplotypes of *P. falciparum* are observed in Lambaréné – Gabon, and proximity. Additionally, the parasite population structure has been shown to be different across age groups, geographical regions and occasionally between peripheral blood and bone marrow of one individual, however, the association between parasite diversity and severity of malarial anemia was not found.

SUMMARY

Understanding the population structure of malaria parasites and the host-parasite interaction is of fundamental interest and may help in the development of improved anti-malarial interventions. This thesis contributes to the improvement of procedures and methods for the characterization of the malaria parasite and reports current epidemiological characteristics of *Plasmodium falciparum* populations in Lambaréné, Gabon.

The result of this thesis encourages the use of rapid diagnostic tests (RDT) not only as a diagnostic tool but also as a source of DNA that can be subsequently used for molecular assays to assess parasite diversity, species distribution, and genetic polymorphisms (e.g. mutations associated with drug resistance) as well as diagnostic performance of RDTs on large scale. In addition, performance and resolution of a genotyping method based on size polymorphisms of the *msp1* gene, by using conventional polymerase chain reaction (PCR) followed by automated capillary electrophoresis (CE) (QIAxcel system, Qiagen) has been improved. Furthermore, in order to overcome the comparatively low sensitivity of conventional PCR, a barcoding assay with 9 single nucleotide polymorphisms (SNPs) for genotyping of low-density *P. falciparum* infection by Taqman-probe-based end-point PCR was adapted from a published method. The alternative use of these two approaches will help to improve the accuracy of parasite genotyping, covering different types of samples, applications, and lab settings.

The improved techniques were applied in two case scenarios: i) an epidemiological survey and ii) to assess parasite population structure in different compartments of the body.

It was observed that in Lambaréné and surroundings, the multiplicity of infection (MOI) and prevalence of chloroquine-resistance-associated mutations remain high; particularly, in the most rural areas and despite a change in malaria control recommendations, including withdrawal of chloroquine from the market. Decreasing MOIs in central areas of Lambaréné may be the result of urbanization and its effect on transmission intensity and spread of drug resistance, which results in less diverse malaria parasite populations.

Within its human host, malaria parasite population structure in both severely anemic and control patients was shown to be similar between bone marrow and peripheral blood. If the differences observed in some patients is of pathophysiological importance remains to be further investigated.

My thesis highlights the crucial role of adequate genotyping approaches in the development of malaria eradication tools, identifies a commonly available source of DNA for retrospective studies and suggests an improvement in the guidelines for sample collection and molecular analyses for studying malaria in endemic regions.

ZUSAMMENFASSUNG

Zur Entwicklung und Verbesserung der Behandlung von Malaria ist es von großem Interesse die Populationsstruktur des Parasiten und die Wirt-Parasit Mechanismen zu verstehen. Diese Dissertation trägt dazu bei, bisherige Methoden zur Charakterisierung des Malariaparasiten zu verbessern und anzuwenden um aktuelle epidemiologischen Daten zu *Plasmodium falciparum* Populationen in Lambaréné, Gabun zu gewinnen.
Die Ergebnisse dieser Arbeit zeigen, dass diagnostische Schnelltests (RDT) nicht nur zur unmittelbaren Diagnose, sondern auch als eine Quelle zur Isolierung von DNA geeignet sind. Diese kann für die Untersuchung der Verteilung unterschiedlicher Parasitenspezies und -stämme mittels molekularbiologischer Verfahren genutzt werden. Des Weiteren können die diagnostische Leistungsfähigkeit von RDTs und genetische Polymorphismen, wie z.B. Mutationen zu Resistenzen analysiert werden. Außerdem konnte die Methode der Genotypisierung des *msp1* Gens anhand von Größenpolymorphismen verbessert werden. Dazu wurde eine konventionelle Polymerase Ketten Reaktion (PCR) durchgeführt und das PCR-Produkt anschließend mittels automatisierter Kapillarelektrophorese (QIAxcel System Qiagen) aufgetrennt. Um Detektionsprobleme durch die verhältnismäßig geringe Sensitivität der konventionellen PCR zu überwinden wurde ein Barcode-Assay mit 9 SNPs (*single nucleotide polymorphism*) zur Genotypisierung von Proben mit geringer *P. falciparum*-Parasitämie auf Basis eines kommerziell erhältlichen Assays entwickelt und dessen Leistungsfähigkeit überprüft. Die so verbesserte Methodik erhöht die Genauigkeit der Genotypisierung, auch bei unterschiedlichen Arten von Probenmaterial, Anwendungen und Laborvoraussetzungen. Die im Rahmen der Dissertation etablierten Techniken wurden an zwei verschiedenen Kohorten angewendet: 1) Im Rahmen einer epidemiologischen Studie und 2) zur Untersuchung der Struktur von Parasitenpopulationen in verschiedenen Kompartimenten des Körpers.
In Lambaréné und Umgebung ist die beobachtete Multiplizität der Infektion (MOI) und das Auftreten von mit Chloroquinresistenz assoziierten Mutationen immer noch hoch; insbesondere in den ländlichen Gegenden und trotz der aktuellen Empfehlungen zur Malariakontrolle, die unter anderem die Anwendung von Chloroquin verbieten. Die Reduktion der Anzahl von Mehrfachinfektionen in städtischen Gebieten Lambarénés, können das Ergebnis von Urbanisierung sein, die einen Effekte auf

