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Ex vivo drug sensitivity assay on Plasmodium malariae in Lambaréné

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“Gesundheit ist nicht alles, aber ohne Gesundheit ist alles nichts”

- Arthur Schopenhauer

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List of abbreviations

ACT	Artemisinin combination therapy
AE	Adverse event
API	Annual parasite incidence
AS	Artesunate
ATQ	Atovaquone
BMM	Blood medium mixture
BSA	Bovine albumin serum
CCM	Complete culture medium
CERMEL	Centre de Recherches Médicales de Lambaréné
CI	Confidence interval
CML	CoMal, used as abbreviation on CoMal slides
CoMal	Plasmodium species co-infections in *Anopheles* mosquitoes: A pilot study of parasite-vector interactions that define transmission in Africa
CQ	Chloroquine
DDT	Dichlorodiphenyltrichloroethane
DHA	Dihydroartemisinin
dhfr	Dihydrofolate reductase
dhps	Dihydropteroate synthase
DNA	Deoxyribonucleic acid
EIR	Entomological inoculation rate
ELISA	Enzyme-linked immunosorbent assay
et al.	Et alii / et aliae
GTS	Global Technical Strategy for Malaria 2016–2030
HCT	Haematocrit
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP2	Histidine-rich protein 2
IBSM	Induced blood-stage malaria
IC_{50}	Inhibiting drug concentration where 50% of parasites died

IC_{90}	Inhibiting drug concentration where 90% of parasites died
IC_{99}	Inhibiting drug concentration where 99% of parasites died
IPT	Intermittent preventive treatment
IRS	Indoor residual spraying
l	Litre
LLIN	Long-lasting insecticide-treated net
LUM	Lumefantrine
M	Molar
mg	Milligram
min	Minutes
ml	Millilitre
mM	Millimolar
nM	Nanomolar
PADH	Post-AS delayed haemolysis
PCE	Parasite clearance estimator
PCR	Polymerase chain reaction
PCT	Parasite clearance time
pLDH	Parasite specific lactate dehydrogenase
PmMSP1	*P. malariae* merozoite surface protein 1
PR	Parasite rate
PRR	Parasite reduction ratio
PVM	Parasitophorous vacuole membrane
RBC	Red blood cells
RDT	Rapid diagnostic test
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
SSA	Sub-Saharan Africa
WHO	World Health Organization
WS	Working solution
µl	Microliter
µM	Micromolar

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1. Introduction

The following study was scheduled to be carried out from October 2019 to September 2020 in Lambaréné, Gabon. Because of the global SARS-CoV-2 pandemic, the sample collection ended in April 2020. Only initial data is presented here. In order to confirm the obtained findings, more experiments must be conducted.

1.1 The global health burden of malaria

Malaria is a mosquito-borne infectious disease. It is caused by single-celled pathogens called *Plasmodium*. Six human-pathogenic *Plasmodium* species are known: *P. falciparum, P. vivax, P. malariae, P. ovale curtisi, P. ovale wallikeri* and *P. knowlesi.* Approximately half of the world´s population in 91 countries and territories is threatened by malaria transmission (World Health Organization, 2019). According to the World Health Organization (WHO), an estimated 228 million malaria cases occurred in 2018 of which 405,000 people died. In the same year, children under five years old accounted for 67 % (272,000) of all deaths worldwide. From 2014 to the present, the incidence rate of malaria remained at approximately 57 cases per 1,000 population at risk (World Health Organization, 2019). Greenwood *et al.* stated that climate instability, global warming, poor health education, civil disturbances, drug resistance and increasing travel between endemic and non-endemic areas are hampering malaria eradication (Greenwood et al., 2005). The amount of money invested in malaria control and elimination programs was US$ 2.3 billion in 2018. This is far less than is needed to reach the ambitious goal of the Global Technical Strategy for Malaria (GTS) to reduce malaria case incidence and mortality rates by 90% by 2030 (World Health Organization, 2019).

1.2 Rationale

Of the six human-pathogenic *Plasmodium species, P. malariae* contributes little to the global malaria health burden. Nevertheless, the pathogen needs to be addressed in order to eradicate malaria worldwide. A plethora of *in vivo* and *ex vivo* tests with the common anti-malarials were conducted on *P. falciparum* and *P. vivax*. *P. malariae* is scientifically neglected because it is considered to be benign, has a low burden on public health, occurs in sub-microscopic concentrations and is thus often not even recognized in microscopic surveys. *P. malariae* is the most abundant non-*falciparum* malaria parasite in Gabon (Woldearegai *et al.*, 2019). Therefore, it is beneficial to learn about the nature of its drug responsiveness.

In order to observe the drug sensitivity of *Plasmodium* species, it is crucial to collect solid data on the IC_{50}-values of those antimalarials that are frequently administered for *Plasmodium* infections. To date, only a single analysis on *P. malariae* was reported from Africa in 1997 by Ringwald *et al.* The WHO continues to recommend CQ as treatment against *P. malariae* mono-infections in addition to ACT combinations. A chemo-susceptibility profiling is necessary as evidence-based guidance for subsequent *in vivo* studies that scientifically prove the effectiveness of CQ treatment regimes. Furthermore, nobody has yet obtained a long-term *in vitro* culture of *P. malariae*. The cultivation of the *P. malariae* parasite would enable standardized drug sensitivity testing and experimental work on the biology of the parasite.

1.3 Purpose of the presented work

This study aims to

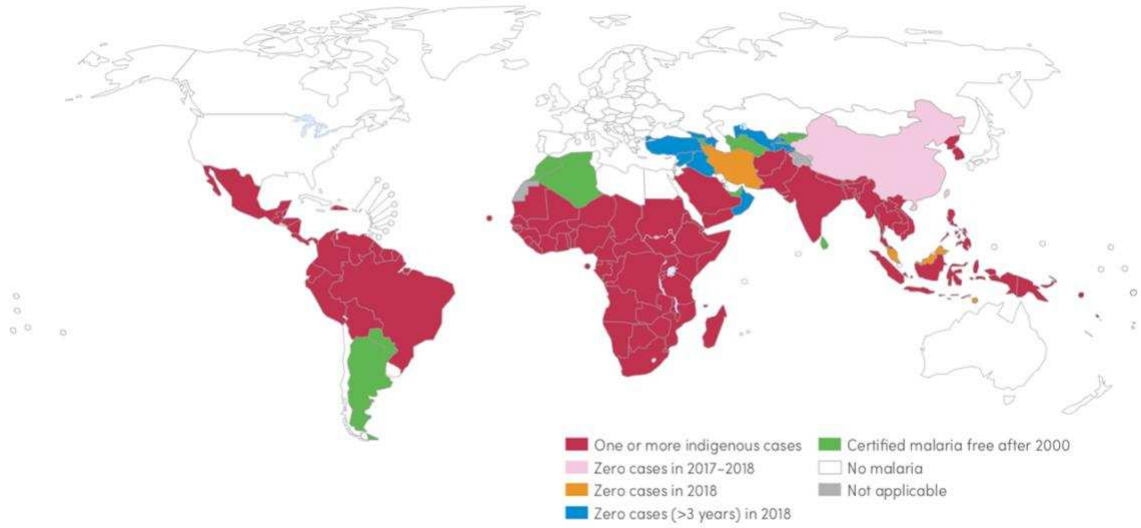
1. assess the susceptibility of *P. malariae* to the antimalarial drugs chloroquine, artesunate, atovaquone, and lumefantrine, using the WHO schizont maturation test, and
2. establish a long-term *in vitro P. malariae* culture.

2. Background

2.1 Distribution of malaria and its impact on global health

Throughout recorded time, human migrations and population growth coincided with the spread of malaria. In the 19th century, malaria was not only restricted to tropical areas but was also common in Central Europe (Hay *et al.*, 2004)**.** In the last century, malaria control strategies in industrialized countries and in some countries of Asia and Latin America successfully eradicated the disease (World Health Organization, 2019). Today, the focus is on tropical developing countries, especially in sub-Saharan Africa (SSA) (**Fig. 1)**. In 2018, only six African countries accounted for more than half of the global malaria burden. Nigeria had the most cases (25%) followed by the Democratic Republic of the Congo (12%), Uganda (5%), Côte d'Ivoire, Mozambique and Niger (4% respectively) (World Health Organization, 2019). One reason why malaria is most prevalent in SSA regions might be the low socio-economic status in those countries (Hay *et al.*, 2004).

Fig. 1: Countries with indigenous cases in 2000 and their status by 2018 (World Health Organization, 2019).



Reports of the distribution of *P. malariae* overlap with *P. falciparum* distribution (Autino *et al.*, 2012). Several studies observed that *P. malariae* usually occurs as

co-infection with *P. falciparum* while mono-infections a rarely seen (Roucher *et al.*, 2014; Woldearegai *et al.*, 2019). *P. malariae* is prevalent in India (Mohapatra *et al.*, 2008; Dhangadamajhi, Kar and Ranjit, 2009), Indonesia (Langford *et al.*, 2015), Papua New Guinea (Rosanas-Urgell *et al.*, 2010) and on the Thai-Myanmar border (M. Zhou, 1998). It is common in South American countries such as Brazil (Oliveira-Ferreira *et al.*, 2010), French Guiana (Carme *et al.*, 2009) and Venezuela (Metzger *et al.*, 2008). Additionally, *P. malariae* is present in Yemen (Al-Mekhlafi *et al.*, 2010). Culleton *et al.* showed that *P. malariae* is widely distributed in a range of nine sub-Saharan countries (Gabon, the Republic of Congo, Rwanda, Kenya, Sao Tome, Burkina Faso, Ghana, Mozambique and Angola) (Culleton *et al.*, 2008).

Over the last decades, *P. falciparum* prevalence has decreased significantly (World Health Organization, 2019). Nevertheless, in some endemic areas a persistent transmission of non-*falciparum* malaria species was discovered (Yman *et al.*, 2019). This highlights the importance of focusing on neglected malaria species as well, in order to eliminate malaria around the world. However, malaria case numbers are difficult to assess due to several confounders. The incidence of malaria is seasonal. The figures can vary considerably depending on the time of data collection. Self-assisted treatment, microscopy, which has a moderate sensitivity (Doderer-Lang *et al.*, 2014), and limited diagnostic resources particularly in rural settings (Autino *et al.*, 2012) make surveillance of malaria prevalence difficult.

2.2 Epidemiology of *P. malariae* in Gabon

In Gabon, as in all other SSA countries, only *P. falciparum, P. ovale* and *P. malariae* are consistently endemic. On the African continent, *P. vivax* is endemic at a low level (Howes *et al*., 2015), but is most frequently imported by travellers (Mukhtar *et al.*, 2019). After *P. falciparum, P. malariae* is the most prevalent malaria parasite in Gabon (Maghendji-Nzondo *et al.*, 2016). By PCR, a 23% prevalence of *P. malariae* was revealed in Moyen-Ogooué Province (Woldearegai *et al.*, 2019). The transmission of *P. malariae* is perennial. The

incidence rate is reduced during the dry season between June and August and peaks during the rainy season in October (Manego *et al.*, 2017).

2.3 Comparison of the sensitivity of microscopy versus RDT and PCR for malaria detection

P. malariae frequently presents with sub-microscopic levels of parasitaemia, occurs as a co-infection with *P. falciparum,* and closely resembles *P. vivax* morphologically (M. Zhou, 1998). Consequently, it is not surprising that case numbers of *P. malariae* are regularly underestimated (Doderer-Lang *et al.*, 2014). The measurement of malaria prevalence critically depends on the sensitivity of the diagnostic tool used (N. Hofmann *et al.,* 2014). Microscopy requires good training and a high level of proficiency to ensure a good quality slide reading. A. Moody showed that microscopy is a weak diagnostic tool for malaria detection due to a sensitivity of 50 parasites / µl of blood (A. Moody, 2002).

Rapid diagnostic tests are another type of malaria diagnosis. By detecting antigens of *Plasmodium* spp., it is possible to obtain a test result within 15–20 minutes, depending on the manufacturer. Each *Plasmodium* species has a different isomer of parasite-specific lactate dehydrogenase (pLDH). The enzyme is part of the organism's glycolytic pathway and can be used for the detection of all malaria species. The technique is easy to apply and is suitable for field conditions. The sensitivity is estimated to be around 100 parasites / µl and is comparable to the sensitivity of microscopy (Moody, 2002). However, due to moderate specificity, it is highly recommended that RDT results be confirmed by microscopy if possible (Bwire *et al.*, 2019).

The most reliable method for the diagnosis of malaria is PCR. By amplifying the parasite's DNA/RNA, it is possible to detect parasite infections at low densities. The sensitivity of PCR assays depends on the template molecule, the target gene and the level of quantification of DNA/RNA (N. Hofmann *et al.,* 2014). While the standard 18S rRNA qPCR assay has a moderate limit of detection of 0.02–3 parasites / µl of blood (M. Rougemont *et al.,* 2004; L. Cnops, J. Jacobs, M. Van Esbroeck, 2010), the 18S rRNA qRT-PCR is at least 10 times more sensitive with

a limit of detection of 0.002 - 0.02 parasites / µl of blood (R. Wampfler *et al*., 2013). The latter approach was applied in this study. Due to the higher number of target molecules in the sample, the RNA-based techniques have a higher sensitivity compared to their DNA-based counterparts (N. Hofmann *et al.,* 2014). However, it should be noted that PCR assays are relatively slow for initial malaria diagnosis and are not feasible under basic field conditions.

2.3.1 Microscopic features of *P. malariae*

In microscopy, the most frequently observed stages of *P. malariae* in stained blood smears are trophozoites or schizonts. Characteristic for all stages is the scattered yellow pigment, which can be observed to a greater extent in mature parasites. Infected host cells are typically not enlarged in comparison to *P. ovale* or *P. vivax* infected red blood cells (World Health Organization 2010). Rings are rarely seen in blood smears. They are small and have a ring to round shape. The cytoplasm is dense and tends to be thicker than in the ring stages of *P. falciparum*. *P. malariae* rings show one nucleus depicted by a single dot in the microscopic field (**Fig. 2**).

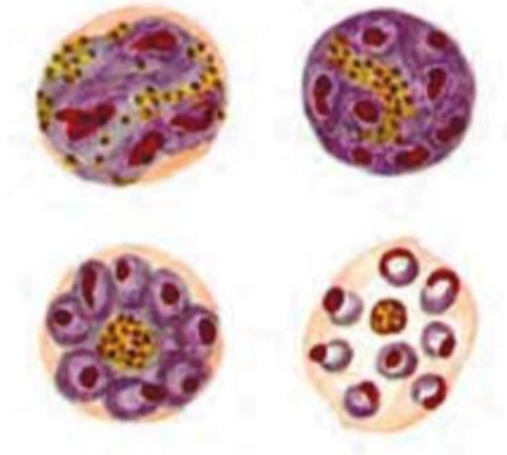
Fig. 2: *P. malariae* ring stages. The right one shows the yellow pigment (World Health Organization 2010).

Trophozoites usually consist of 30 to 50 jet-black granules. As the parasite grows, it extends across the host cell and forms the typical band shape (World Health Organization 2010) (**Fig. 3**).

Fig. 3: *P. malariae* trophozoite with band shape and yellow pigment (World Health Organization 2010).

In schizonts, the yellow pigment is concentrated in the centre of the cell and surrounded by more or less symmetrically arranged merozoites. The average number of merozoites is estimated to be eight. The parasite fills the entire erythrocyte (World Health Organization 2010) (**Fig. 4**).

Fig. 4: *P. malariae* schizonts (World Health Organization 2010).



The mature gametocyte generally has a round, compact shape. The nucleus appears to be dense and red-stained, the cytoplasm deep blue. The pigment is scattered and limited to the cytoplasm of the parasite (World Health Organization 2010). Microgametocytes are more frequently observed than macrogametocytes (Collins and Jeffery, 2007; World Health Organization, 2010)

2.4 Biology of *P. malariae*

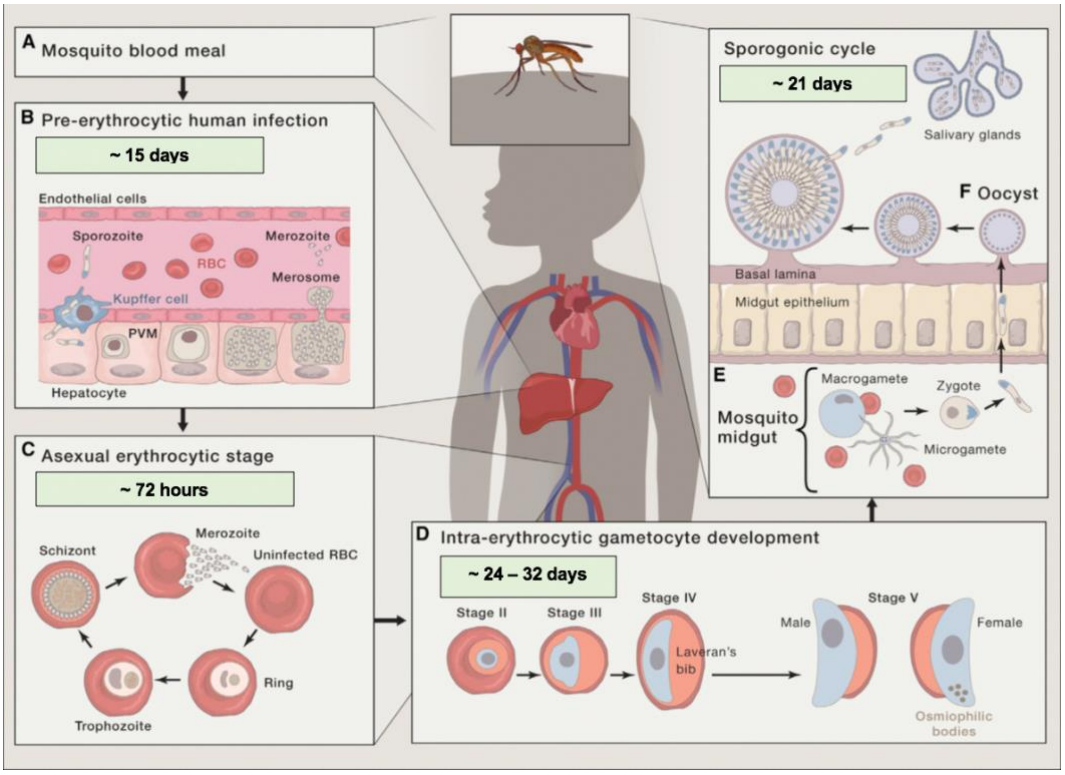
The word malaria is related to the outdated theory of its aetiology, namely that bad air from swamps was the agent of "swamp fever" (an ancient synonym for

malaria). In the 5th century AD, the Indian physician Susruta proposed that malaria might be a vector-borne disease transmitted by mosquitoes (Mukhopadhyaya, 2003). However, it was not until 1889 that Alphonse Laveran and Camillo Golgi discovered and proved that malaria was caused by a monocellular organism, the sporozoan of the genus *Plasmodium*.

2.4.1 Life cycle in humans

P. malariae has a 72-hour life cycle. As they develop during their life cycle, all *Plasmodium* species alternate between female *Anopheles* mosquitoes and human hosts (**Fig. 5**).

Fig. 5: The life cycle of *P. malariae* (adopted from Cowman *et al.*, 2016).
(A) During mosquito blood feeding, some sporozoites invade the peripheral blood system of the human host.
(B) The liver stage: As soon as the sporozoites infected a hepatocyte, they form a PVM to be protected from phagolysosomes of the host cell. Schizogony starts.
(C) The erythrocytic cycle.
(D) Gametocytes develop for sexual replication.
(E and F) The sporogonic cycle.



After blood feeding by a female *Anopheles,* male micro- and female macrogametocytes get ingested. A process called exflagellation occurs. The microgametes transform into multiple flagellates, the "sperm" of the malaria parasites, and fertilize the macrogamete. After fertilization, a mobile ookinete develops. The latter penetrates the epithelial wall of the mosquito midgut and forms under the basal membrane an oocyst. After two to three weeks, hundreds of sporozoites are released from the oocysts. The sporozoites are transmitted to the salivary glands by the circulation of the haemolymph. Approximately 100 sporozoites are injected into the human venules per blood meal to initiate the liver cycle (Collins and Jeffery, 2007). Following inoculation, the parasites migrate to the liver within one hour. The sporozoites infect the hepatocytes by crossing the sinusoidal barrier. This process is called *traversal* (Cowman *et al.*, 2016). Further evolution into the liver stage takes around 15 days. Asexual reproduction produces merozoites, leading to the release of up to 40,000 merozoites per hepatocyte. Vesicles are formed that leave the hepatocyte as merosomes (A. Sturm *et al*., 2006).

The intra-erythrocytic cycle begins when the merozoites invade the erythrocytes within minutes after being released into the hepatic blood circulation. In the erythrocytes, the merozoites transform into ring stages. More mature forms are called trophozoites and these develop into schizonts. Six to 14 merozoites are reproduced by schizogony (Collins and Jeffery, 2007). The merozoites egress from their host cells and reinvade other erythrocytes. The merozoites continue to develop, either into asexual schizonts or into sexual gametocytes. If a female *Anopheles* is blood feeding and gametocytes are transmitted, the cycle is repeated (World Health Organization, 2010). The morphology of the intra-erythrocytic stages of *P. malariae* resembles that of *P. falciparum*. This makes microscopic differentiation between the species challenging.

It was suggested that the development of *P. falciparum* gametocytes largely depends on environmental conditions. Cowman *et al.* assumed that exposure to anti-malarial medication causes the parasite to develop into its sexual stage (the gametocyte) much more quickly than would normally be the case. This increases

the chances of successful transmission before the parasite can be killed (Cowman *et al.*, 2016). However, this hypothesis was not yet examined for *P. malariae*.

Hypnozoites are pre-erythrocytic, dormant liver stages of *Plasmodium* that can persist over years. Their existence is proven in *P. ovale* and *P. vivax*. It is confirmed that hypnozoites are responsible for the so-called relapse of *P. vivax* and probably *P. ovale* spp. (Markus, 2011). In the case of *P. malariae*, various observations disqualified this hypothesis. As stated by P. Garnham, hypnozoites were found neither in an experimentally infected chimpanzee nor in the liver of a man who died accidentally three months after a *P. malariae* infection. As was observed, the most decisive argument against the existence of *P. malariae* hypnozoites is that primaquine treatment for hypnozoites is not necessary to prevent quartan malaria recurrence (Garnham, 1981).

2.4.2 Persistence and recrudescence of *P. malariae*

By coining the term recrudescence, P. Garnham tried to differentiate the recurrence of *P. malariae* from the relapse of *P. vivax* and *ovale* that is caused by hypnozoites (Garnham, 1981). Vinetz *et al.* observed in 1998 the case of a 74-year-old Greek woman who tested positive for *P. malariae.* Clinical examinations gave reason to suspect that her infection most likely persisted for more than 40 years. The case demonstrates that *P. malariae* can cause asymptomatic infections that persist and reactivate decades later (Vinetz *et al.*, 1998) due to the pathogen's very slow growth and low metabolic activity. The latter is depicted by the unique duration of a 72-hour cell cycle and a reproduction rate of only eight merozoites per schizont (Collins and Jeffery, 2007).

It was observed that quartan fever recurrences appear although patients received a standard antimalarial treatment regime (Müller-Stöver *et al.*, 2008). In this context, an episode of malaria can still be triggered after a couple of months. Therefore, the study design of *in vivo* clinical trials on *P. malariae* must include an extended follow-up. A follow-up would provide evidence of whether the respective drug compound is efficacious despite the long prepatent period of

development in the liver. Franken *et al.* explained the chronicity of infection by referring to the slow cell cycle of the pathogen. Because of the long pre-erythrocytic hepatic development period of at least 15 days (see above), a standard treatment regime of three days would not eliminate the infection (Franken *et al.*, 2012).
Another reason for persisting quartan fever might be the subtherapeutic plasma levels of antimalarials. Insufficient plasma levels can be caused either by inadequate intake of medication or by counterfeit antimalarials sold on the black market.

Recrudescence needs to be distinguished from reinfection. It is morphologically impossible to differentiate between them by microscopy. Instead, molecular diagnostic tools need to be applied. Length polymorphisms in *P. malariae* populations are an applicable target for genotyping assays (Ariey, Gay and Ménard, 2013). *P. malariae* merozoite surface protein 1 (PmMSP1), an extremely diverse parasite locus, was found to be a reliable marker for differentiation (Elizardez *et al.*, 2019).
P. malariae is well adapted to its host organism. From the perspective of an optimal host-parasite relationship, it is beneficial to contain the severity of the disease. Kerlin and Gatton stated that *P. malariae* has the propensity to infect mainly mature RBCs. This leads to a self-limiting blood stage density because merozoites compete for the limited number of mature erythrocytes. It is believed that this self-regulation contributes to a low reproduction rate and a long erythrocytic cycle, culminating in years of parasitic survival. Due to the slow replication rate of *P. malariae*, the host has enough time to mount an appropriate immune response, which keeps the virulence of infection low (Kerlin and Gatton, 2013). Interestingly, the immune response does not necessarily eliminate all the parasites. The infection can persist asymptomatically without being noticed by the carrier but may result in sequelae decades later (Elizardez *et al*., 2019).

2.5 Clinical features

The clinical disease that is caused by *P. malariae* is quartan fever. It was described by Hippocrates as “the least dangerous of all [fevers], and the mildest and most protracted” (Adams, 1886). Due to the fact that *P. malariae* is considered a benign parasite, very few systematic studies were conducted on the clinical burden of infection by the pathogen. Most of the data were collected from returnee, non-immune travellers admitted to hospitals in Europe, or from neuro-syphilis patients that were treated with *P. malariae* between 1940 and 1960 (Collins and Jeffery, 1999). In general, *P. malariae* infections are asymptomatic in adolescents and healthy adults. Severe cases were rarely reported. Semi-immune individuals from malaria-endemic countries regularly harbour low parasitaemia without showing any clinical symptoms (Roucher *et al.*, 2014).
However, the chronic character of the disease should not be neglected. The clinical hallmark of malaria is fever caused by the oscillating release of new populations of parasites into the blood stream. Other symptoms are headache, chills, fatigue, arthralgia and myalgia, which quickly disappear after treatment (Grande *et al.*, 2019). In 2007, Collins and Jeffery described 72-hour oscillating fever paroxysms due to the cell cycle of *P. malariae.* Interestingly, the quartan fever pattern cannot be observed in every infected carrier (Yavne *et al*., 2017). Anaemia is the most common and most severe long-term consequence of a chronic infection (Langford *et al.*, 2015). Besides, few reports of insidious weight loss and splenomegaly exist (Joseph M. Vinetz, Jun Li, Thomas F. McCutchan, 1998). The discussion of nephrotic syndrome is highly controversial. It was regularly detected over time (Hendrickse and Adeniyi, 1979; Ehrich and Eke, 2007; Hedelius *et al.*, 2011). Despite the anecdotal character of the reports on renal failure, *P. malariae* is significantly more likely to cause nephrotic syndrome than any other *Plasmodium* species (Langford *et al.*, 2015).

In conclusion, *P. malariae* infections trigger an immunological response in their host and cause morbidity, such as anaemia. If other parasitic infections are already present, the patient is malnourished or has a pre-existing anaemia, *P.*

malariae can be one cause of mortality (Collins and Jeffery, 2007; Langford *et al.,* 2015).

2.6 Malaria control strategies

Before malaria control interventions took place, environmental improvements were responsible for the decrease in malaria prevalence (Omran, 2005). The gains made in malaria control were coincidental with economic and social development. Malaria is not so much an obligate tropical disease but rather one that became restricted to the tropics as living conditions evolved (Kitron, 1987). Mosquito breeding sites started being controlled in the course of the 19th century. The eradication of *Anopheles gambiae* in Brazil (Killeen *et al.*, 2002) and Egypt (Shousha, 1948) are excellent examples of what vector elimination can achieve. In the 1940s, insecticides such as dichlorodiphenyltrichloroethane (DDT) were used for vector control. Intermittent preventive treatment (IPT) was specifically aimed at infants and pregnant women. Sulfadoxine-pyrimethamine was administered in the absence of symptoms to suppress further transmission. DDT proved not to be sustainable because of its poisoning effects and the emerging resistance to it in mosquitoes (Greenwood *et al.*, 2005). Long-lasting insecticide-treated nets (LLIN) became more common after the turn of the millennium and were distributed for free. By implementing ACTs and indoor residual spraying (IRS), various countries came closer to their goal of malaria elimination (Hay *et al.*, 2004). In Zanzibar, the use of ACTs and LLINs decreased the mortality rate of children under five years old by 71% (Björkman *et al.*, 2019). Health-care education is also an important tool for controlling malaria. LLINS are used more frequently in households where people are aware of mosquitoes as malaria vectors (Maghendji-Nzondo *et al.*, 2016).

Over the last decade, there was intensive research into the development of a potential malaria vaccine that can provide 100% long-term protection. So far, RTS,S/AS01E is the most promising candidate that guarantees modest protection against clinical malaria in children (Dobaño *et al.*, 2019). However, partial protection is only given three to four years (The RTS, S Clinical Trials

Partnership 2015). Therefore, malaria vector contraction as well as fast and effective treatment remain the most important control strategies. For malaria treatment, the WHO recommends artemisinin-based combination therapies (ACT) in all endemic countries for uncomplicated *P. falciparum* malaria and non-*falciparum* malaria, if the species is not known (World Health Organization, 2015).

2.7 Antimalarial drugs

The life cycle of *Plasmodium* spp. offers various targets for chemo-intervention. The majority of anti-malarials acts on the intra-erythrocytic stages of the parasite (blood schizonticides). 8-aminochinolines like piperaquine and tafenoquine inhibit both replicating and dormant *Plasmodium* liver stages and, as a result, are used for chemoprophylaxis (tafenoquine) and to eliminate hypnozoites of *P. ovale* and *P. vivax* (primaquine).

In the 19th century, the first compound that was widely used to fight malaria was quinine, a short-acting alkaloid drug extracted from cinchona bark. At this time, it was common to use gin, which contains quinine, as a malaria prophylactic (Shanks, 2016). Due to the frequent occurrence of side effects, the resulting poor compliance, and the difficulties of quinine extraction, the drug is rarely used today (Ridley, 2002). After its discovery during the Second World War, chloroquine (CQ) became the main antimalarial compound for the next two decades. However, CQ resistance arose simultaneously in South America and South-East Asia in the early 1960s (D'Alessandro and Buttiëns, 2001). When chloroquine, the world's most-administered drug regimen, proved ineffective after such a short time, research into the development of alternatives intensified. The chemical structure of CQ was modified and related substances as amodiaquine, ferroquine, pyronaridine, and piperaquine were produced in an attempt to overcome CQ resistance (Ridley *et al.*, 1996; Foley and Tilley, 1998; Ridley, 2002). Further development of the active anti-malarial metabolite quinine and quinidine resulted in drugs like mefloquine, lumefantrine and halofantrine. The substances artemether and artesunate (the most commonly prescribed

antimalarials nowadays) are derived from the Asian herb known as sweet wormwood (*Artemisia annua* L) (Tu, 2011).

The anti-malarial drugs that are used today are divided in the following categories:

- Aryl-aminoalcohols (related to quinoline from the cinchona tree) such as quinine, quinidine, chloroquine, amodiaquine, mefloquine, halofantrine, lumefantrine, piperaquine, primaquine, tafenoquine, ferroquine, pyronaridine.
- Antifolates such as pyrimethamine, proguanil, chlorproguanil, trimethoprim.
- Artemisinins such as artemisinin, dihydroartemisinin (DHA), artemether, artesunate (AS).

Since the chemo-susceptibility profiling conducted is based on CQ, AS, ATQ and LUM, the following paragraphs are dedicated to the different characteristics of these compounds.

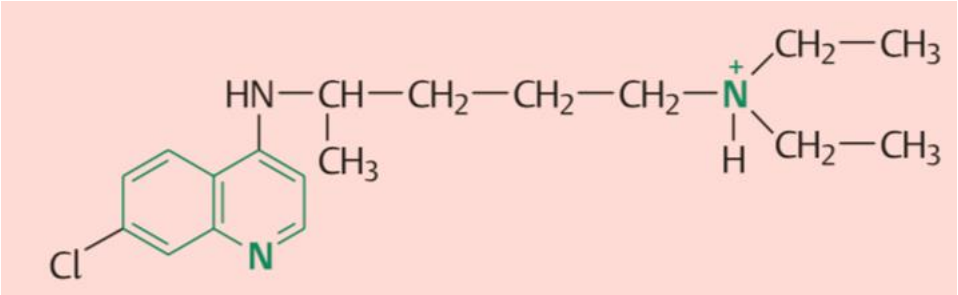
2.7.1 Chloroquine

CQ used to be the front-line anti-malarial thanks to its low cost and high efficacy. After the *P. falciparum* and *P. vivax* strains developed resistance, the application of CQ decreased drastically. CQ is a 4-aminoquinoline derivative and interferes in haemozoin polymerisation. Toxic haeme gets accumulated, a product from the erythrocyte´s haemoglobin metabolism. This leads to lysis of the host cell (Sullivan *et al.*, 1996). Siswantoro *et al.* showed *in vivo* that the drug is still quite effective against *P. malariae* even though CQ-resistance was reported in patients in Indonesia infected by *P. malariae* (Maguire *et al.*, 2002). Adverse events such as headaches, gastrointestinal problems and visual disorders were seldom reported (H. Lüllmann, K. Mohr, L. Hein, 2016), whereas pruritus and vomiting

seem to be frequent side effects of the drug intake (Bakshi *et al.*, 2000). Ursing *et al.* confirmed that CQ prolongs the QT interval significantly (Ursing *et al.*, 2020).

The chemical structure of CQ is depicted in **Fig. 6**. In this study, the efficacy of CQ against *P. malariae* was assessed.

Fig. 6: CQ chemical structure (H. Lüllmann, K. Mohr, L. Hein, 2016).



2.7.2 Artemisinins

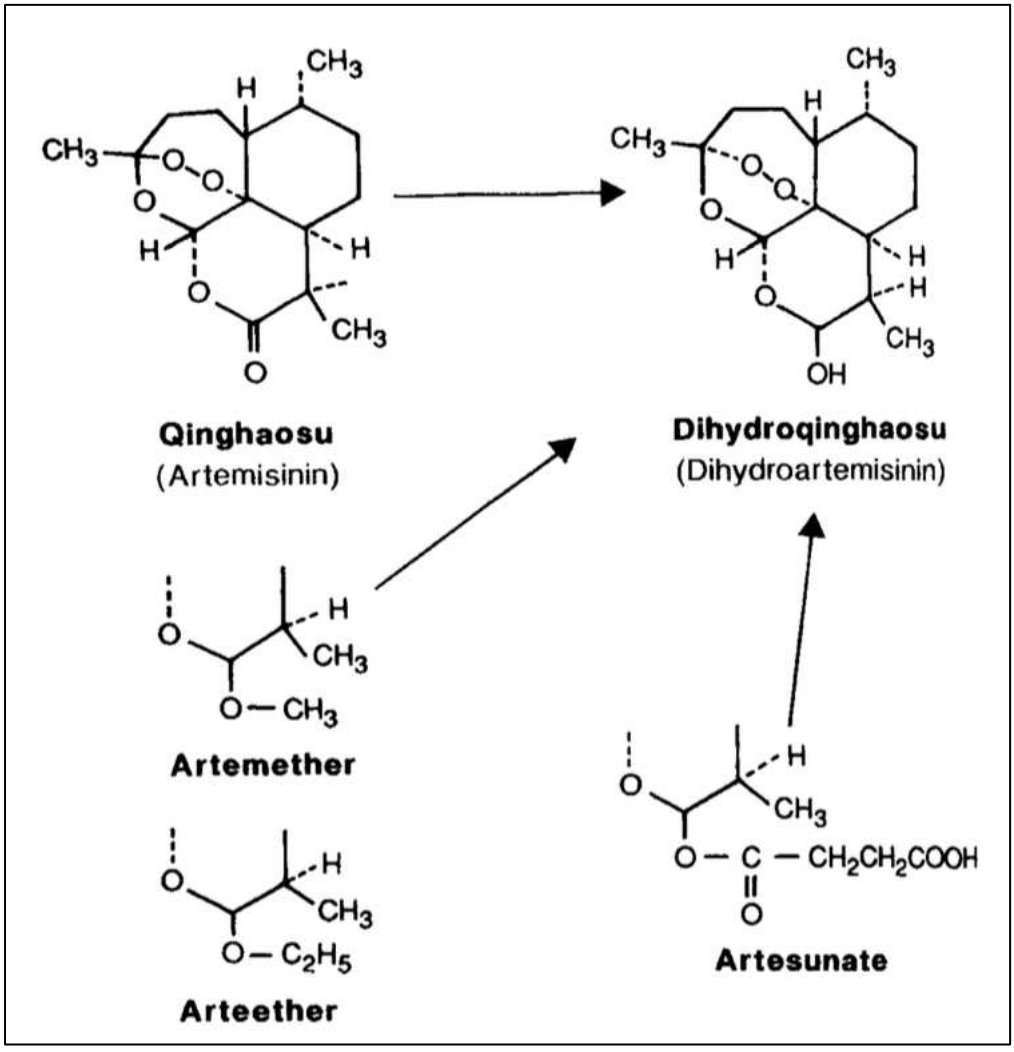
After *P. falciparum* developed resistance to CQ, artemisinin derivatives became the most efficient and most frequently administered antimalarial compounds worldwide. Artemisinin was isolated from *Artemisia annua*, a plant well known in traditional Chinese medicine for its antipyretic effect. In 1972, Y. Tu and colleagues were able to purify qinghaosu (artemisinin), the active component of the extract (Tu, 2011). By modifying artemisinin, they stabilised the compound and observed a tenfold increase in antimalarial activity. This work was awarded with the Nobel Prize in Physiology or Medicine to Youyou Tu in 2015 (Roussel *et al.*, 2016).