Übertragungsintensität und Verbreitung von Resistenzbildung haben und mit der Abnahme der Diversität von Infektionen assoziiert ist.
Die Untersuchung von zwei Kompartimenten (peripheres Blut und Knochenmark) des menschlichen Wirts zeigte, dass die Struktur der Parasitenpopulationen ähnlich ist und sich in anämischen nicht wesentlich von Kontrollpatienten unterscheidet. Ob die Unterschiede, die in einzelnen Patienten beobachtet wurden, von pathophysiologischer Relevanz sind wird in weitergehenden Arbeiten untersucht werden.
Meine Arbeit stellt die Wichtigkeit adäquater, gut etablierter und validierter Methoden zur Genotypisierung von Malariaparasiten dar und zeigt neue Quellen zur Gewinnung von DNA für retrospektive Studien auf. Dies ist entscheidend für die Entwicklung neuer Interventionen zur Kontrolle der Malaria. Außerdem lassen sich anhand der Arbeit Verbesserung zu den bisherigen Empfehlungen zur Probengewinnung und der molekularen Analyse zur Untersuchung von Malaria in endemischen Gebieten ableiten.

Reference

1. World Health Organization. World Malaria Report [Internet]. 2018. 238 p. Available from: https://www.who.int/malaria/publications/world_malaria_report/en/
2. Le PV V, Kumar P, Ruiz MO, Mbogo C, Muturi EJ. Predicting the direct and indirect impacts of climate change on malaria in coastal Kenya. PLoS One [Internet]. 2019 Feb 6;14(2):e0211258. Available from: https://doi.org/10.1371/journal.pone.0211258
3. Woldearegai TG, Lalremruata A, Nguyen TT, Gmeiner M, Veletzky L, Tazemda-Kuitsouc GB, et al. Characterization of Plasmodium infections among inhabitants of rural areas in Gabon. Sci Rep. 2019 Jul;9(1):9784.
4. Centers for Disease Control and prevention - CDC. DPDx - Laboratory Identification of Parasites of Public Health Concern: Malaria [Internet]. 2017 [cited 2019 Oct 12]. Available from: https://www.cdc.gov/dpdx/malaria/index.html
5. Juliano JJ, Porter K, Mwapasa V, Sem R, Rogers WO, Ariey F, et al. Exposing malaria in-host diversity and estimating population diversity by capture-recapture using massively parallel pyrosequencing. Proc Natl Acad Sci U S A. 2010 Nov;107(46):20138–43.
6. Beck S, Mockenhaupt FP, Bienzle U, Eggelte TA, Thompson WN, Stark K. Multiplicity of Plasmodium falciparum infection in pregnancy. Am J Trop Med Hyg. 2001 Nov;65(5):631–6.
7. Kwiek JJ, Alker AP, Wenink EC, Chaponda M, Kalilani L V, Meshnick SR. Estimating true antimalarial efficacy by heteroduplex tracking assay in patients with complex Plasmodium falciparum infections. Antimicrob Agents Chemother [Internet]. 2006/11/20. 2007 Feb;51(2):521–7. Available from: https://www.ncbi.nlm.nih.gov/pubmed/17116685
8. Lee SA, Yeka A, Nsobya SL, Dokomajilar C, Rosenthal PJ, Talisuna A, et al. Complexity of Plasmodium falciparum infections and antimalarial drug efficacy at 7 sites in Uganda. J Infect Dis. 2006 Apr;193(8):1160–3.
9. Kun JF, Schmidt-Ott RJ, Lehman LG, Lell B, Luckner D, Greve B, et al. Merozoite surface antigen 1 and 2 genotypes and rosetting of Plasmodium falciparum in severe and mild malaria in Lambarene, Gabon. Trans R Soc Trop Med Hyg. 1998;92(1):110–4.
10. Ghanchi NK, Hasan Z, Islam M, Beg MA. MAD 20 alleles of merozoite surface protein-1 (msp-1) are associated with severe Plasmodium falciparum malaria in Pakistan. J Microbiol Immunol Infect. 2015 Apr;48(2):213–8.
11. Bushman M, Morton L, Duah N, Quashie N, Abuaku B, Koram KA, et al. Within-host competition and drug resistance in the human malaria parasite Plasmodium falciparum. Proc R Soc B Biol Sci [Internet]. 2016;283(1826):20153038. Available from:

http://rspb.royalsocietypublishing.org/content/283/1826/20153038

12. Walk J, Reuling IJ, Behet MC, Meerstein-Kessel L, Graumans W, van Gemert G-J, et al. Modest heterologous protection after Plasmodium falciparum sporozoite immunization: a double-blind randomized controlled clinical trial. BMC Med [Internet]. 2017 Sep 13;15(1):168. Available from: https://www.ncbi.nlm.nih.gov/pubmed/28903777

13. Neafsey DE, Juraska M, Bedford T, Benkeser D, Valim C, Griggs A, et al. Genetic Diversity and Protective Efficacy of the RTS,S/AS01 Malaria Vaccine. N Engl J Med [Internet]. 2015;373(21):2025–37. Available from: http://www.nejm.org/doi/abs/10.1056/NEJMoa1505819%5Cnhttp://www.ncbi.nlm.nih.gov/pubmed/26488565

14. Miller LH, Good MF, Milon G. Malaria pathogenesis. Science. 1994 Jun;264(5167):1878–83.

15. Smalley ME, Abdalla S, Brown J. The distribution of Plasmodium falciparum in the peripheral blood and bone marrow of Gambian children. Trans R Soc Trop Med Hyg. 1981;75(1):103–5.

16. Genrich GL, Guarner J, Paddock CD, Shieh W-J, Greer PW, Barnwell JW, et al. Fatal malaria infection in travelers: novel immunohistochemical assays for the detection of Plasmodium falciparum in tissues and implications for pathogenesis. Am J Trop Med Hyg. 2007 Feb;76(2):251–9.

17. Seydel KB, Milner DAJ, Kamiza SB, Molyneux ME, Taylor TE. The distribution and intensity of parasite sequestration in comatose Malawian children. J Infect Dis. 2006 Jul;194(2):205–8.

18. Farfour E, Charlotte F, Settegrana C, Miyara M, Buffet P. The extravascular compartment of the bone marrow: a niche for Plasmodium falciparum gametocyte maturation? Malar J. 2012 Aug;11:285.

19. De Niz M, Meibalan E, Mejia P, Ma S, Brancucci NMB, Agop-Nersesian C, et al. Plasmodium gametocytes display homing and vascular transmigration in the host bone marrow. Sci Adv. 2018 May;4(5):eaat3775.

20. Mbouamboua Y, Koukouikila-Koussounda F, Ntoumi F, Adukpo S, Kombo M, Vouvoungui C, et al. Sub-microscopic Plasmodium falciparum infections in matched peripheral, placental and umbilical cord blood samples from asymptomatic Congolese women at delivery. Acta Trop. 2019 May;193:142–7.