Common derivatives of *A. annua*, which are used clinically, are artesunate, arthemeter and dihydroartemisinin (**Fig. 7**) (Hien and White, 1993). The active metabolite of the artemisinin compound is dihydroartemisinin (DHA). With a half-life of 45 minutes, the elimination rate of DHA is relatively high (Teja-Isavadharm *et al.*, 1996; Batty *et al.*, 1998). The exact mode of action of artemisinins has yet to be fully elaborated. However, it is believed that the endoperoxide-mediated release of oxygen and carbon-centred radicals directly damage the parasite (Klonis, Creek and Tilley, 2013). Today, artemisinin as part of artemisinin-based combination therapies (ACT) is the standard treatment of uncomplicated *P.*

falciparum and non-*falciparum* infections. As artemisinins have a short elimination half-life, they account for a fast onset of parasite clearance (White, 2004). The WHO recommends using artemisinin derivatives together with a partner drug in order to prevent the development of resistances (White, 1999). The slow-acting partner drug eventually clears the indolent parasites that remain and functions as a prophylactic against new infections by yielding high serum levels over a longer period of time.

The use of artesunate (AS) as one derivative of artemisinin was reviewed by Roussel *et al.* in 2016. They evaluated the safety and efficacy of AS in travellers with severe malaria that were treated in Europe. AS was shown to have a wide window of antimalarial effect from early ring stages to schizonts. A frequently observed AE was the so-called post-AS delayed haemolysis (PADH). PADH is

Fig. 7: Chemical structure of artemisinin and derivatives (Hien and White, 1993)**.**



caused by a decrease in haemoglobin and increase in lactate dehydrogenase. PADH is often treated by blood transfusion if necessary. No deaths associated with the AE were observed (Roussel *et al.*, 2016).

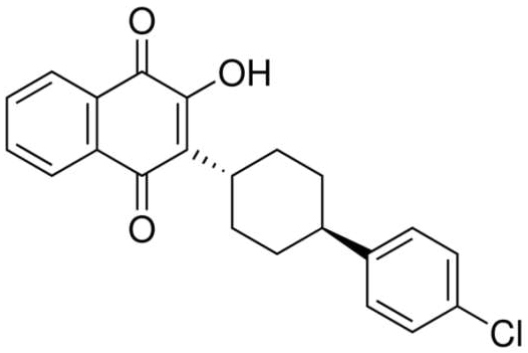
ACTs are frequently used against *P. malariae* mono- or co-infections. AS, as an artemisinin derivative, was therefore chosen to monitor the efficacy of artemisinins in Gabon.

2.7.3 Atovaquone

Atovaquone (ATQ) is a hydroxy-naphthoquinone derivative (**Fig. 8**). Its effect is probably based on the inhibition of electron transport in the cytochrome B1 complex of the protozoan mitochondrial membrane, which alters its membrane potential. Additionally, ATQ reduces pyrimidine synthesis (Müller-Stöver *et al.*, 2008). Looareesuwan *et al.* observed that *Plasmodium* infections under ATQ mono-treatment are often recurrent. The recurrent parasites were less susceptible to the drug than prior to drug intake. This was seen as a reminder that drugs should not be administered as single entities because doing so accelerates the emergence of resistance (Looareesuwan *et al.*, 1996). To prevent recurrence of *Plasmodium*, a combination with proguanil is required (Malarone®). An *in vitro* drug interaction study revealed that the biguanide is the most promising partner drug for ATQ (Canfield, Pudney and Gutteridge, 1995). The latter compound acts synergistically with atovaquone by reducing the activity of dihydrofolate reductase and thus inhibiting pyrimidine synthesis (Srivastava *et al.*, 1999). Radloff *et al.* studied seven patients with *P. malariae* on an atovaquone-proguanil combination and did not observe any AEs (Radloff *et al.*, 1996).

As a common part of antimalarial prophylaxis in non-immune travellers, it was worth including ATQ in the *ex vivo* test of this study.

Fig. 8: Chemical structure of ATQ. [1]

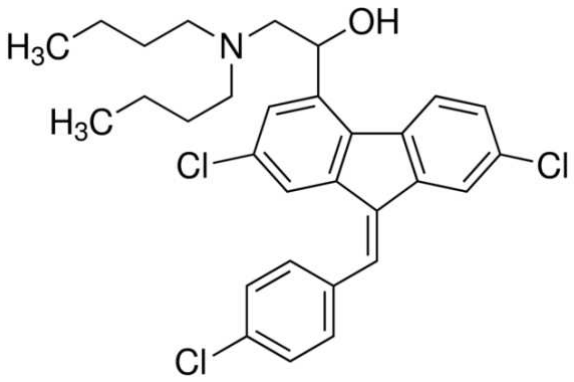


2.7.4 Lumefantrine

Lumefantrine (LUM) belongs to the aryl-amino-alcohol group of antimalarials. It is only available in combination with artemether under the name COARTEM®. The combination of artemether-lumefantrine is an ACT that dominates the sub-Saharan treatment policy (Groger and Ramharter, 2018). LUM is a slow-acting drug that eliminates the indolent stages that stay in circulation for a prolonged time period after the plasma level of the fast-acting artemisinin derivative was quickly eliminated. It can be considered a prophylactic that shields the patient against a reinfection or parasite recurrence from exo-erythrocytic or indolent intra-erythrocytic *Plasmodium* stages. The only AE that was observed by Mombo-Ngoma *et al.* was vomiting one day after treatment administration. The drug is lipophilic and is better absorbed when administered with fatty food (Ezzet, Mull and Karbwang, 1998). Ezzet *et al.* determined the half-life of LUM to be about three to four days (Ezzet *et al.*, 2000). **Fig. 9** shows the chemical structure. The disadvantage of LUM is that, after a certain threshold of time, the plasma concentration is no longer high enough for complete parasite clearance from the blood. This leads to parasites that are only exposed to low drug concentrations, which entails the risk of developing resistance. Therefore, drug surveillance should not only be conducted on the fast-acting artemisinin but also on the partner drug LUM.

[1] https://www.sigmaaldrich.com (April 30th, 2020)

Fig. 9: Chemical structure of LUM. [2]



3. Antimalarial drug tolerance and resistance

Drug resistance is defined as the ability of a malaria species to survive and continue its development despite administration and absorption of a drug at a dose equal to or higher than usually recommended and tolerated by the individual (World Health Organization, 1973). *P. malariae* is regarded to be susceptible to all known antimalarials, regardless of whether they are used for prophylaxis or therapy. Nevertheless, little is known about drug sensitivity since treatment of patients is based more on empirical evidence than on systematic *in vivo* and *in vitro* studies.

Several case reports can be found that document *P. malariae* recrudescence despite antimalarial prophylaxis or treatment (Grobusch, Göbels and Teichmann, 2004; Müller-Stöver *et al.*, 2008). This provokes the question of whether resistance against the respective compounds is a possible cause of the relapse of clinical symptoms. However, the most obvious reason for a return of malaria in the patient is a re-exposure to the malarial vector.

In local markets in Asian and African countries, counterfeit antimalarials are frequently sold that do not have a sufficient effect on the pathogen (Gaudiano *et al.*, 2007). If the patient is non-compliant or the drug resorption is inadequate, the plasma concentration of the compound will be too low for parasite elimination. In this case, antimalarial medication can even prolong the latency of *P. malariae*

[2] https://www.sigmaaldrich.com (April 30th, 2020)

infection and delay clinical breakthrough (Müller-Stöver *et al.*, 2008). Therefore, measuring the drug plasma concentration is an appropriate tool if malaria episodes reoccur despite anti-malarial intake (Grobusch, Göbels and Teichmann, 2004).

P. malariae has an exceptionally long duration of pre-patency (time of parasite maturation in the liver before the intra-erythrocytic cycle starts). Only derivatives of 8-aminoquinolines like primaquine are able to kill liver stages of *Plasmodium*. If the treatment took place at an inappropriate time, the drug may have already exceeded its half-life when the parasite entered the bloodstream. This implicates that the plasma level of the drug was no longer sufficient to kill the parasite (Franken *et al.*, 2012). Kugasia *et al.* explained a case of *P. malariae* recrudescence after quinine treatment by pointing out the indolence of erythrocytic stages in blood circulation (Kugasia, Polara and Assallum, 2014). The accumulation of chloroquine or quinine depends on the acidity in parasitic food vacuoles due to the basic nature of the drugs. Quinine and chloroquine lyse trophozoites by interfering in the haematin polymerization. As *P. malariae* has a remarkably slow metabolism, the acidity in the vacuoles is low and the antimalarial drug becomes concentrated in lower levels. This circumstance may shield the parasite. A latent parasitaemia remains until further propagation leads to a clinical breakthrough of symptoms.

When evaluating anecdotal reports of CQ resistance in *P. malariae* strains (Maguire *et al.*, 2002), consideration should be given to the fact that infected patients generally have longer parasite clearance times (PCT). Collins and Jeffery revealed that in contrast to *P. vivax* and *P. falciparum*, clearance times for *P. malariae* are frequently longer than seven days. They reviewed several studies and reported one patient that needed 16 days to eliminate all parasites (Collins and Jeffery, 2002). However, as anti-malarial resistance was already widespread for decades in *P. falciparum* (D'Alessandro and Buttiëns, 2001) and *P. vivax* (Sumawinata *et al.*, 2003) it cannot be excluded that *P. malariae* also developed resistance to common drugs. Therefore, it is important to carry out chemo-susceptibility tests on a regular basis in order to offer compelling proof that certain anti-malarials are still effective.

3.1 Assessing drug sensitivity of malaria parasites

Drug efficacy can be defined as the propensity of a drug to interrupt further parasite propagation by inhibiting essential life functions. Damaged parasites are recognized and cleared from blood circulation by the immune system. Drug efficacy depends on the genetic properties of each *Plasmodium* strain. Therefore, it is also reported as drug sensitivity (Noedl, Wongsrichanalai and Wernsdorfer, 2003). There are two tools available to monitor the ongoing drug efficacy of antimalarial compounds: chemo-susceptibility profiling *ex vivo* or *in vitro* and drug efficacy testing inside an infected patient (*in vivo*). *In vitro* and *ex vivo* tests are simple tools for epidemiological surveillance and for monitoring changes of drug sensitivity after a period of clinical use. Performing ex vivo tests is easier because it requires no patient management and can be done at any time in the laboratory. However, *in vitro* testing cannot be related directly to *in vivo* results. *In vivo* testing is the only tool for measuring essential parameters of drug tolerability and efficacy under “real life” conditions. The clinical outcome depends on patient-related factors such as drug metabolism, immunity, adverse events etc., which need to be involved in the final assessment of whether the drug can actually be used in humans.

3.1.1 *In vitro / ex vivo* drug testing

In vitro and *ex vivo* testing are commonly used tools for the evaluation of new drugs. Regardless of the question of treatment failure or success, it allows the quantitative monitoring of drug sensitivity. While *ex vivo* chemo-susceptibility tests are performed directly on fresh parasite isolates, *in vitro* assays are done with cell culture-adapted parasite isolates. Various techniques are applicable for chemo-susceptibility profiling today. In general, all of them are performed by exposing the parasite, at a pre-determined stage, to anti-malarial compounds in different concentrations over a specific period of time. The inhibition by the drug is measured by observing the effect on growth and multiplication.

The WHO schizont maturation test, which was applied in this study, is a simple but labour-intensive method for assessing drug sensitivity. Schizont maturation is observed over a certain period of time. The number of schizonts in the drug cultivation is compared against the drug-free control sample on prepared Giemsa-stained thick films. The number of multinucleate schizonts correlates directly with the drug dilution in each well. The method is applicable to low densities of parasites and simple field conditions. However, it is difficult to compare the results of several microscopists because of the interindividual variability. The technique requires a high degree of experience in counting and assessing the developmental stages of the parasites. When determining the incubation time, the time of onset of drug action needs to be considered. If incubation time is too short, slow-acting drugs cannot be evaluated properly (Nodari *et al.*, 2020).

Antigen-based malaria detection is another common method. Parasite-specific lactate dehydrogenase (pLDH) is a central enzyme in the parasitic glycolysis and differs from the human isomer. The pLDH of *P. falciparum* and non-*falciparum* parasites are distinguishable. By measuring its activity, the number of parasites in the host can be quantified (Piper *et al.*, 1999). The detection of histidine-rich protein II was shown to be efficacious as well (Noedl *et al.,* 2002a). The alanine- and histidine-rich protein is expressed by *P. falciparum* trophozoites proportional to their growth. The measurement of the antigen by ELISA is highly sensitive and far less labour-intensive than the WHO schizont maturation assay. The test is carried out for a longer incubation time and thus allows testing of slow-acting drugs (Burkhardt *et al.,* 2007). However, this method is not applicable for *P. malariae* testing. As mentioned above, presence of HRPII was only proven in *P. falciparum* (Noedl, Wongsrichanalai and Wernsdorfer, 2003).

In the case of *P. malariae*, science still relies on schizont maturation tests. As explained above, this approach is extremely time-consuming and cannot be standardised. Because a standardized *in vitro* culture model of *P. malariae* is lacking and antigens were not yet determined, an ELISA cannot be applied to *P. malariae.* Suboptimal culture conditions are likely the reason for failure in culturing the parasite (Lingnau, Doehring-Schwerdtfeger and Maier, 1994;

Ringwald *et al.*, 1997). The low number of mono-infections and the difficulties with diagnosis further limited the number of studies performed on *P. malariae*.

4. Materials and methods

4.1 Study site

The Centre de Recherches Médicales de Lambaréné (CERMEL, **Fig. 10**) is located in the Moyen-Ogooué province in Gabon. Moyen-Ogooué is one of the nine provinces of Gabon situated in extensive rainforests and equatorial climate. It covers an area of 18,535 km^2 with a total population of about 70,000 people and its provincial capital is Lambaréné (Woldearegai *et al.*, 2019). Lambaréné is a small town around 250 km southeast of Libreville, the capital of Gabon. It is characterized by a rural setting with a population of approximately 35,000 people. Malaria transmission in Lambaréné is perennial (Sylla, Kun and Kremsner, 2000; Grobusch *et al.*, 2007).

CERMEL is a non-profit institution that closely collaborates with the Albert Schweitzer Hospital in Lambaréné and the University of Tübingen. The research focus of CERMEL lays in infectious diseases like tuberculosis, soil-transmitted helminths and malaria. In the latter case, CERMEL is strongly involved in vaccine development.

Fig. 10: The new CHU campus of CERMEL. [3]

[3] Author

4.2 The CoMal project

This analysis was an ancillary study to the COMAL project “Plasmodium species co-infections in *Anopheles* mosquitoes: A pilot study of parasite-vector interactions that define transmission in Africa” (CoMal, study ID number: DFG BO 2494/3-1). CoMal is a multinational study conducted in Benin, Cameroon, Congo and Gabon. The period determined for the study was 2018 to 2021. It is designed to gain more detailed information about the biology of *P. malariae* transmission through the observation of host-parasite and parasite-vector interactions in order to provide a better understanding of the genetic and molecular basis of *P. malariae* transmission to key malaria vectors in Gabon.

The study has three main objectives:

1. Identify competent *P. malariae* vectors for subsequent experimental transmission assays:
    a. Characterise in time and space co-infections in vector and human populations at selected locations in four countries.
    b. Compare co-infection rates in vector populations and human populations as quality control for mosquito collection strategy.

2. Establish the experimental transmission of *P. malariae* in selected malaria vectors from *P. malariae* single- and co-infections with *P. falciparum*.

3. Characterise the genetic and molecular basis of differential susceptibility / refractoriness to *P. malariae* in key malaria vectors.

The long-term goal of CoMal is to contribute to the establishment of a standardized experimental human blood-stage model for *P. malariae*. Induced blood-stage models allow the implementation of parasitological and pharmacological studies under a controlled environment.

4.3 Study design

This work is an experimental evaluation of the *ex vivo* activity of chloroquine, artesunate, atovaquone and lumefantrine to target asexual blood stages of *P. malariae.* Blood samples were collected together with the CoMal team from January to April 2020. The study protocol was submitted to and approved by the CERMEL institutional ethics committee. The main objective was to determine the drug response of *P. malariae* isolates in Gabon. A secondary objective was to estimate the metabolic needs of the pathogen as an essential basis for the establishment of an *in vitro* culture of *P. malariae*. It was planned to perform at least ten drug susceptibility tests on ten *P. malariae* mono- or co-infected isolates. It was decided to use as many isolates as possible for establishing a *P. malariae* culture. A *P. falciparum* culture, that is well adapted to *in vitro* culture conditions, was not used as a positive control.

Appendix 1 provides a flowchart of all study activities.

4.4 Field screening

The blood samples for the experiments were obtained from participants enrolled within the cross-sectional study of CoMal who met the inclusion criteria (**Appendix 2**). Twice a week, a team of four people screened up to 30 individuals for malaria after obtaining written informed consent or a signed assent form for minors. Baseline characteristics like body temperature, weight, age and sex were taken. A malaria rapid diagnostic test was made *in situ* as well as a blood spot on a Whatman filter paper for PCR analyses. Thick and thin smears were made for microscopic examination of the slides for the presence of *Plasmodium* spp. species. 10 µl of finger-prick blood was taken for the thick smear and 5 µl for the thin smear. Each participant was given a specific identification number (CML xxxx) related to the CoMal study that was noted together with the time and date on the microscopic slide. The slides were stained and double read by the clinical laboratory at CERMEL. When the slide reading was completed, all patients who

tested positive for malaria were treated with Coartem®. If participants were found to be positive for *P. malariae,* they were asked to donate blood for the experiments before treatment.

4.5 Material

4.5.1 Lab equipment

Table 1: Lab equipment with correspondent manufacturer.

Name	Manufacturer
Autoclave	Melag, Euroclav 29 VS, France
Biological Safety Cabinet	Thermo Scientific, Hera Safe KSP, USA
Cell culture flasks, 25cm^2	Greiner Bio-One GmbH, Germany
Centrifuge	Thermo Scientific, Heraeus Megafuge 8, USA
CO_2-Incubator	Mytrom, Germany
Falcon tubes, 15 ml and 50 ml	BD Falcon™, USA
Filter papers	Macherey-Nagel, Germany
Heparin tubes	Becton Dickinson, United Kingdom
LD-column	Miltenyi Biotec, USA
LightCycler 480 II	Roche, Switzerland
MACS multi stand	Miltenyi Biotec, USA
Microscope	Leika DM 750, Germany
Microscope slides	Nuova Aptaca SRL, Italy
Multi-channel pipette	Eppendorf, Germany
Pipette controller	Brand, Germany
Pipette tips	Greiner Bio-One GmbH, Germany
Quick-DNA/RNA Microprep Plus Kit, D7005	Zymo Research, USA
Rapid test for the antigen detection of malaria (RDT)	Access Bio, USA

Reagent Reservoir	VWR, USA
Safe-lock tubes, 2 ml	Eppendorf, Germany
Single-channel pipettes	Eppendorf, Germany
Slide box	Heathrow Scientific, China
Stripettes, 1 ml, 5 ml, 10 ml, 25 ml	Corning Incorporated, USA
Syringes, 10 ml	Braun, Germany
Tissue Culture Plate, 96 well	Corning Incorporated, USA
Vortex mixer	neoLab, Germany
Water bath	Thermo Scientific, Haake, SC 150, USA

4.5.2 Software

Table 2: Software with correspondent manufacturer.