21. World Health Organization (WHO). Guidelines for the treatment of malaria. Third edition [Internet]. 2015. Available from: https://www.who.int/malaria/publications/atoz/9789241549127/en/

22. Douglas NM, Lampah DA, Kenangalem E, Simpson JA, Poespoprodjo JR, Sugiarto P, et al. Major Burden of Severe Anemia from Non-Falciparum Malaria Species in Southern Papua: A Hospital-Based Surveillance Study. PLOS Med [Internet]. 2013 Dec 17;10(12):e1001575. Available from: https://doi.org/10.1371/journal.pmed.1001575

23. World Health Organization. Guideline for the treatment of malaria. 3rd, editor. Geneva; 2015.

24. Calis JCJ, Phiri KS, Faragher EB, Brabin BJ, Bates I, Cuevas LE, et al. Severe Anemia in Malawian Children. N Engl J Med [Internet]. 2008 Feb 28;358(9):888–99. Available from: https://doi.org/10.1056/NEJMoa072727

25. White NJ. Anaemia and malaria. Malar J [Internet]. 2018;17(1):371. Available from: https://doi.org/10.1186/s12936-018-2509-9

26. Looareesuwan S, Davis TM, Pukrittayakamee S, Supanaranond W, Desakorn V, Silamut K, et al. Erythrocyte survival in severe falciparum malaria. Acta Trop. 1991 Feb;48(4):263–70.

27. Nguyen TT, Nzigou Mombo B, Lalremruata A, Koehne E, Zoleko Manego R, Dimessa Mbadinga LB, et al. DNA recovery from archived RDTs for genetic characterization of Plasmodium falciparum in a routine setting in Lambarene, Gabon. Malar J. 2019 Oct;18(1):336.

28. Wongsrichanalai C, Barcus MJ, Muth S, Sutamihardja A, Wernsdorfer WH. A review of malaria diagnostic tools: microscopy and rapid diagnostic test (RDT). AmJTrop Med Hyg. 2007;77.

29. World Health Organization (WHO). Rapid diagnostic tests [Internet]. 2018. Available from: https://www.who.int/malaria/areas/diagnosis/rapid_diagnostic_tests/en/

30. Kain KC, Lanar DE. Determination of genetic variation within Plasmodium falciparum by using enzymatically amplified DNA from filter paper disks impregnated with whole blood. J Clin Microbiol. 1991 Jun;29(6):1171–4.

31. Edoh D, Steiger S, Genton B, Beck HP. PCR amplification of DNA from malaria parasites on fixed and stained thick and thin blood films. Trans R Soc Trop Med Hyg. 1997;91(3):361–3.

32. Mischlinger J, Pitzinger P, Veletzky L, Groger M, Zoleko-Manego R, Adegnika AA, et al. Use of Capillary Blood Samples Leads to Higher Parasitemia Estimates and Higher Diagnostic Sensitivity of Microscopic and Molecular Diagnostics of Malaria Than Venous Blood Samples. J Infect Dis. 2018 Sep;218(8):1296–305.

33. Scopel KKG, Fontes CJF, Nunes AC, Horta MF, Braga EM. Low sensitivity of nested PCR using Plasmodium DNA extracted from stained thick blood smears: an epidemiological retrospective study among subjects with low parasitaemia in an endemic area of the Brazilian Amazon region. Malar J [Internet]. 2004 Mar 31;3:8. Available from: https://www.ncbi.nlm.nih.gov/pubmed/15056392

34. Collins WJ, Greenhouse B, Rosenthal PJ, Dorsey G. The use of genotyping in antimalarial clinical trials: a systematic review of published studies from 1995-2005. Malar J. 2006 Dec;5:122.

35. Zhong KJ, Salas CJ, Shafer R, Gubanov A, Gasser Jr RA, Magill AJ, et al. Comparison of IsoCode STIX and FTA Gene Guard collection matrices as

whole-blood storage and processing devices for diagnosis of malaria by PCR. J Clin Microbiol [Internet]. 2001 Mar;39(3):1195–6. Available from: https://www.ncbi.nlm.nih.gov/pubmed/11230458

36. Coleman RE, Sattabongkot J, Promstaporm S, Maneechai N, Tippayachai B, Kengluecha A, et al. Comparison of PCR and microscopy for the detection of asymptomatic malaria in a Plasmodium falciparum/vivax endemic area in Thailand. Malar J [Internet]. 2006 Dec 14;5:121. Available from: https://www.ncbi.nlm.nih.gov/pubmed/17169142

37. Veron V, Carme B. Recovery and use of Plasmodium DNA from malaria rapid diagnostic tests. Am J Trop Med Hyg. 2006 Jun;74(6):941–3.

38. Papa Mze N, Ndiaye YD, Diedhiou CK, Rahamatou S, Dieye B, Daniels RF, et al. RDTs as a source of DNA to study Plasmodium falciparum drug resistance in isolates from Senegal and the Comoros Islands. Malar J. 2015;14(1):1–8.