Name	**Manufacturer**
Graph Pad Prism 8.0	Graph Pad Software Inc., CA, USA
ICEstimator	(Le Nagard *et al.*, 2011)
LightCycler® 480 Software, Version 1.5	Roche, Switzerland
Microsoft Excel 2016	Microsoft, USA
Microsoft Word 2016	Microsoft, USA
STATA 11.2	StataCorp., TX, USA

4.5.3 Chemicals and reagents

Table 3: Chemicals and reagents with correspondent manufacturer.

Name	**Manufacturer**
HEPES 1M H0887	Sigma Aldrich, USA
Albumax II Lipid-Rich BSA	Gibco Invitrogen, New Zealand
Artesunate	Sigma Aldrich, USA
Atovaquone	Sigma Aldrich, USA

Cellulose	Sigma Aldrich, USA
Chloroquine diphosphate salt	Sigma Aldrich, USA
Dimethyl sulfoxide (DMSO)	Sigma Aldrich, USA
DNA / RNA shield	Zymo Research, USA
Gentamicin 50 mg / ml	Sigma Aldrich, USA
Giemsa R solution	RAL Diagnostics, France
Human AB serum	Universitätsklinikum Tübingen, Germany
L-glutamine 200 mM	Sigma Aldrich, USA
Lumefantrine	Sigma Aldrich, USA
Merck phosphate buffer tablets	Sigma Aldrich, USA
Methanol	VWR chemicals, France
RPMI 1640 without L-glutamine R0883	Sigma Aldrich, USA

4.5.3.1 Complete culture medium (CCM)

6 ml of 200 mM / 100x L-glutamine, 12 ml of 1 M HEPES, 500 µl of 50 mg / mL gentamicin, 50 ml Albumax II and 10 ml human AB serum are added to a 500 ml bottle RPMI 1640 without glutamine. The CCM is stored at 4°C.

4.5.3.2 70 % ethanol

To produce 1 l of 70% ethanol, 700 ml of pure ethanol is mixed with 300 ml of distilled water in a spray bottle. The mixture is stored at room temperature and used to clean the laminar flow and all working surfaces in the lab before and after work.

4.5.3.3 Phosphate buffer

To prepare the phosphate buffer, one Merck Phosphate Buffer Tablet is dissolved in 1 l of distilled water in a glass bottle. The buffer solution is stored at 4°C in order to prevent fungal growth.

4.5.3.4 10 % Giemsa stain solution

To prepare 20 ml of 10 % Giemsa solution, 2 ml of Giemsa R solution is diluted with 18 ml of phosphate buffer in a 50 ml falcon tube. The mixture is transferred to another container through a filter paper. The Giemsa dye is freshly prepared before slide staining and stored for a maximum of four hours.

4.6 Methods

4.6.1 Determination of parasite density in the microscope

Parasite density for the experimental outcomes was determined according to the applicable Lambaréné slide reading method. To determine a slide as negative, 100 fields were counted, and no parasite was seen. If five parasites were counted in 30 fields, the slide reading was stopped. If 50 parasites were counted in 20 fields, the slide reading was stopped. The parasitaemia was expressed as the number of asexual parasites per microliter of blood and calculated with the following formula:

$$Parasite\ density\ per\ \mu l\ of\ blood = \frac{Number\ of\ parasites\ counted\ \times\ microscopic\ factor}{Number\ of\ fields\ examined}$$

4.6.2 Drug stock solutions

The antimalarial drugs used for the *ex vivo* drug assay were delivered in powder form. The concentration of the stock solution was set at 100 mM for each drug. 3–5 mg of each drug was collected and diluted. DMSO was used for the dilution of AS, ATQ and LUM, distilled water for the dilution of CQ. In order to obtain

working solutions (WS), the drugs were diluted with RPMI 1640 and stored at 4°C. **Table 4** shows how the WSs were created. Each WS had a volume of 200 µl.

Table 4: Preparation of WS.

Drug	Concentration of WS (nM)	Drug stock solution (µl)	RPMI 1640 medium (µl)
CQ	250	20	180
AS	100	8	192
ATQ	100	8	192
LUM	500	40	160

4.6.3 Preparation of a CF11 column

Inside the laminar flow a 10 ml syringe was placed on top of a 15 ml falcon tube by using adhesive tape. The plunger was taken out of the syringe and the latter was filled with cellulose powder until the 4 ml mark. 8 ml RPMI 1640 was poured on top of the cellulose. After the entire RPMI passed through and a new falcon tube was placed under the syringe, the CF11 was ready for the subsequent sample processing. It was essential to keep the cellulose wet. Otherwise, the blood got stuck in the column and did not drip into the falcon tube.

4.6.4 Processing of the samples

10 ml of venous blood from adults and 5 ml of venous blood from children between three to ten years old were drawn respectively into heparin tubes. Thin and thick smears were made. The slides were dried, fixed with methanol and stained with 10% Giemsa for 15 min. In order to discard the serum, the blood was centrifuged and subsequently washed two times with RPMI 1640 at 2000 g for 5 min. The blood pellet was diluted in equal parts with RPMI 1640 and run through a CF11 column to deplete leucocytes. After the RBC pellet was obtained it was washed three times with RPMI 1640 to discard the remaining cellulose.

4.6.5 Establishing a *P. malariae* culture

After the blood was processed, 400 µl of RBC pellet was mixed with 9.6 ml of complete culture medium in a 25 cm^2 culture flask to obtain a haematocrit of 4%. The culture flask was maintained in an incubator at 37°C, 80% humidity and a gas atmosphere that constituted 5% CO_2, 5% O_2 and 90% N_2. Every 72 hours the medium was changed. Smears were taken daily to follow the cell cycle of the parasite.

4.6.6 Drug sensitivity testing *ex vivo*

The *ex vivo* drug susceptibility testing was based on the WHO schizont maturation test. The drugs chosen for testing were CQ, AS, ATQ, and LUM. The serial dilution was performed for each drug in a 96-well plate. To determine the highest drug solution needed for full inhibition, IC_{50}-values from previously published drug assays with CQ and AS tested on *P. malariae* were considered (Ringwald *et al.*, 1997; Siswantoro *et al.*, 2011). Because there was no data found on ATQ and LUM, IC_{50}-values of tests on *P. falciparum* were taken from the literature (Tahar *et al.*, 2014; Diawara *et al.*, 2017).

4.6.6.1 Blood preparation

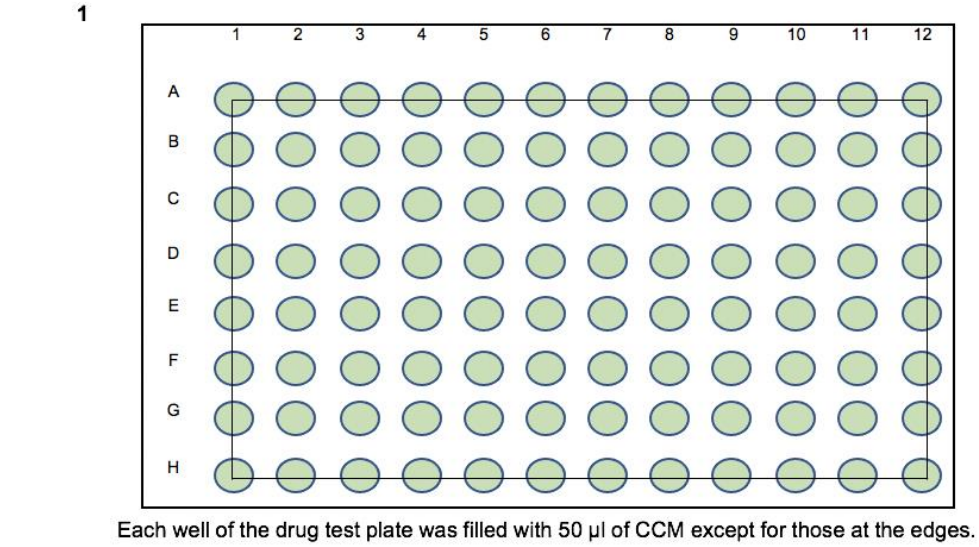
A blood medium mixture (BMM) was prepared with a 4% HCT. 400 µl of washed RBC-pellet was mixed with 600 µl of 20% human AB-serum in a 2 ml tube. Subsequently, the blood was diluted in a reagent reservoir with 9 ml of CCM.

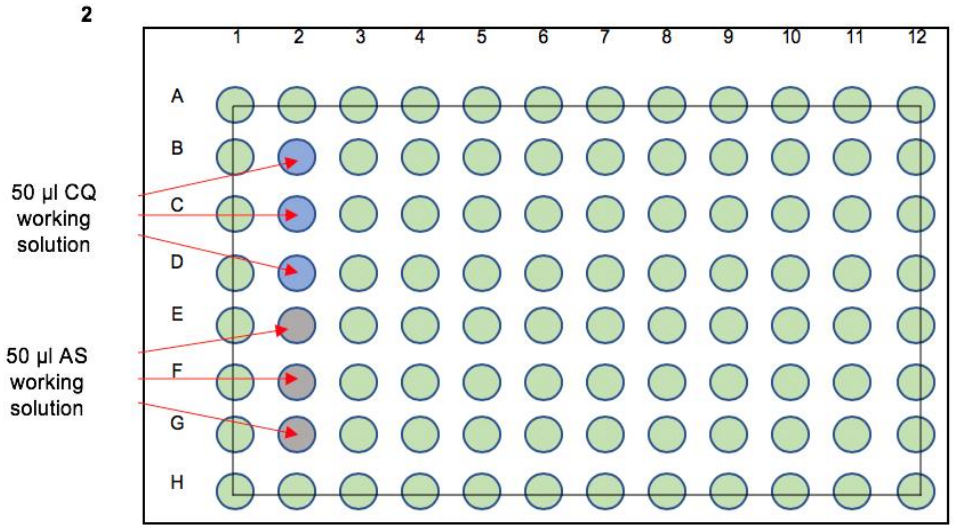
4.6.6.2 Handling of the 96-well plate

Evaporation of the cell culture medium plays a crucial role in multi-well cellular assays as it can degrade assay performance. The longer the drug plates are incubated the more the distribution of evaporation and the loss of liquid varies (Walzl *et al.*, 2012). Due to the so-called edge effect the outer wells were left

empty. A misinterpretation of data caused by variations in growth rates was thus prevented. The plates were incubated for a maximum of 72 hours. **Fig. 11** shows the plate layout.

Fig. 11: Handling procedure for the 96-well drug plates. Drug sensitivity was observed in triplicates. Numbers in **(6)** and **(7)** display the drug concentration in nmol / l.





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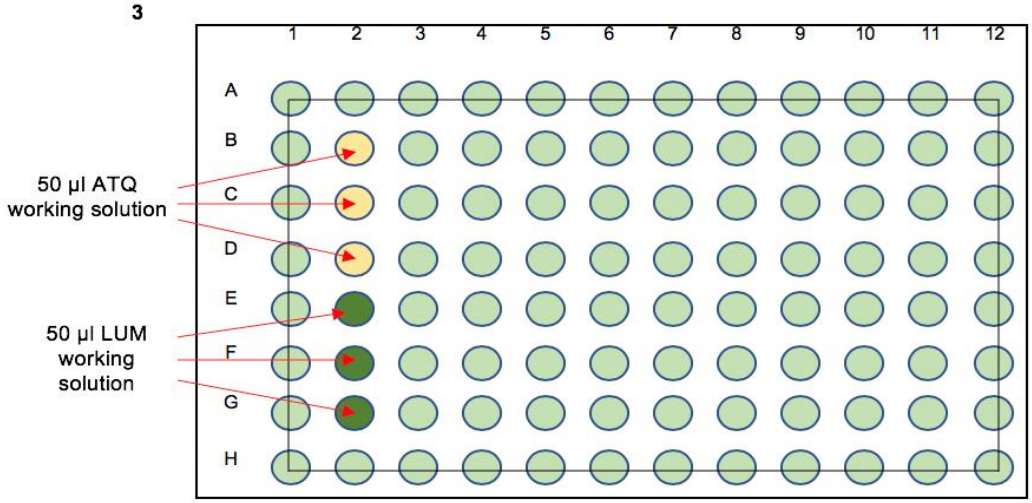
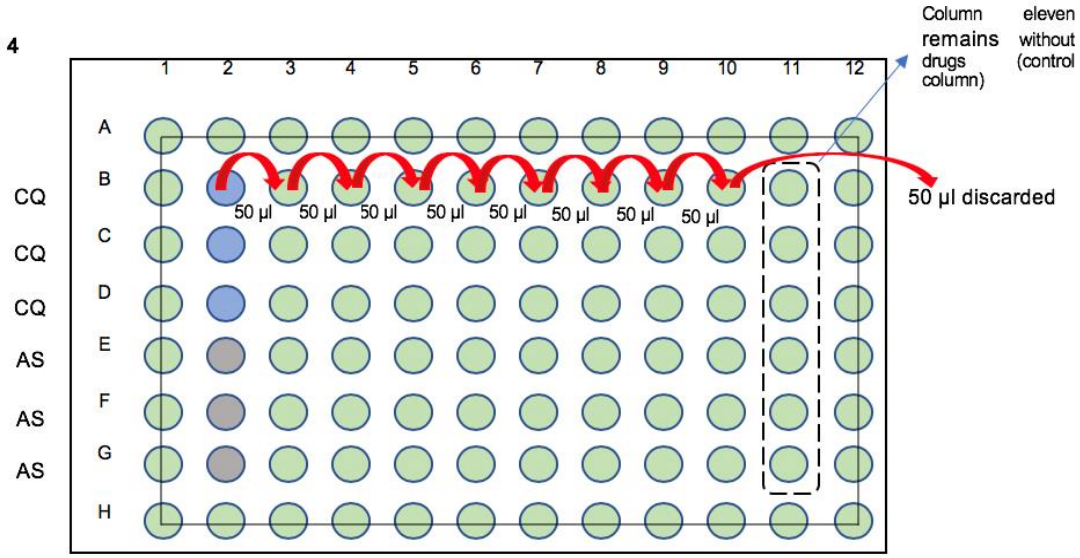
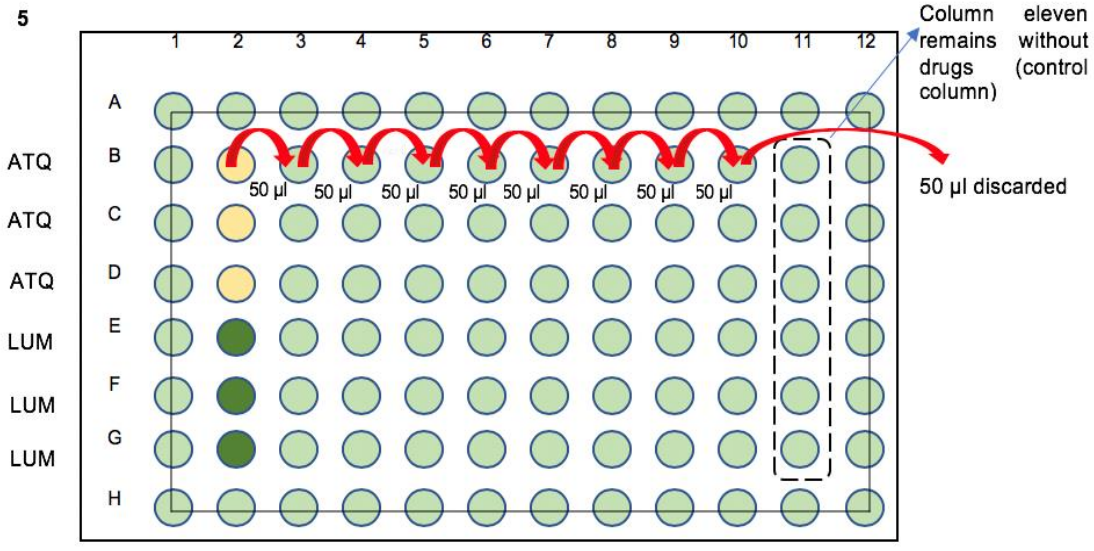

1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
50 µl ATQ working solution
50 µl LUM working solution

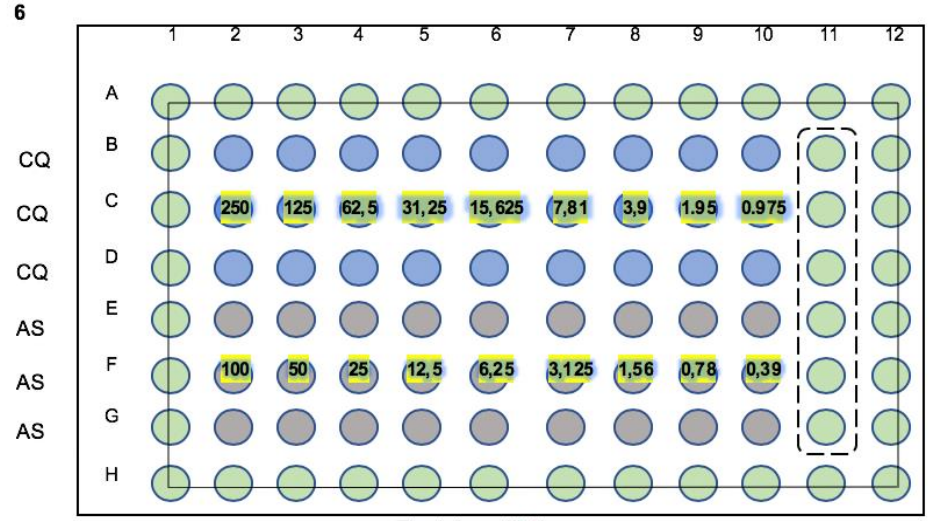
4


1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
CQ
CQ
CQ
AS
AS
AS
50 µl 50 µl 50 µl 50 µl 50 µl 50 µl 50 µl 50 µl
50 µl discarded
Column eleven remains without drugs (control column)

5

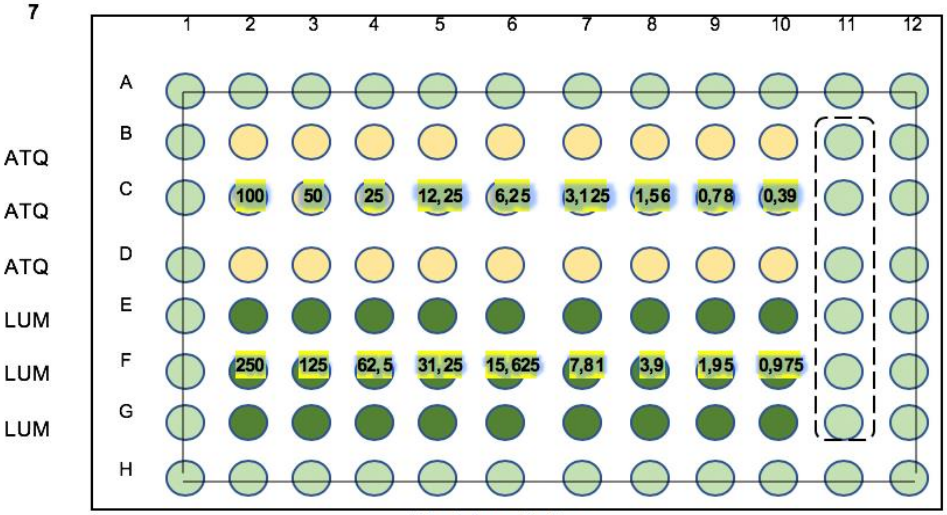

1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
ATQ
ATQ
ATQ
LUM
LUM
LUM
50 µl 50 µl 50 µl 50 µl 50 µl 50 µl 50 µl 50 µl
50 µl discarded
Column eleven remains without drugs (control column)

6



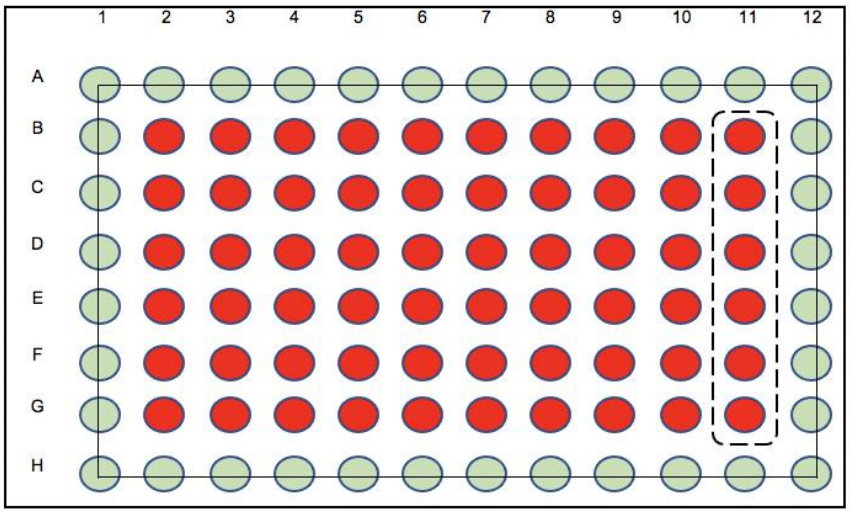
Final drug dilutions

7



Final drug dilutions

8



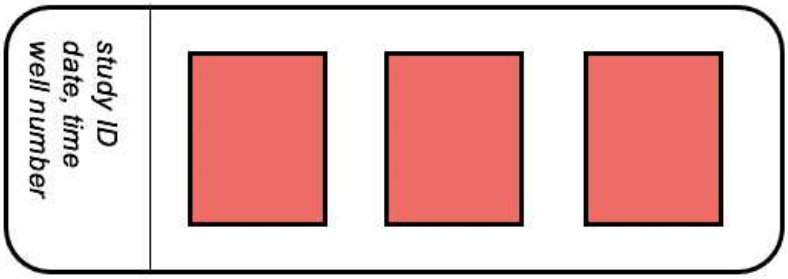
To each applicable well 50 µl of BMM were added. Each well contained a volume of 100 µl.