39. Ishengoma DS, Lwitiho S, Madebe RA, Nyagonde N, Persson O, Vestergaard LS, et al. Using rapid diagnostic tests as source of malaria parasite DNA for molecular analyses in the era of declining malaria prevalence. Malar J [Internet]. 2011;10(1):6. Available from: http://www.malariajournal.com/content/10/1/6

40. Cnops L, Boderie M, Gillet P, Esbroeck M Van, Jacobs J. Rapid diagnostic tests as a source of DNA for Plasmodium species-specific real-time PCR. 2011;1–11. Available from: http://www.malariajournal.com/content/10/1/67

41. Morris U, Aydin-Schmidt B, Shakely D, Mårtensson A, Jörnhagen L, Ali AS, et al. Rapid diagnostic tests for molecular surveillance of Plasmodium falciparum malaria -assessment of DNA extraction methods and field applicability. Malar J [Internet]. 2013;12(1):106. Available from: https://doi.org/10.1186/1475-2875-12-106

42. Scherf A, Mattei D, Sarthou JL. Multiple infections and unusual distribution of block 2 of the MSA1 gene of Plasmodium falciparum detected in west African clinical isolates by polymerase chain reaction analysis. Mol Biochem Parasitol. 1991 Feb;44(2):297–9.

43. Viriyakosol S, Siripoon N, Petcharapirat C, Petcharapirat P, Jarra W, Thaithong S, et al. Genotyping of Plasmodium falciparum isolates by the polymerase chain reaction and potential uses in epidemiological studies. 1995;73(6):85–95.

44. WHO. Methods and Techniques for Clinical Trials on Antimalarial Drug Efficacy: Genotyping to Identify Parasite Populations. World Heal Organ Geneva, Switz. 2008;

45. Messerli C, Hofmann NE, Beck HP, Felger I. Critical evaluation of molecular monitoring in malaria drug efficacy trials and pitfalls of length-polymorphic markers. Antimicrob Agents Chemother. 2017;61(1):1–11.

46. Daniels R, Volkman SK, Milner DA, Mahesh N, Neafsey DE, Park DJ, et al. A general SNP-based molecular barcode for Plasmodium falciparum identification and tracking. Malar J. 2008 Oct;7:223.

47. Bankole BE, Kayode AT, Nosamiefan IO, Eromon P, Baniecki ML, Daniels RF, et al. Characterization of Plasmodium falciparum structure in Nigeria with malaria SNPs barcode. Malar J [Internet]. 2018 Dec 17;17(1):472. Available from: https://www.ncbi.nlm.nih.gov/pubmed/30558627

48. Preston MD, Campino S, Assefa SA, Echeverry DF, Ocholla H, Amambua-Ngwa A, et al. A barcode of organellar genome polymorphisms identifies the geographic origin of Plasmodium falciparum strains. Nat Commun [Internet]. 2014 Jun 13;5:4052. Available from: https://doi.org/10.1038/ncomms5052

49. Baniecki ML, Faust AL, Schaffner SF, Park DJ, Galinsky K, Daniels RF, et al. Development of a Single Nucleotide Polymorphism Barcode to Genotype Plasmodium vivax Infections. PLoS Negl Trop Dis [Internet]. 2015 Mar 17;9(3):e0003539. Available from: https://doi.org/10.1371/journal.pntd.0003539

50. Tanabe K, Zollner G, Vaughan JA, Sattabongkot J, Khuntirat B, Honma H, et al. Plasmodium falciparum: genetic diversity and complexity of infections in an isolated village in western Thailand. Parasitol Int. 2015 Jun;64(3):260–6.

51. Escalante AA, Grebert HM, Isea R, Goldman IF, Basco L, Magris M, et al. A study of genetic diversity in the gene encoding the circumsporozoite protein (CSP) of Plasmodium falciparum from different transmission areas--XVI. Asembo Bay Cohort Project. Mol Biochem Parasitol. 2002;125(1–2):83–90.

52. Srisutham S, Saralamba N, Sriprawat K, Mayxay M, Smithuis F, Nosten F, et al. Genetic diversity of three surface protein genes in Plasmodium malariae from three Asian countries. Malar J. 2018 Jan;17(1):24.

53. Liljander A, Wiklund L, Falk N, Kweku M, Mårtensson A, Felger I, et al. Optimization and validation of multi-coloured capillary electrophoresis for genotyping of Plasmodium falciparum merozoite surface proteins (msp1 and 2). Malar J [Internet]. 2009 Apr 23;8:78. Available from: https://www.ncbi.nlm.nih.gov/pubmed/19386138

54. Gupta V, Dorsey G, Hubbard AE, Rosenthal PJ, Greenhouse B. Gel versus capillary electrophoresis genotyping for categorizing treatment outcomes in two anti-malarial trials in Uganda. Malar J. 2010 Jan;9:19.

55. Applied Biosystems. Applied Biosystems 7900HT Fast Real-Time PCR System: Allelic Discrimination Getting Started Guide [Internet]. 2007 [cited 2019 Oct 5]. Available from: http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_042114.pdf

56. Fulakeza J, McNitt S, Vareta J, Saidi A, Mvula G, Taylor T, et al. Comparison of msp genotyping and a 24 SNP molecular assay for differentiating Plasmodium falciparum recrudescence from reinfection. Malar J [Internet]. 2019;18(1):84. Available from: https://doi.org/10.1186/s12936-019-2695-0

57. Lalremruata A, Jeyaraj S, Engleitner T, Joanny F, Lang A, Belard S, et al. Species and genotype diversity of Plasmodium in malaria patients from Gabon analysed by next generation sequencing. Malar J. 2017 Oct;16(1):398.

58. Adjuik M, Agnamey P, Babiker A, Borrmann S, Brasseur P, Cisse M, et al. Amodiaquine-artesunate versus amodiaquine for uncomplicated Plasmodium falciparum malaria in African children: a randomised, multicentre trial. Lancet [Internet]. 2002;359(9315):1365–72. Available from: http://www.sciencedirect.com/science/article/pii/S0140673602083484

59. Ohrt C, Mirabelli-Primdahl L, Looareesuwan S, Wilairatana P, Walsh D, Kain KC. Determination of failure of treatment of plasmodium falciparum infection by using polymerase chain reaction single-strand conformational polymorphism fingerprinting. Clin Infect Dis. 1999 Apr;28(4):847–52.

60. Duffy F, Bernabeu M, Babar PH, Kessler A, Wang CW, Vaz M, et al. Meta-analysis of Plasmodium falciparum var Signatures Contributing to Severe Malaria in African Children and Indian Adults. MBio [Internet]. 2019 Apr 30;10(2):e00217-19. Available from: https://www.ncbi.nlm.nih.gov/pubmed/31040236

61. Beck HP, Felger I, Huber W, Steiger S, Smith T, Weiss N, et al. Analysis of multiple Plasmodium falciparum infections in Tanzanian children during the phase III trial of the malaria vaccine SPf66. J Infect Dis. 1997 Apr;175(4):921–6.

62. Carlton JM, Fidock DA, Djimde A, Plowe C V, Wellems TE. Conservation of a novel vacuolar transporter in Plasmodium species and its central role in chloroquine resistance of P. falciparum. Curr Opin Microbiol. 2001 Aug;4(4):415–20.

63. Fidock DA, Nomura T, Talley AK, Cooper RA, Dzekunov SM, Ferdig MT, et al. Mutations in the P. falciparum digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. Mol Cell. 2000 Oct;6(4):861–71.

64. WHO | Status report on artemisinin resistance and ACT efficacy (August 2018). WHO [Internet]. 2018 [cited 2019 Aug 14]; Available from: https://www.who.int/malaria/publications/atoz/artemisinin-resistance-august2018/en/

65. Takala-harrison S, Laufer MK. Antimalarial drug resistance in Africa : key lessons for the future. Ann N Y Acad Sci. 2015;1342:62–7.

66. Kremsner PG, Radloff P, Metzger W, Wildling E, Mordmuller B, Philipps J, et al. Quinine plus clindamycin improves chemotherapy of severe malaria in children. Antimicrob Agents Chemother. 1995 Jul;39(7):1603–5.