The 96-well plate was dosed. 50 µl of CCM was added to each well except for the outer wells. 50 µl of working solution of the drugs was transferred into the first wells in column 2. Serial dilution was performed with a multi-channel pipette. Starting in column 2, the drug-medium mixture was mixed by pipetting up and down and 50 µl was transferred from column 2 to column 3. After mixing, 50 µl was removed from column 3 to column 4. This procedure was repeated for each column going from left to right, resulting in a 9-fold dilution per row. 50 µl was discarded after column 10 to allow each well to contain the same amount of 50 µl. Each drug dilution was performed in triplicate series. Column 11 was skipped and used as negative control without drug exposure. Finally, 50 µl of BMM was added to every well except the outer wells. The drug plates were placed in an incubator at 37°C, 80% humidity and a gas atmosphere that constituted 5% CO_2, 5% O_2 and 90% N_2. The parasite maturity was controlled for the first time after 12 hours post-incubation. Incubation was stopped when mature schizonts were seen in the control wells.

4.6.7 WHO schizont maturation test

The schizont maturation test was developed by the WHO in order to provide a simple instrument for drug sensitivity testing that is also applicable under primitive field conditions. The test focuses on the comparison between the number of schizonts in drug wells with the number of schizonts in control wells. The drug plates were incubated until schizonts were seen in the control. After incubation, the experiment was stopped. The supernatant medium was discarded and 10 µl of BMM from each well was transferred onto a microscopic slide in order to make thick smears. Each slide contained the BMM from three wells (**Fig. 12**). The thick films were stained for 10 min in Giemsa dye.

Fig. 12: Three thick smears were made on one microscopic slide. Each thick smear represents one well with 10 µl of BMM.



A little immersion oil was put on the slide to increase the resolving power of the microscope. The number of schizonts was counted against 100 asexual parasites in each well. The mean number of schizonts in the control wells and in the triplicate drug dilution wells was calculated. Using the following formula, the proportion of schizonts in the drug dilution wells was calculated:

$$Proportion\ of schizonts\ in\ \% = \frac{Mean\ number\ of\ schizonts\ in\ the\ respective\ drug\ dilution}{Mean\ number\ of\ schizonts\ in\ the\ control}$$

The result indicated how many schizonts survived in the drug-exposed well compared to the non-exposed control. Using the WHO schizont maturation test, the IC_{50}-value for each drug was calculated.

4.6.8 Blood preservation

In order to confirm the presence of *Plasmodium* spp. species by qPCR, blood samples were preserved from each blood isolate. 500 µl of RBC-pellet was mixed 1:2 with DNA/RNA shield in a 2 ml tube. The tube was labelled with the study ID, date and time, and stored at -20°C.

4.6.9 DNA and RNA extraction

For DNA/RNA extraction, the Quick-DNA/RNA Microprep Plus Kit, D7005 was used. All centrifugation steps were performed at 13.000 g for 30 seconds at room temperature, unless specified.
12 µl of Proteinase K was added to 600 µl of reagent / blood mixture. The mixture was incubated for 60 minutes. An equal volume of isopropanol was added and mixed by vortexing. The sample was transferred into a Zymo-Spin-Column in a collection tube and centrifuged. The flow-through was discarded. The spin-column was transferred into a clean microcentrifuge tube. 200 µl of DNA/RNA lysis buffer was added directly to the filter matrix of the spin-column and left for 5 minutes. The sample was centrifuged again. For DNA purification, the spin-column was transferred into a clean collection tube. For RNA-purification, an equal volume of ethanol was added to the flow-through that was obtained by centrifugation. The sample was transferred into a new Zymo-Spin-Column in a collection tube and centrifuged. The flow-through was discarded. To both spin-columns 400 µl of DNA/RNA prep buffer was added. The columns were centrifuged and the flow-through discarded. After, 700 µl of DNA/RNA wash buffer was added. The columns were centrifuged and the flow-through discarded. 400 µl of DNA/RNA wash buffer was added and the columns were centrifuged for 2 minutes to ensure complete removal of the wash buffer. The columns were transferred into microcentrifuge tubes. 15 µl of preheated DNase/RNase-free water was directly added to the filter matrix in the spin-columns. The columns were left for 5 minutes. The columns were then centrifuged to elute the DNA and RNA from the respective columns. The eluted DNA and RNA was stored at -20°C.

4.6.10 18S rRNA qRT-PCR

A real-time PCR (qRT-PCR) was performed using the Roche Light Cycler 480 II. The target gene was 18S rRNA. Two assays were made:

a. Detection of *P. falciparum* and / or non-*falciparum* human *Plasmodium*, which are *P. ovale, P. vivax* and *P. malaria.* Referred to in the following as Pf / Povm assay.
b. Detection of *P. malariae,* referred to in the following as Pm assay.

For assay (a), the following primers and probes were used:

- Plas F Primer: 5'-GCT TAG TTA CGA TTA ATA GGA GTA GCT TG-3'
- Plas R Primer: 5'-GAA AAT CTA AGA ATT TCA CCT CTG ACA-3'
- Pf Probe: [FAM] 5'-TCT GAA TAC GAA TGT C-3' [MGBEQ]
- Povm Probe: [HEX] 5'-CTG AAT ACA AAT GCC-3' [MGBEQ]

P. falciparum-RNA from cell culture was used as positive control.

For assay (b), the following primers and probes were used:

- PmT for Primer: 5'-GGT GTT GGA TGA TAG AGT AA-3'
- PmT rev Primer: 5'-CCC AAA GAC TTT GAT TTC TC-3'
- PmT Probe: [HEX] 5'-AGG AAG CTA TCT AAA AGA AAC ACT CAT-3' [MGBEQ]

RNA from a *P. malariae* mono-infected sample was used as positive control. The negative control for both assays was nuclease-free water.

Ten samples were run in duplicates with two positive and one negative control. **Table 5** shows the reagents and corresponding volumes that were used to prepare the master mix for assay (a) and assay (b).

Table 5: Reagents and corresponding volumes for the master mix of assay (a) and (b).

Assay (a)			Assay (b)		
Reagent	Volume (µl)	Final concentration	Reagent	Volume (µl)	Final concentration
2× OneStep-SensiFAST mix	5	1×	2× OneStep-SensiFAST mix	5	1×

Transcriptase	0.1		Transcriptase	0.1	
Pf-Fam probe	0.4	400 nM	Pm-Hex probe	0.15	150 nM
Povm-Hex probe	0.4	400 nM	Pm for	0.4	400 nM
PlasF	0.8	800 nM	Pm rev	0.4	400 nM
PlasR	0.8	800 nM	PCR water	2.95	
PCR water	1.5				

4.6.11 Statistical analyses

The relative inhibitory effect of the respective drug dilution was calculated as follows: In order to determine the IC_{50}-value, the maximum inhibition was determined as 100% inhibition of schizont maturation. The minimum inhibition was achieved in those wells that did not contain any medication. The *P. malariae* asexual blood-stage inhibitory concentrations (i.e. IC_{50}, IC_{90} and IC_{99}) for CQ, AS and ATQ were determined using the free software ICEstimator (available online at http://www.antimalarial-icestimator.net/). The system used the inhibitory sigmoid E_{max} model that constituted a non-linear least-squares algorithm (Le Nagard *et al.*, 2011). Before calculation, the concentrations of the respective drug (x-axis) and the respective overall mean number of schizonts (y-axis) were entered in two lists. The relative effect of the drug on the number of schizonts was calculated automatically by the software. In the subsequently obtained graphic, the x-axis showed the drug concentrations in nM and the y-axis showed the number of schizonts in %. The ICEstimator indicated for each IC-value the associated confidence interval. Additionally, the standard error was calculated.

Data were entered in GraphPad Prism 8 (GraphPad Software, USA) and statistically analysed by Stata 11.2 (Data Analyses and Statistical Software, Texas, USA). The normality of the data was validated by the Shapiro-Wilk test. The number of schizonts per drug concentration was compared to the control by one-way ANOVA or Kruskal-Wallis test. For Post-hoc analysis, the paired t-test or Kolmogorov-Smirnov test were applied. P-values <0.05 were considered to be statistically significant.

The output of ultrasensitive q-RT-PCR reactions was analysed by visual inspection and by calculating the crossing point (Cp) using LightCycler 480 Software (version 1.5.1.62) via the second derivative maximum method. A Cp-value below the threshold of 40 indicated a positive sample.

5. **Results**

5.1 Study participants

555 participants were screened for malaria positivity between January and April 2020 in the CoMal-study. **Table 6** shows the baseline characteristics of the study participants.

Table 6: Baseline characteristics of participants.

Male / female	235/320
Median age in years	18
Median axillary temperature in °C	36.9

According to microscopy analysis, the overall *Plasmodium* prevalence in the study cohort was 29% (162 positive cases out of 555 participants). In 21 household surveys, 162 participants were found positive for *Plasmodium* spp. Microscopy revealed that 148 (91%) were mono-infected with *P. falciparum*, seven (4%) with *P. malariae,* one (1%) with *P. ovale,* and six (4%) were co-infected with *P. malariae* and *P. falciparum* (**Table 7**). In total, 13 people were found to be infected with *P. malariae*. However, two took anti-malarial medication before the blood-drawing, and one participant did not give consent for our study**.** Microscopically, the asexual parasitaemia of *P. malariae* infections showed nearly synchronous stages consisting of either schizonts or trophozoites.

Table 7: Microscopic results of n=555 slides.

Total number of *Plasmodium* spp. infections	162
P. falciparum mono-infections	148 (91 %)

Mean asexual parasite density / µl	1916
Mixed infection with *P. falciparum* and *P. malariae*	6 (4 %)
Mean asexual parasite density / µl	1408
P. malariae mono-infections	7 (4 %)
Mean asexual parasite density / µl	1772
P. ovale mono-infections	1 (1 %)
Mean asexual parasite density / µl	2485

Participants were between 1 and 89 years old. The distribution of malaria prevalence among the different age cohorts is shown in **Table 8**. Intra-cohort *Plasmodium* prevalence was highest in participants aged between 11–15. In this cohort, 31 of 60 participants (52%) were found positive for *Plasmodium* spp.

Table 8: The malaria prevalence of the study participants is distributed among the individual age cohorts. The relative figures were calculated in relation to the total number of participants.

Age (range)	Total number of participants	Positive for *Plasmodium* spp. (%)	Positive for *P. malariae* (%)	Positive for *P. falciparum* and *P. malariae* (%)	Positive for *P. falciparum* (%)	Positive for *P. ovale* (%)
1 - 5	93	29 (31)	1 (1)	1 (1)	26 (28)	1 (1)
6 - 10	94	39 (41)	-	3 (3)	36 (38)	-
11 - 15	60	31 (52)	2 (3)	1 (2)	28 (47)	-
16 - 20	29	12 (41)	1 (3)	1 (3)	10 (35)	-
21 - 89	279	51 (18)	3 (1)	-	48 (17)	-

In total, ten experiments were conducted. Out of the participants that were included in the study, seven were male and three female. The median age of the participants infected with *P. malariae* was 22. Only participants CML 1063 and 1157 were febrile (**Table 9**). The qPCR analyses on the ten isolates revealed that four participants were mono-infected with *P. malariae* and five participants had a co-infection with *P. malariae* and *P. falciparum.* The isolate CML 1161 was determined as a co-infection with *P. falciparum* and *P. ovale*.

Table 9: Characteristics of the participants with *P. malariae* isolates used in the experiments.

Nr.	CML - ID	Sex	Age (years)	Temperature in °C	Parasitaemia (parasites / µl)	Species (Microscopy)	Species (q-RT-PCR)
1	1037	M	8	36.7	803	*P.f. + P.m.*	*P.f. + P.m.*
2	1063	F	32	39	387	*P. malariae*	*P. malariae*
3	1077	M	10	36.5	383	*P.f. + P.m.*	*P.f. + P.m.*
4	1157	M	2	38.7	8439	*P. malariae*	*P. malariae*
5	1161	F	29	37.2	233	*P. malariae*	*P.f. + P.o.*
6	1179	M	28	36.6	462	*P. malariae*	*P.f. + P.m.*
7	1194	M	18	37.5	406	*P. malariae*	*P. malariae*
8	1320	F	76	36	715	*P. malariae*	*P. malariae*
9	1337	M	12	36.5	439	*P.f. + P.m.*	*P.f. + P.m.*
10	1351	M	11	37.1	358	*P. malariae*	*P.f. + P.m.*

Participant CML 1157 showed an extraordinarily high parasitaemia in comparison to all other isolates (**Fig. 13** and **14**).

Fig. 13: Eleven *P. malariae* trophozoites in a thick smear of unprocessed fresh blood of participant CML 1157.

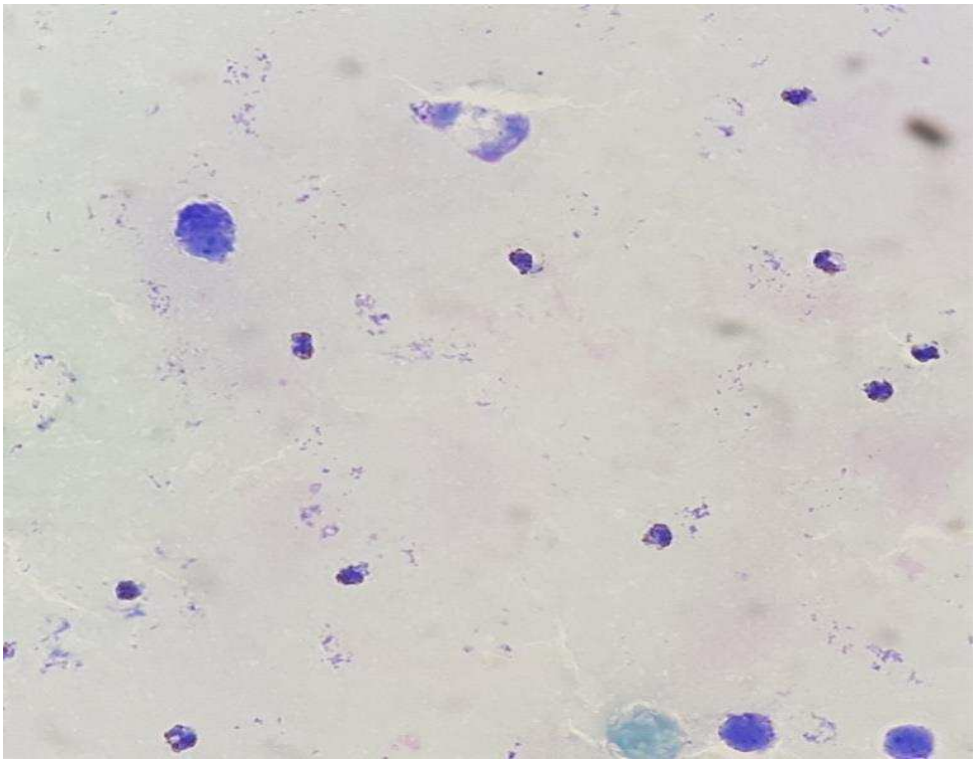
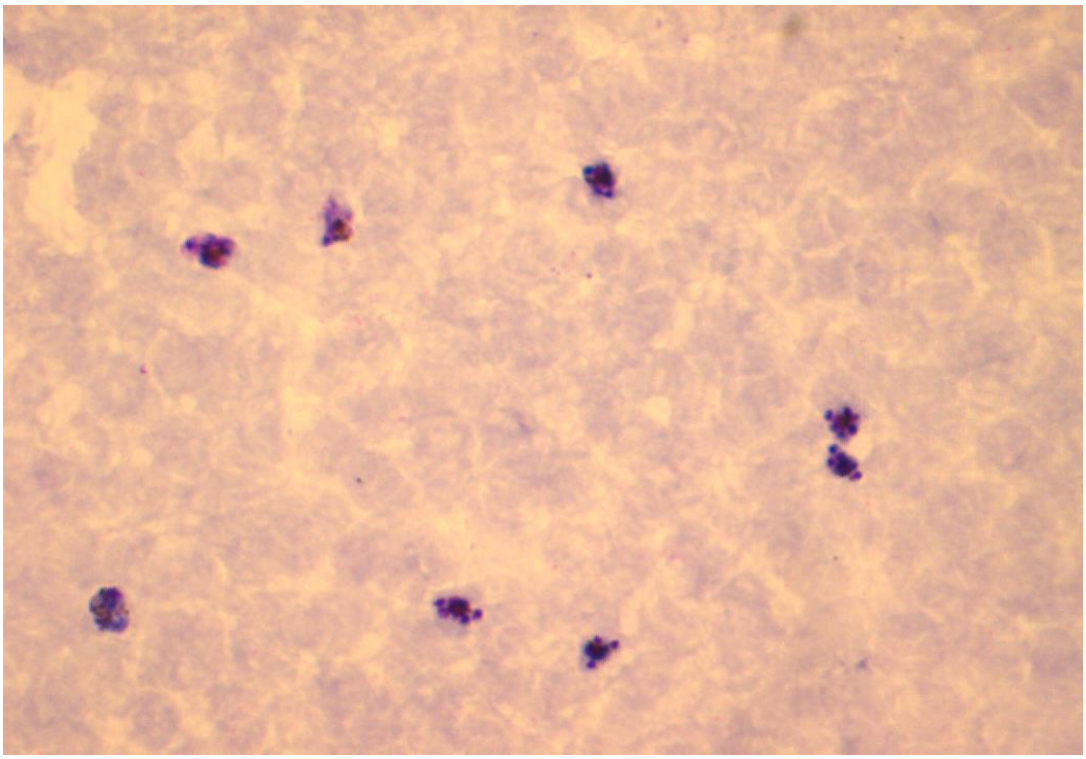


Fig. 14: Eight *P. malariae* schizonts, 36th hour of incubation, thick smear (CML 1157).