67. WWARN. DNA Extraction: Preparing Rapid Diagnostic Tests [Internet]. 2014. Available from: http://www.wwarn.org/tools-resources/procedures/dna-extraction-preparing-rapid-diagnostic-tests

68. Mordmüller B, Surat G, Lagler H, Chakravarty S, Ishizuka AS, Lalremruata A, et al. Sterile protection against human malaria by chemoattenuated PfSPZ vaccine. Nature [Internet]. 2017;542(7642):445–9. Available from: http://dx.doi.org/10.1038/nature21060

69. Luchavez J, Lintag ME, Coll-Black M, Baik F, Bell D. An assessment of various

blood collection and transfer methods used for malaria rapid diagnostic tests. Malar J [Internet]. 2007 Nov 15;6:149. Available from: https://www.ncbi.nlm.nih.gov/pubmed/18001481

70. Moncunill G, Mayor A, Jiménez A, Nhabomba A, Puyol L, Manaca MN, et al. Cytokine and antibody responses to Plasmodium falciparum in naïve individuals during a first malaria episode: effect of age and malaria exposure. PLoS One [Internet]. 2013/02/21. 2013;8(2):e55756–e55756. Available from: https://www.ncbi.nlm.nih.gov/pubmed/23437061

71. Doolan DL, Dobaño C, Baird JK. Acquired immunity to Malaria. Clin Microbiol Rev. 2009;22(1):13–36.

72. Tatura SNN, Gunawan S, Bernadus J, Sandjoto S. Plasmodium falciparum found in the bone marrow of a child in Manado City, East Indonesia: A case report. Asian Pac J Trop Med. 2017 Oct;10(10):1015–7.

73. Manrique P, Hoshi M, Fasabi M, Nolasco O, Yori P, Calderón M, et al. Assessment of an automated capillary system for Plasmodium vivax microsatellite genotyping. Malar J [Internet]. 2015 Aug 21;14:326. Available from: https://www.ncbi.nlm.nih.gov/pubmed/26293655

74. Dean DA, Wadl PA, Hadziabdic D, Wang X, Trigiano RN. Analyzing microsatellites using the QIAxcel system. Methods Mol Biol. 2013;1006:223–43.

75. Ndong Ngomo JM, M'Bondoukwe NP, Yavo W, Bongho Mavoungou LC, Bouyou-Akotet MK, Mawili-Mboumba DP. Spatial and temporal distribution of Pfmsp1 and Pfmsp2 alleles and genetic profile change of Plasmodium falciparum populations in Gabon. Acta Trop [Internet]. 2018 Feb 1 [cited 2018 Dec 19];178:27–33. Available from: https://www.sciencedirect.com/science/article/abs/pii/S0001706X17301547?via%3Dihub

76. Aubouy A, Migot-Nabias F, Deloron P. Polymorphism in two merozoite surface proteins of Plasmodium falciparum isolates from Gabon. Malar J. 2003;2:1–6.

77. Conway DJ, Cavanagh DR, Tanabe K, Roper C, Mikes ZS, Sakihama N, et al. A principal target of human immunity to malaria identified by molecular population genetic and immunological analyses. Nat Med. 2000;6(6):689–92.

78. Mayengue P, Ndounga M, Malonga F, Bitemo M, Ntoumi F. Genetic polymorphism of merozoite surface protein-1 and merozoite surface protein-2 in Plasmodium falciparum isolates from Brazzaville, Republic of Congo. Malar J [Internet]. 2011;10(1):276. Available from: http://www.malariajournal.com/content/10/1/276

79. Juliano JJ, Trottman P, Mwapasa V, Meshnick SR. Detection of the dihydrofolate reductase-164L mutation in Plasmodium falciparum infections from Malawi by heteroduplex tracking assay. Am J Trop Med Hyg. 2008 Jun;78(6):892–4.

80. Liu S, Mu J, Jiang H, Su X. Effects of Plasmodium falciparum mixed infections on in vitro antimalarial drug tests and genotyping. Am J Trop Med Hyg. 2008 Aug;79(2):178–84.

81. Chang H-H, Worby CJ, Yeka A, Nankabirwa J, Kamya MR, Staedke SG, et al. THE REAL McCOIL: A method for the concurrent estimation of the complexity of infection and SNP allele frequency for malaria parasites. PLOS Comput Biol [Internet]. 2017;13(1):e1005348. Available from: http://dx.plos.org/10.1371/journal.pcbi.1005348

82. Volkman SK, Sabeti PC, DeCaprio D, Neafsey DE, Schaffner SF, Milner Jr DA, et al. A genome-wide map of diversity in Plasmodium falciparum. Nat Genet [Internet]. 2006 Dec 10;39:113. Available from: https://doi.org/10.1038/ng1930

83. Lekana-Douki JB, Dinzouna Boutamba SD, Zatra R, Zang Edou SE, Ekomy H, Bisvigou U, et al. Increased prevalence of the Plasmodium falciparum Pfmdr1 86N genotype among field isolates from Franceville, Gabon after replacement of chloroquine by artemether-lumefantrine and artesunate-mefloquine. Infect Genet Evol. 2011 Mar;11(2):512–7.