5.2 *In vitro P. malariae* culture

The attempt was made to establish a long-term *in vitro* culture from all ten participants that tested positive microscopically for *P. malariae* and were successfully involved in our study. Two experiments failed due to fungal growth in the culture flask (CML 1037 and 1320), and in another two, *P. falciparum* overgrew *P. malariae* (CML 1161 and 1337). In five *in vitro* experiments, the parasites died during the second cell cycle and only debris was observed in the subsequent follow-up (CML 1063, 1077, 1179, 1194 and 1351).

In isolates CML 1157 and 1063, parasites were observed for the longest period of time. *P. malariae* ring stages were observed after 24 (CML 1157) and 36 hours (CML1063), respectively (**Table 10, Fig. 15 – 17**), indicating full completion of the cell cycle and re-invasion of erythrocytes. In the isolate CML 1157, parasites were still observed after the 12th day of incubation (four completed cell cycles). The yellow pigment, typical for *P. malariae*, was still clearly visible, the nuclei appeared dense and red stained, and the cytoplasm appeared dark blue (**Fig. 18 – 21**). In isolate 1063, parasites were observed until the 5th day of incubation.

Table 10: Where feasible, 100 parasites were counted per thick smear. The respective asexual stages are indicated proportionately in %.

CML - ID	Hours of incubation (number of cell cycles)	Rings	Trophozoites	Schizonts
1063	0 (1)	-	98	2
1063	24 (1)	-	88	12
1063	36 (1)	3	11	86
1063	58 (1)	60	-	40
1063	72 (1)	18	81	1
1063	96 (2)	-	45	55
1063	120 (2)	-	12	88
1063	144 (2)	-	-	-
1063	216 (3)	-	-	-
1157	0 (1)	-	100	-
1157	24 (1)	3	-	97
1157	36 (1)	78	17	5
1157	58 (1)	30	70	-
1157	72 (1)	-	100	-
1157	96 (2)	-	19	81
1157	108 (2)	66	30	4
1157	144 (2)	-	100	-
1157	216 (3)	8	45	1
1157	288 (4)	12	52	3

Fig. 15: One *P. malariae* schizont (*) next to two ring stages (**), 36th hour of incubation, thick smear (CML 1157).

Double infections of erythrocytes by *P. malariae* ring stages were observed (**Fig. 16** – **17**).

Fig. 16: Two *P. malariae* rings in one erythrocyte, 58th hour of incubation, thin smear (CML 1063).

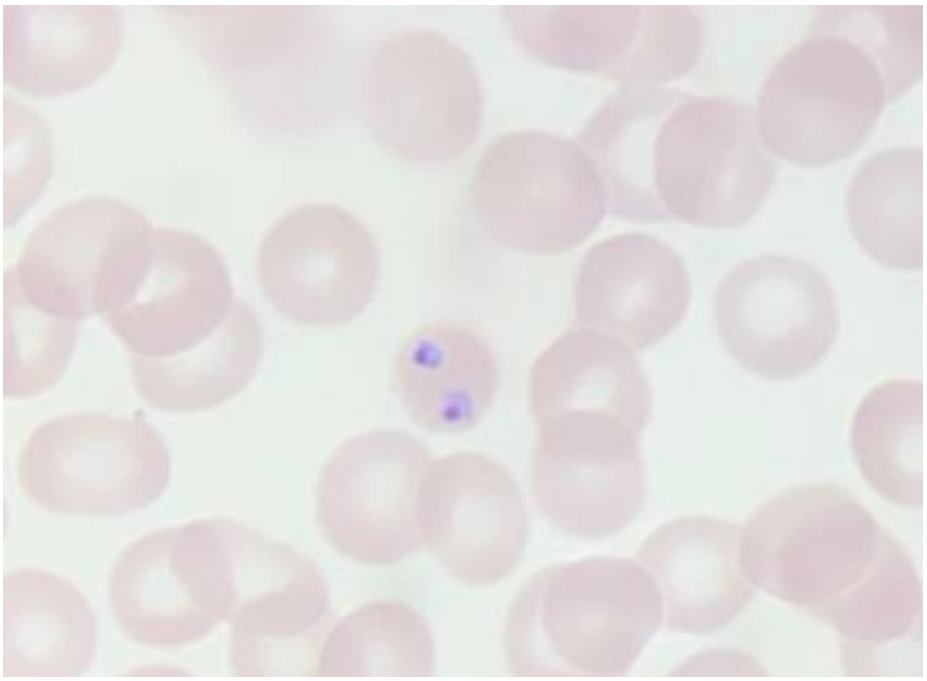


Fig. 17: Double infection of one erythrocyte by two *P. malariae* rings, 58th hour of incubation, thin smear (CML 1063).

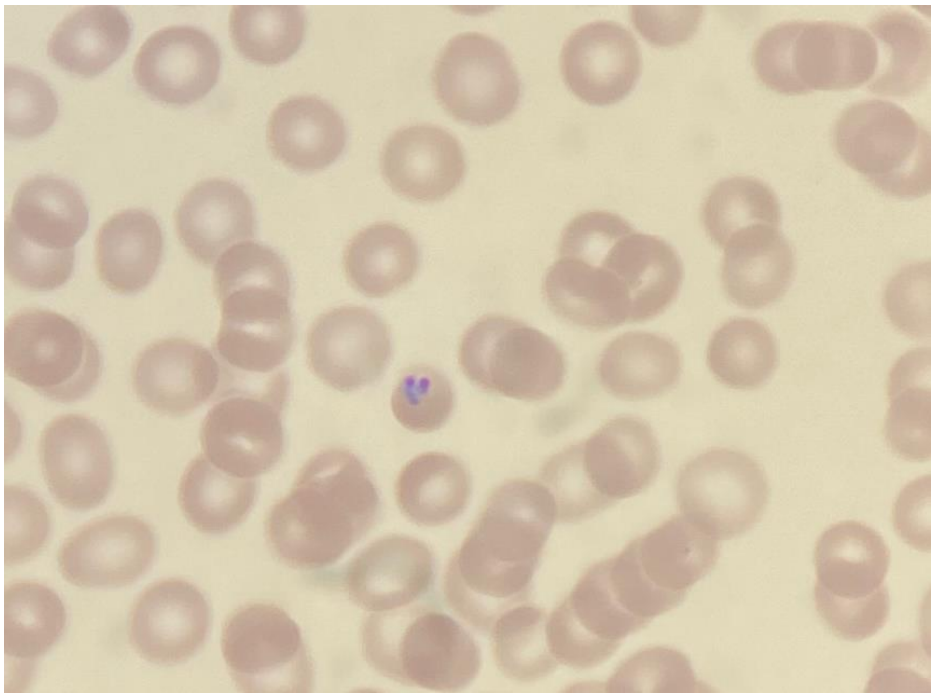


Fig. 18: One clearly visible *P. malariae* ring (*), remnants of schizonts with yellow pigment (**), 4th cell cycle, 288th hour of incubation, thick smear (CML 1157).

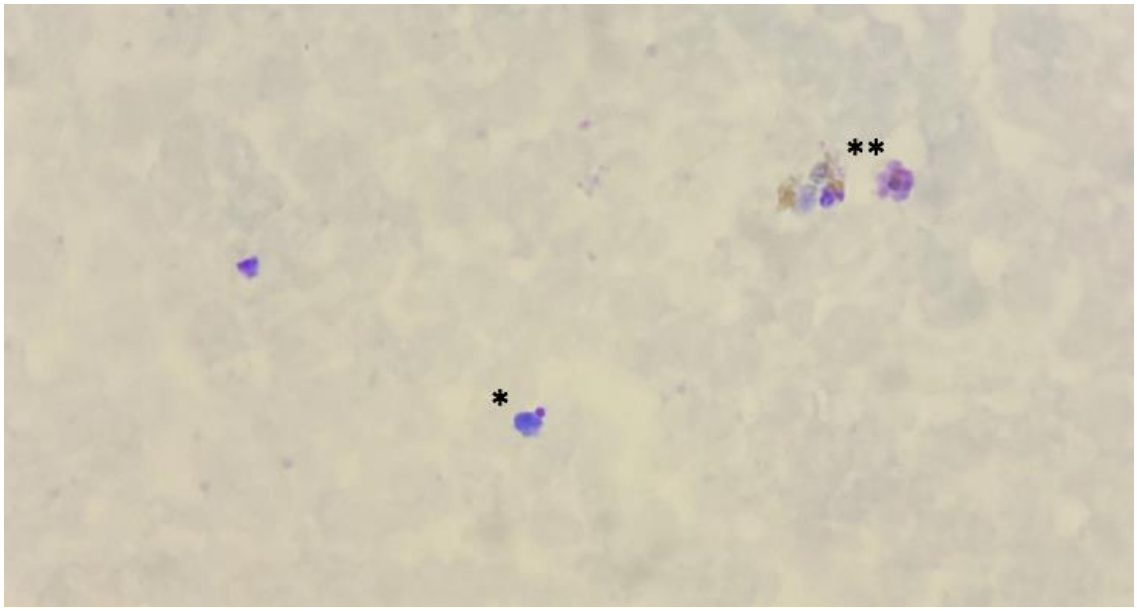


Fig. 19: Remnants of *P. malariae* schizonts with clearly distinguishable chromatin and scattered yellow pigment, 4th cell cycle, 288th hour of incubation, thick smear (CML 1157).

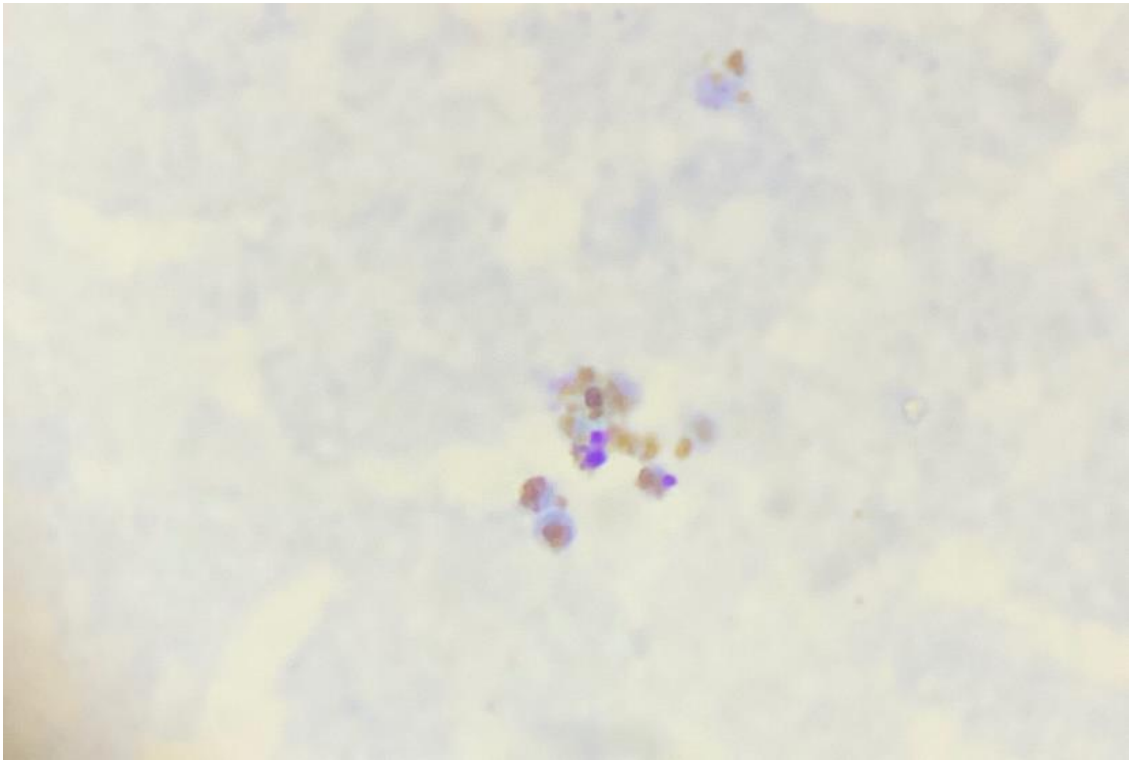


Fig. 20: Two *P. malariae* schizonts, chromatin is dense and red stained, the inferior schizont has at least three merozoites, 4th cell cycle, 288th hour of incubation, thick smear (CML 1157).

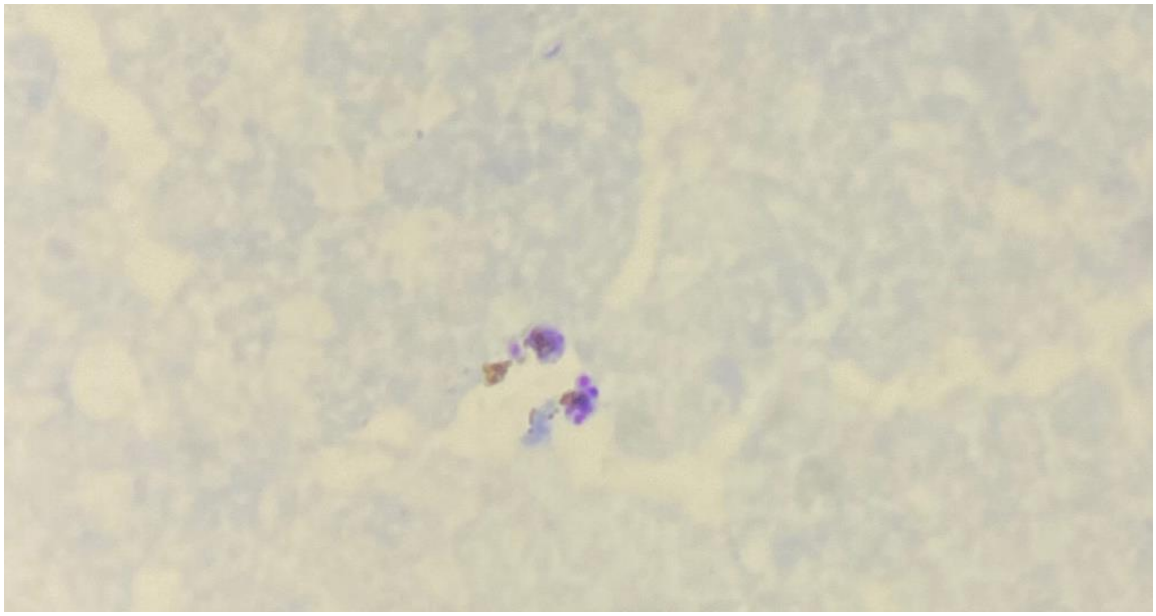
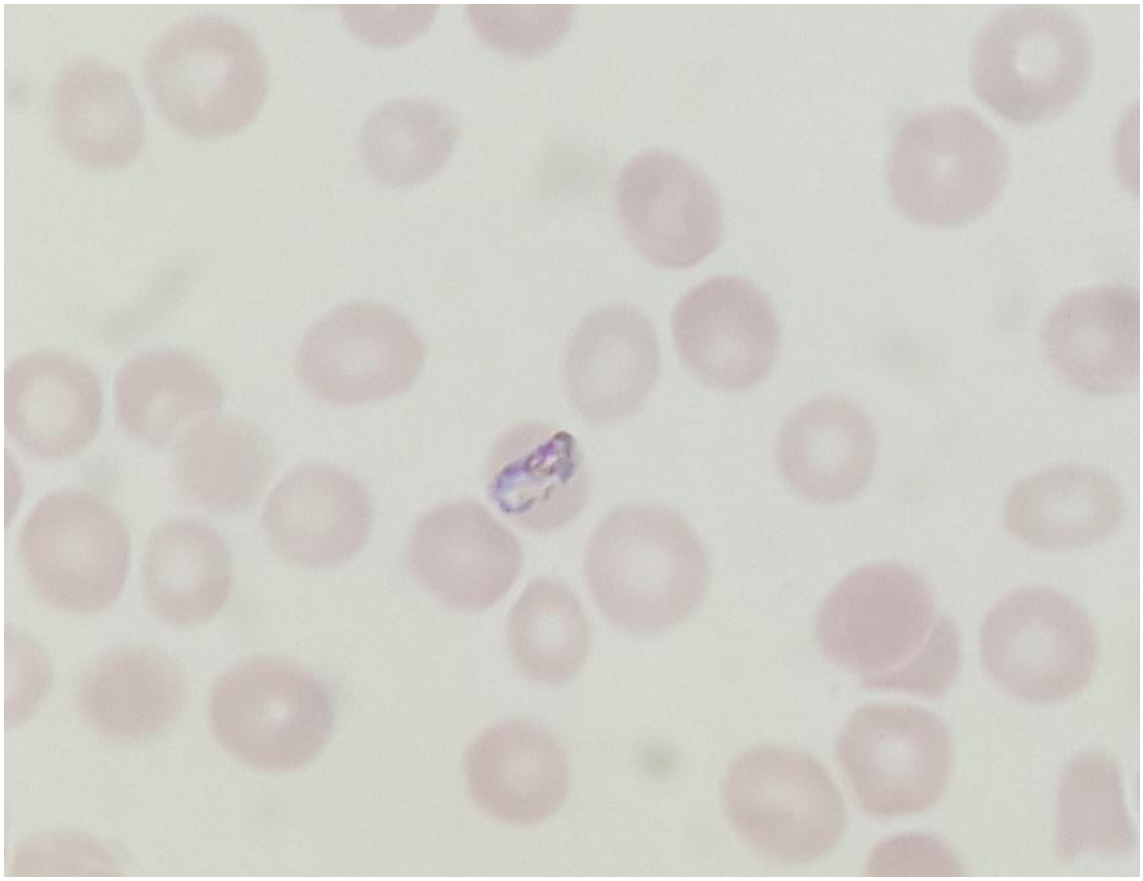


Fig. 21: Typical band form of a trophozoite, 4th cell cycle, 288th hour of incubation, thin smear (CML 1157).

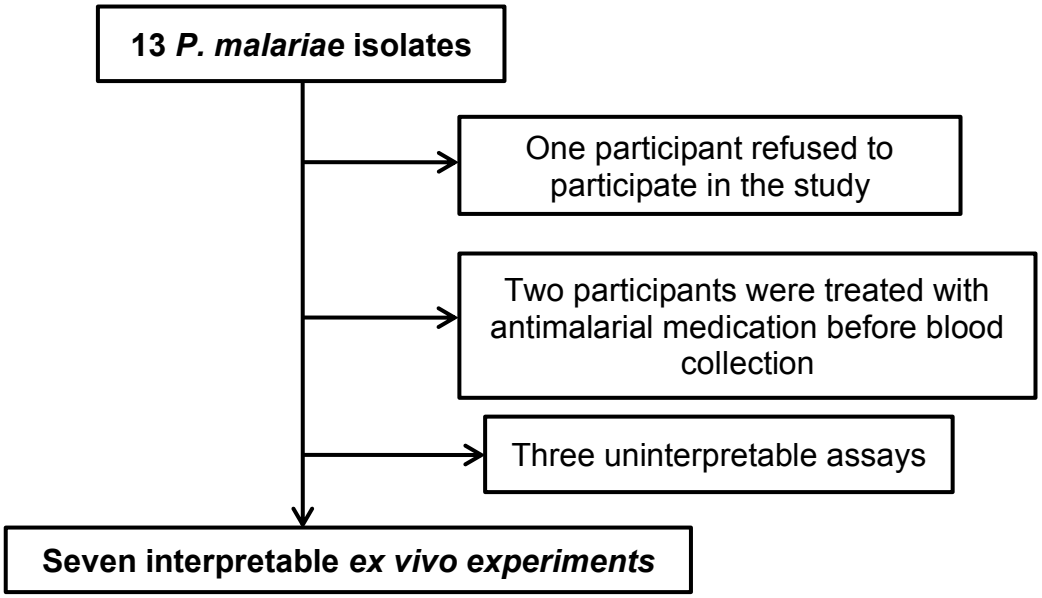


5.3 *Ex vivo* drug-susceptibility assay

Fig. 22 illustrates the study profile of the drug susceptibility testing. 13 participants matched the inclusion criteria. From ten assays set up, seven showed successfully inhibition of schizont maturation by CQ, AS and ATQ. In all experiments, LUM did not show an inhibitory effect on *P. malariae* schizont maturation. No significant difference in the number of mature schizonts between control and drug wells was observed.

In isolates co-infected with *P. malariae* and *P. falciparum,* asexual *P. falciparum* parasites were counted. During the experimental course, *P. falciparum* was only observed in isolate CML 1037. Ring stages of the latter parasite were detected in five of 120 drug wells. The respective number of *P. falciparum* parasites in each well was less than ten. In all other experiments, asexual *P. falciparum* stages were not present microscopically.

Fig. 22: Study profile of the *ex vivo* drug susceptibility testing.



Using the WHO schizont maturation assay, the *ex vivo* IC_{50}-values of CQ, AS and ATQ were determined (**Table 11**). The geometric mean of the CQ IC_{50}-value was 10 nM, the median 8 nM. Geometric mean and median for AS were both 3 nM. For ATQ, a geometric mean and a median of both 3 nM were revealed. **Fig. 23**

displays the distribution of the IC_{50}-values of each individual drug assay in a bar chart. No difference in IC_{50}-values was found between single-species and double-species infections.

Fig. 23: Distribution of the IC_{50}-values of the individual drug assays. For each drug, seven assays were performed.

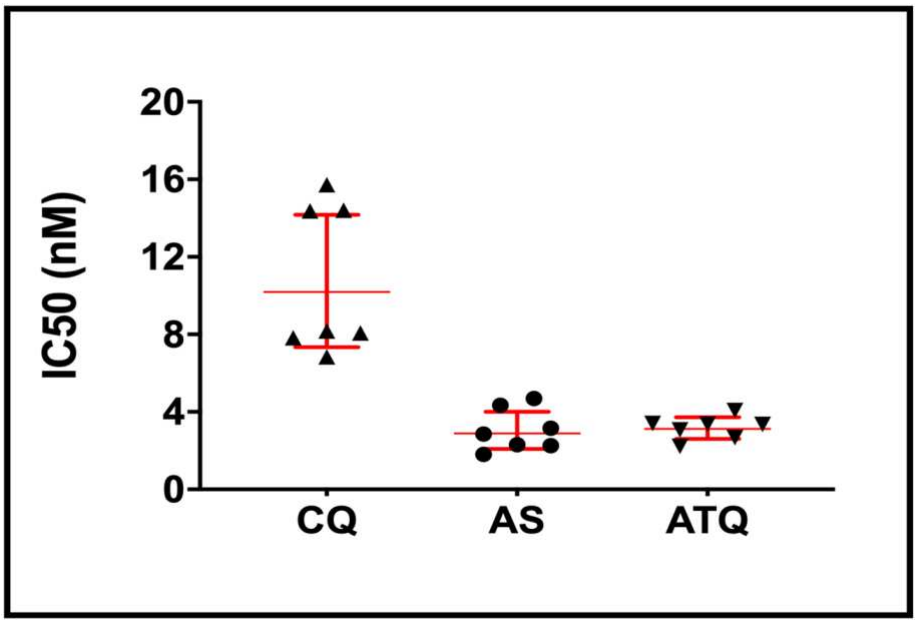


Fig. 24, **26** and **28** show the logarithmic plot of the number of schizonts at CQ, AS and ATQ concentrations, respectively.

Fig. 25, **27** and **29** depict the proportion of schizont maturation under the influence of the respective drug concentration compared to the control.

Table 11: Ex vivo susceptibility (in nM) of *P. malariae* isolates to CQ, AS and ATQ.

Ex vivo susceptibility	CQ (CI)	AS (CI)	ATQ (CI)
Number of isolates	7	7	7
Median of IC_{50}-value	8	3	3
Geometric mean of IC_{50}-value	10 (8.88 – 10.94)	3 (2.25 – 3.46)	3 (2.01 – 4.11)
Standard error	0.44	0.26	0.44
Geometric mean of IC_{90}-value	47 (45.93 – 48.44)	15 (13.73 – 15.2)	23 (21.47 – 24.02)

Standard error	0.54	0.32	0.55
Geometric mean of IC_{99}-value	259 (257.38 – 260.58)	85 (84.06 – 85.92)	203 (201.42 – 204.66)
Standard error	0.69	0.4	0.7

Fig. 24: Logarithmic plot of the number of schizonts at the respective CQ concentration.

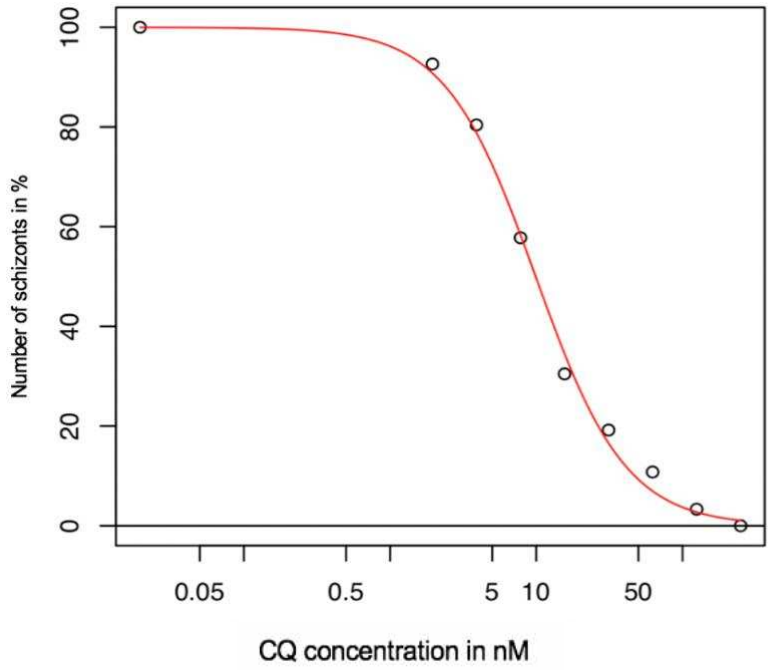


Fig. 25: (***: schizont maturation is significantly different to the control at CQ concentrations of 7,81 nM and higher, p < 0,001)

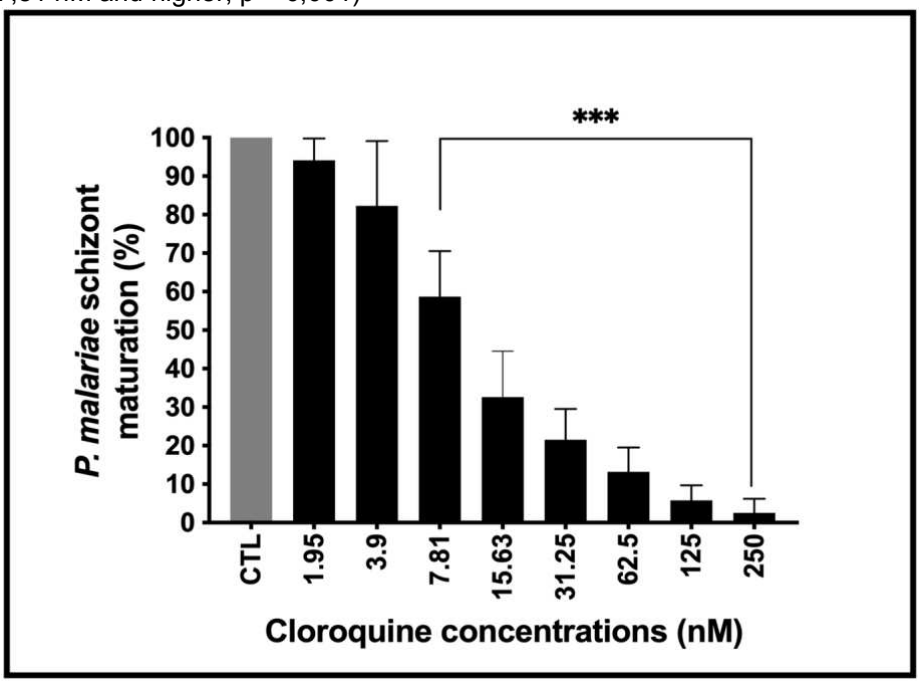


Fig. 26: Logarithmic plot of the number of schizonts at each AS concentration.

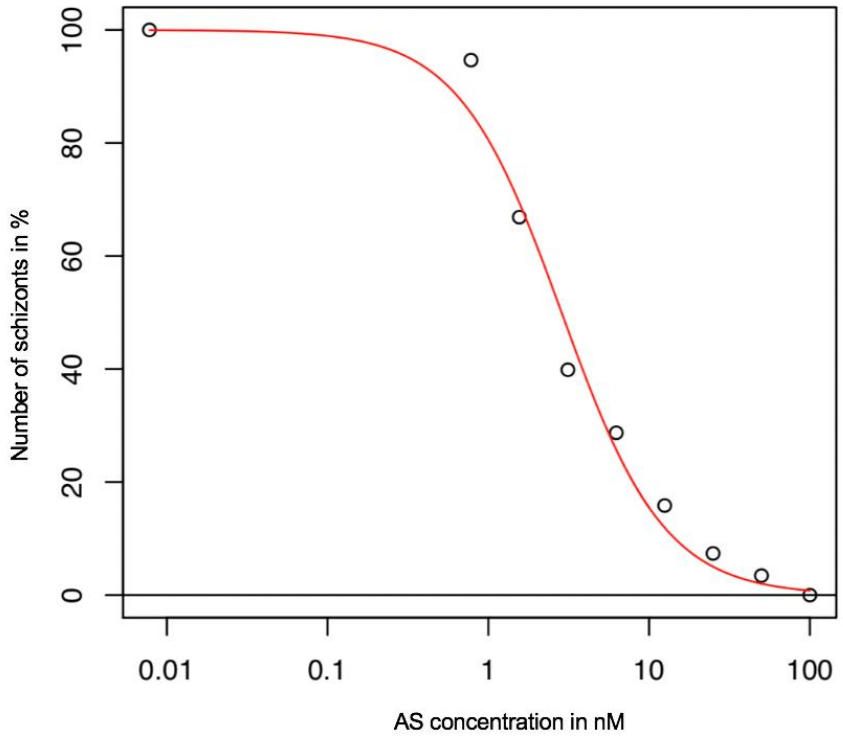


Fig. 27: (***: schizont maturation is significantly different to the control at AS concentrations of 1,56 nM and higher, $p < 0.001$)

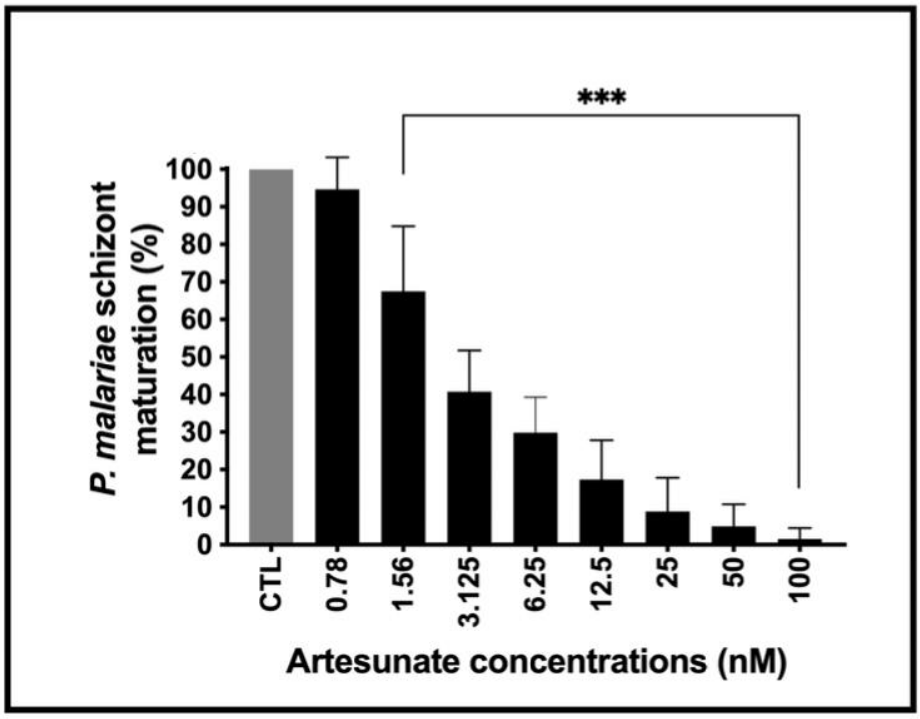


Fig. 28: Logarithmic plot of the number of schizonts at each ATQ concentration.

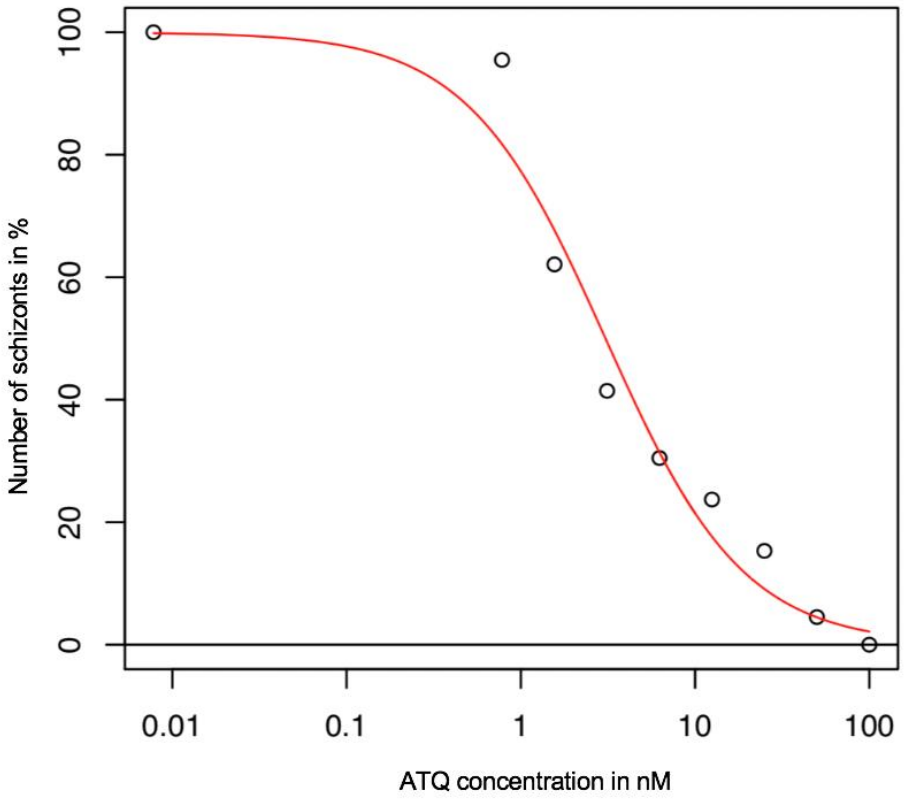
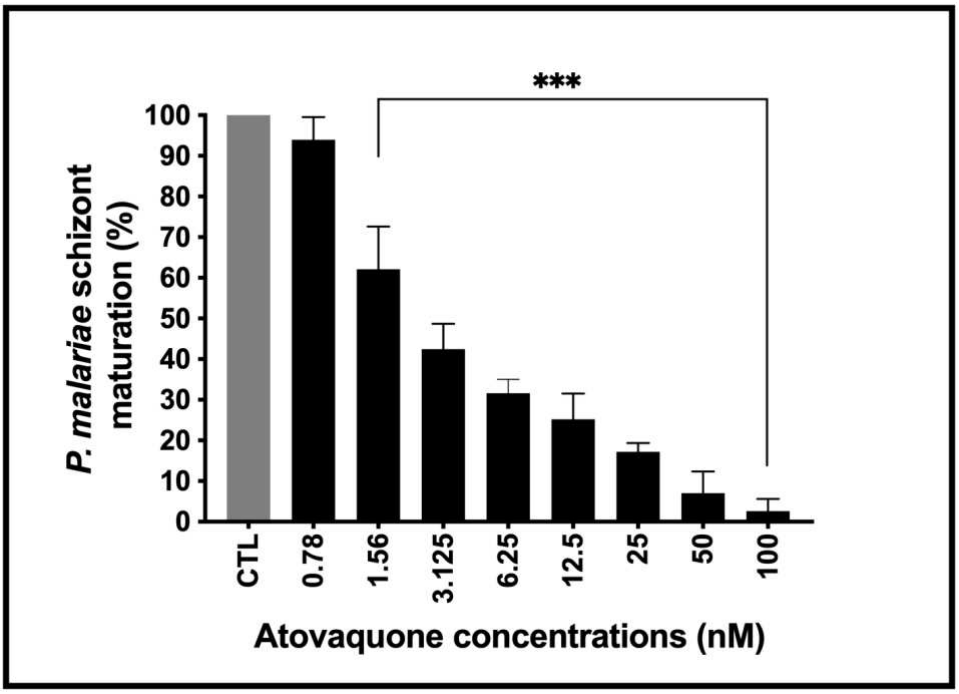


Fig. 29: (***: schizont maturation is significantly different to the control at ATQ concentrations of 1,56 nM and higher, p < 0,001)



6. Discussion

6.1 Biological features

Febrile courses in *P. malariae* mono-infected individuals are rarely seen (Collins and Jeffery, 2007). In our study, two participants were febrile due to a *P. malariae* mono-infection. The parasitaemia of the respective participants were far apart; a 32-year-old woman with 387 parasites / µl of blood and a two-year-old girl with 8439 parasites / µl of blood. After assessing *P. malariae* infections in a 20-year longitudinal study, Roucher *et al.* found that parasitaemia observed in patients with *P. malariae* attacks was only minimally higher compared to asymptomatic patients (Roucher *et al.,* 2014). This implies that there might be no correlation between parasitaemia and pyrogenicity. The latter is confirmed by our study results. No correlation was observed between the degree of parasitaemia and the development of clinical symptoms.