84. Zatra R, Lekana-douki JB, Lekoulou F, Bisvigou U, Ngoungou EB, Ndouo FST. In vitro antimalarial susceptibility and molecular markers of drug resistance in Franceville, Gabon. BMC Infect Dis. 2012 Nov;12:307.

85. Voumbo-Matoumona DF, Kouna LC, Madamet M, Maghendji-Nzondo S, Pradines B, Lekana-Douki JB. Prevalence of Plasmodium falciparum antimalarial drug resistance genes in Southeastern Gabon from 2011 to 2014. Infect Drug Resist. 2018;11:1329–38.

Publications

Parts of this thesis have been published in form of the following scientific article:

Nguyen TT, Nzigou Mombo B, Lalremruata A, Koehne E, Zoleko Manego R, Dimessa Mbadinga LB, et al. **DNA recovery from archived RDTs for genetic characterization of *Plasmodium falciparum* in a routine setting in Lambarene, Gabon**. Malar J. 2019 Oct;18(1):336.

Declaration of contributions to the dissertation

The dissertation work was carried out at the Institute for Tropical Medicine under the supervision of Prof. Benjamin Mordmüller.

The dissertation is comprised by 3 work packages:

1. **The work package on the excellence of RDT as a source of DNA for molecular assays**

The study was designed by me under the supervision of Dr. Ghyslain Mombo-Ngoma (Head of the Clinical Operations Department - CERMEL) and Prof. Benjamin Mordmüller (Deputy Director of Institute for Tropical Medicine – University of Tübingen).

I have written the study proposal, corresponded to the submission of this study to the scientific review committee and the local ethics committee of Centre de Recherches Médicale de Lambaréné – CERMEL. The samples were collected by the study team (PYRAMAX) in Lambaréné during the time of a clinical trial (NCT03201770). I carried out DNA extraction with the assistance of Brice Nzigou Mombo (a lab technician). The other experiments were carried out myself independently.

Statistical analysis was carried out independently by myself.

The results of this work have been published and I am the first and the corresponding author of the paper (DOI: 10.1186/s12936-019-2972-y). Tables 4, 9, 11, 12 and figures 5A, 6, 8, 9 of this thesis were cited from that paper (the citation was appropriately mentioned in the thesis).

2. **The work package on the establishment of a minimal SNP barcode**

The work was designed by me under the supervision of Prof. Benjamin Mordmüller.

I carried out all the experiments independently.

Statistical analysis was carried out independently by myself.

3. **The work package on the diversity of *Plasmodium falciparum* in malarial anemia**

This work was an exploratory part of the main study (completed) that was designed by Prof. Benjamin Mordmüller. The idea of using samples from anemic malaria cases to study parasite diversity was initiated by Prof. Benjamin Mordmüller and myself.

I carried out all the DNA extraction and genotyping experiments independently.

Statistical analysis was carried out by myself

I confirm that I wrote the manuscript myself under the supervision of Prof. Benjamin Mordmüller and that any additional sources of information have been duly cited.

Signature 

Tübingen, 05 November 2019

Acknowledgements

I would like to acknowledge Centre de Recherches Médicales de Lambaréné (CERMEL) and Institute for Tropical Medicine – University of Tuebingen for financially and scientifically supporting me during my PhD time. I would like to thank 108 Military Central Hospital and Vietnamese-German Center for Medical Research (VG-care) for supporting me in organizing transportation.

I would also like to thank:

- Prof. Peter G. Kremsner
- Prof. Benjamin Mordmüller
- Prof. Samuel Wagner
- Prof. Leonard Kaysser
- Prof. Thirumalaisamy P. Velavan
- Prof. Le Huu Song
- Albert Lalremruata
- Dr. Ghyslain Mombo-Ngoma
- Erik Koehne
- Jutta Kun
- Annette Knoblich