P. malariae is reported as a parasite that occurs commonly as a co-infection, most frequently with *P. falciparum* (Roucher *et al.*, 2014). This is also supported by our results. Using 18S rRNA qRT-PCR to confirm the presence of *P. malariae*, it was revealed that 56% (5 out of 9) of our *P. malariae* infected participants were co-infected with *P. falciparum*.

The age of *P. malariae*-infected individuals is indicated differently in the literature. Langford *et al.* determined a median age of 21.7 for 5,000 cases that tested positive in a hospital-based surveillance study in southern Papua, Indonesia (Langford *et al.*, 2015). In contrast, Woldearegai and colleagues found a median age of 60 in 18 mono-infected individuals in a cross-sectional study in a rural area of Gabon. However, in this study, the median age of 22 for participants infected with *P. malariae* confirms the results of Langford *et al*.

6.2 *In vitro P. malariae* culture

As previously stated by other research groups, the metabolic needs of *P. malariae* remain undetermined (Lingnau, Doehring-Schwerdtfeger and Maier, 1994; Ringwald *et al.*, 1997). No standard operating procedure exists for the cultivation of the parasite. In this study, it was possible to maintain one *P.*

malariae isolate over four cell cycles (CML 1157). Evidence for the completion of one cell cycle was the observation that merozoites re-infected erythrocytes after their release from the schizonts. *In vitro*, vivid *P. malariae* parasites were never kept alive longer than in this study (twelve days). Lingnau *et al.* observed only low numbers of *P. malariae* parasites over six days (Lingnau, Doehring-Schwerdtfeger and Maier, 1994). Ringwald *et al.* obtained insufficient parasite growth and harvest due to unfavourable metabolic conditions (Ringwald *et al.*, 1997).

6.2.1 Prospects for future attempts to cultivate *P. malariae*

Unfortunately, it was not possible to establish a long-term *in vitro P. malariae* culture during the study period. After parasites in the isolate CML1157 completed four cell cycles (twelve days), mainly debris was observed (as a sign of parasite death). The isolate was discarded. For adaptation of *P. falciparum* to *in vitro* culture conditions, it is known that after one or two cell cycles the parasitaemia decreases drastically. However, after a certain period of recovery, there is increased parasite growth again (White III *et al.,* 2016). It is reasonable to apply this theory to *P. malariae.* After the *P. malariae* field isolate is freshly obtained and put in a culture flask, it would be beneficial in future experiments to keep the *P. malariae* isolate in the incubator for more than one month. Indolent stages might survive in sub-microscopic levels and resurface after several cycles in the culture flask as a sign of adaptation to *in vitro* culture conditions.

As described earlier, it is believed that *P. malariae* only invades mature erythrocytes (Kerlin and Gatton, 2013). A testable hypothesis for explaining the premature death of *P. malariae* in *in vitro* models could be that old erythrocytes die within a few days in cell culture. This results in a lower number of host cells. If the merozoites are released from the schizonts, they cannot suitably infect erythrocytes before death. Also, merozoites probably need to compete with each other for the remaining host cells. This might explain the observed double infections that are common for *P. falciparum* infections. To verify this hypothesis, it would be important to collaborate with haematologists and find characteristic

surface markers on mature erythrocytes. Using flowcytometry, it would then be possible to observe the age distribution of erythrocytes over time. This approach would make it possible to assess whether old erythrocytes actually decrease in number during incubation in blood culture. Favourable results might also be gained from enriching mature erythrocytes. These could be mixed with the *P. malariae* isolate to possibly prolong its *in vitro* lifetime.

If a long-term *in vitro P. malariae* culture model were established, specific *P. malariae* antigens could be identified and used for diagnostic purposes (ELISA). Drug assays could then be standardised and carried out faster and more conveniently. So far, only the WHO schizont maturation test is available, which is both time consuming and rather inaccurate (Rebelo *et al.,* 2013).

6.3 *Ex vivo* drug susceptibility testing

The widespread and uncontrolled use of antimalarial monotherapies quickly leads to parasite resistance. Combination therapies as ACTs were introduced in the hope of slowing down the rate of reduction in parasite susceptibility to the common antimalarial compounds (World Health Organization, 2015). Chemo-susceptibility profiling monitors the response of *Plasmodium* to crucial antimalarial drugs, such as artemisinin derivatives, in order to be able to react promptly to the eventual rise in resistance to these drugs. In this context, drug assays provide important guidance for malaria treatment with existing as well as future drug compounds (Ursing *et al.*, 2020).

The first CQ resistance in *P. falciparum* species developed almost simultaneously in South-East Asia and South America in the early 1960s (Moore and Lanier, 1961; D'Alessandro and Buttiëns, 2001). By 2005, 80% of wild *P. falciparum* strains became resistant to the compound (Ginsburg, 2005). *P. vivax* resistance to CQ was first noted in 1989, when Rieckmann *et al.* reported a persisting *P. vivax* infection after intake of CQ doses higher than those regarded as adequate for parasite clearance (Rieckmann, Davis and Hutton, 1989). In 2002, Maguire *et al.* suspected *P. malariae* resistance to CQ in two patients after CQ intake did not yield a total parasite clearance after eight days of treatment despite adequate

drug plasma levels. However, subsequent *in vivo* studies did not find compelling evidence for evolving CQ resistance (Barnadas *et al.*, 2007; Siswantoro *et al.*, 2011). Instead, it is suspected that the PCT of *P. malariae* is commonly prolonged due to the slow metabolism of the parasite (Collins and Jeffery, 2002). The long parasitic prepatent period of development in the liver (three to four weeks), and the long cell cycle of 72 hours prevent a rapid elimination of the parasite from the bloodstream.

In contrast to our findings, previous *in vitro* drug assays with CQ on *P. malariae* showed seven- to 16-fold higher IC_{50}-values (Tan-ariya and Pasuralertsakul, 1994; Ringwald *et al.*, 1997; Tanomsing *et al.*, 2007; Siswantoro *et al.*, 2011). Our data revealed that with an IC_{50} geometric mean of 10 nM (95% CI 8.9–10.9 nM), CQ susceptibility is still well preserved in Gabon. This could be due to the fact that CQ is hardly used today, and that Gabon's national guideline was recommending the treatment of non-*falciparum* malaria with an ACT agent for more than 20 years (Woldearegai *et al.*, 2019). Nevertheless, Woldearegai *et al.* reported a CQ resistance in *P. falciparum* strains of 89% in Gabon. An *in vitro* study of *P. falciparum* conducted by Borrmann *et al.* in 2002 documented a CQ IC_{50}-value of 4160 nM (Borrmann *et al.*, 2002). This implies that in Gabon, malaria treatment by CQ should be restricted to *P. malariae*.

AS proved to be very efficient for *P. malariae* clearance in the *ex vivo* approach. The obtained IC_{50}-value of 3 nM (95% CI 2.3–3.5) is consistent with the results reported by Siswantoro *et al.* in 2011. AS is an artemisinin derivative that is still used in the treatment of *P. falciparum* infections. In Gabon, two studies on AS sensitivity of *P. falciparum* gave very similar results to what this study revealed in the AS-*P. malariae* drug assay. Kurth *et al.* obtained an IC_{50}-value of 2 nM on *P. falciparum* isolates near Lambaréné (Kurth *et al.*, 2009). Pradines and colleagues reported 3 nM in *P. falciparum* isolates from Libreville (Pradines *et al.*, 2001). This indicates that *P. malariae* and *P. falciparum* populations in Gabon are equally sensitive to AS *ex vivo*. Likewise, a clinical study on AS in Gabon stated a parasitological cure rate of 83% by day 56 in semi-immune patients that tested

positive for *P. malariae* (Borrmann, 2002). This underlines the *in vivo* effectiveness of AS against *P. malariae.*

No other study reported yet *ex vivo* ATQ sensitivity of *P. malariae* strains. In this chemo-susceptibility profiling, the yielded ATQ IC_{50}-value of 3 nM (95% CI 2–4.1) was very similar to that obtained for AS. Pradines *et al.* revealed an ATQ IC_{50}-value of 3 nM on *P. falciparum* in Libreville. Thus, it can be assumed that in *ex vivo* tests, *P. falciparum* and *P. malariae* are equally susceptible to ATQ in Gabon.
A clinical trial with ATQ plus proguanil was carried out on patients found positive for *P. malariae* and *P. ovale* at the Albert Schweitzer Hospital in Lambaréné, Gabon (Radloff *et al.*, 1996). Only three participants were infected with *P. malariae.* The observed PCT in *P. malariae* patients was prolonged in comparison to *P. falciparum* cases. This was attributed to the 72-hour life cycle of *P. malariae* and was not related to mechanisms of resistance. The authors concluded that despite the small number of participants, the combination of ATQ and proguanil constitutes an appropriate alternative to CQ for treatment of *P. malariae* infections.

Unfortunately, it was not possible to obtain results for the inhibitory effect of LUM on the *P. malariae* schizont maturation. The most obvious reason might be that a mistake was made when handling the drug. To prepare a stock solution, 2.6 mg of LUM was diluted with only 49 µl of DMSO in order to obtain a concentration of 100 mM. Because of the small amount of DMSO, it seems likely that the drug did not fully dissolve. Flakes of the drug powder were still observed in the 2 ml tube in which the stock solution was prepared. When preparing the working solution, the undissolved drug might have remained in the stock solution. In this case, the working solution would have contained mainly solvent rather than the drug. To exclude this possibility, the same working solution should have been tested on *P. falciparum* strains. An inhibitory effect on *P. falciparum* would indicate a mechanism of resistance in *P. malariae*.

In the literature, no evidence was found for the hypothesis that LUM has no inhibitory effect on *P. malariae*. Instead, several studies attested the high inhibitory effect of the drug combination artemether-lumefantrine against non-*falciparum* malaria *in vivo* (Mombo-Ngoma *et al.*, 2012; Groger and Ramharter, 2018). Unfortunately, no *ex vivo* drug assay with LUM on *P. malariae* was yet published. Hence, it can be concluded that LUM needs to be tested again *ex vivo* on *P. malariae* in Lambaréné. A test on *P. falciparum* should be conducted as a control. It is advisable to prepare the stock solution in a 15 ml falcon tube to be able to use as much DMSO as needed to completely dissolve LUM.

6.3.1 Limitations of the drug assay

An important limitation is the small number of tests that were conducted. To draw a definite conclusion on the *in vitro* drug sensitivity of *P. malariae* in Gabon, more isolates need to be tested. For statistical significance, it is advisable to perform three assays on each isolate. Inter-individual differences between each test set-up can occur. Pipetting mistakes can be involved in the preparation of the drug solutions and drug plates. This could lead to an unintended deviation in drug concentrations and measured inhibition of schizont maturation. In this study, only one drug assay per isolate was performed due to the difficulty in obtaining *P. malariae* infected blood and the intense labour involved in evaluating drug tests by the WHO method.

Additionally, a reference strain for use as a positive control was absent. *P. falciparum* strains like 3D7 or W2 are known to be sensitive to all drugs that were applied in this study. Reference strains adapt quickly to culture conditions and are easy to maintain. When performing tests on fully sensitive parasites it is possible to monitor whether the stock solutions were prepared properly and whether the drugs show the same effect in repeated tests (Rutvisuttinunt *et al.*, 2012).

After the blood was drawn, the samples were immediately transferred into an incubator set at 37°C, and subsequently used for the experiments. However, it is likely that there are time variations of isolate exposure to the drug plate. Some

samples were processed faster than others. The initial parasitic stage of *P. malariae* in the collected isolates varied between schizonts and trophozoites. The parasites were not synchronized but applied directly to the drug wells. According to Wilson *et al.*, the drug sensitivity of *Plasmodium* depends on the stage of the cell cycle (Wilson *et al.*, 2013). Therefore, the drug response of the parasite isolates may vary depending on the length of time they are exposed to a drug and on the parasite's respective stage in the parasite cell cycle at that time.
Besides, Borrmann *et al.* found that *in vitro* drug assays can show parasite sensitivity, while clinical studies reveal a high level of resistance (Borrmann *et al.*, 2011). This inevitably means that *in vivo* studies must be conducted for a valid statement on parasite sensitivity to be made. *In vitro* studies are a good initial approach, but *in vivo* trials help guarantee that the parasite strains are actually susceptible to the respective drug compound in humans.

6.4 Clinical implications and future directives

This study was the first *ex vivo* approach in Gabon that intended to reveal the drug sensitivity of *P. malariae* to CQ, AS, ATQ and LUM. As the results did not reveal an unexpected drug resistance, it is reasonable to continue indicating CQ, AS and ATQ in the treatment of *P. malariae* infections in Gabon.
Apart from this initial conclusion, the main message of this dissertation is a call for more *ex vivo* and *in vitro* studies on a neglected parasite. Due to various limitations, the results presented here cannot be applied to all *P. malariae* strains in Gabon. Besides, the question of why LUM did not yield any effect on the schizont maturation remains unclear and requires further investigation. In this study, only single drug compounds were tested for their effectiveness against *P. malariae*. In order to comply with current guidelines for malaria treatment, it would be more appropriate to test antimalarial multidrug formulations. ACTs are the most commonly used drug combinations against non-*falciparum* malaria worldwide and should be tested in Gabon on *P. malariae* strains.
The most important basis for obtaining high-value datasets on drug susceptibility would be the implementation of a long-term *in vitro P. malariae* culture. This tool

will allow for standardized chemo-susceptibility profiling. Drug testing will be more reproducible if identical working conditions and the same parasite strain can be used. The time-consuming search for fresh *P. malariae* isolates would become redundant. At the present moment, a long-term *in vitro* culture of *P. malariae* has yet to be obtained. Greater scientific effort is needed in order to better understand the metabolic characteristics and needs of this pathogen.

7. Summary

7.1 English

Background: Malaria is an infectious disease that still has a major impact on public health. In order to eliminate the disease worldwide, the neglected parasite *P. malariae*, considered to be benign, needs to be included in malaria eradication programs. As *P. malariae* is the most prevalent non-*falciparum* pathogen in Gabon, it is worthwhile to gain more knowledge about its drug responsiveness. To the best of our knowledge, no *ex vivo* drug assay was previously conducted on *P. malariae* in Gabon.

Methods: A cross-sectional study monitoring *P. malariae* prevalence was carried out by the CoMal team in Lambaréné and surroundings. Patients who tested positive for *P. malariae* were asked to donate blood after giving their informed consent. The blood samples were used in attempts to establish an *in vitro P. malariae* culture, as well as for an *ex vivo* drug assay. The WHO schizont maturation test was applied to evaluate the inhibitory effect of chloroquine, artesunate, atovaquone and lumefantrine on *P. malariae* blood stages. The analysis was based on microscopy. In order to obtain a definite species identification, PCR analysis was carried out in Tübingen.

Results: 555 participants were recruited between January and April 2020. In total, ten experiments on *P. malariae* isolates were carried out. Seven interpretable drug assays were obtained. The geometric mean of the IC_{50}-values for CQ, AS and ATQ were 10 nM, 3 nM, and 3 nM, respectively. LUM did not show any inhibitory effect on the parasite maturation. It was possible to maintain a *P. malariae* culture for four cell cycles. During this period, it was observed that merozoites reinvaded new erythrocytes and formed *P. malariae* ring stages.

Conclusion: The IC_{50}-values obtained for CQ, AS and ATQ give reason to state that these drug compounds are applicable for *P. malariae* treatment. The fact that living *P. malariae* parasites were still observed after four cell cycles under *in vitro*

culture conditions gives sufficient reason to believe that it is possible to cultivate *P. malariae in vitro*. However, due to the low number of interpretable results obtained and the failure to grow a long-term *P. malariae* culture, conducting more *ex vivo* or *in vitro* studies on the pathogen is strongly recommended. In summary, this study contributed to a better understanding of the biology and drug sensitivity of the scientifically neglected malaria pathogen *P. malariae*.

Anton Heinrich Wilhelm Hoffmann Prof. Dr. med. Steffen Borrmann

7.2 Deutsch

Hintergrund: Malaria ist eine Infektionskrankheit, die weltweit nach wie vor für über 200.000 Todesfälle sorgt. Um die Krankheit global auszulöschen, muss auch der Parasit *P. malariae*, der als gutartig gilt, bekämpft werden. Da *P. malariae* der am weitesten verbreitete nicht-*falciparum* Erreger in Gabun ist, lohnt es sich, mehr über seine Sensibilität gegenüber gängigen Malariamedikamenten zu erfahren. Laut der derzeitigen Literatur wurde in Gabun bisher kein Arzneimitteltest über *P. malariae* durchgeführt.

Methoden: Im Rahmen der CoMal-Studie wurden Menschen in Lambaréné und Umgebung auf Malaria untersucht. Patienten, die positiv auf *P. malariae* getestet wurden, wurden nach ihrer Einwilligung darum gebeten, Blut zu spenden. Die Blutproben wurden für den Versuch der Etablierung einer *in vitro*-Kultur von *P. malariae* sowie für die Durchführung eines *ex vivo*-Arzneimitteltests verwendet. Der Schizonten-Reifetest der WHO wurde benutzt, um die hemmende Wirkung von Chloroquin, Artesunat, Atovaquon und Lumefantrin auf die intra-erythrozytären Stadien von *P. malariae* zu untersuchen. Alle Objektträger wurden mikroskopisch ausgewertet. Um eine eindeutige Identifizierung der Spezies zu gewährleisten, wurde in Tübingen eine PCR-Analyse durchgeführt.

Ergebnisse: 555 Teilnehmer wurden zwischen Januar und April 2020 untersucht. Insgesamt wurden zehn Experimente mit Isolaten von *P. malariae* durchgeführt. Sieben interpretierbare Ergebnisse von Arzneimitteltests konnten erzielt werden. Die berechneten IC_{50}-Werte von CQ, AS und ATQ betrugen 10 nM, 3 nM und 3 nM. LUM zeigte keine hemmende Wirkung auf die Parasitenreifung. Es gelang, eine *P. malariae*-Kultur für vier Zellzyklen aufrechtzuerhalten. Während dieser Zeit wurde beobachtet, wie Merozoiten neue Erythrozyten infizierten und *P. malariae*-Ringstadien bildeten.

Schlussfolgerung: Die in den Experimenten gemessenen IC_{50}-Werte geben Anlass zu der Feststellung, dass die Substanzen CQ, AS und ATQ zur

Behandlung einer *P. malariae*-Infektionen angewendet werden können. Die Tatsache, dass es gelungen ist, lebendige *P. malariae* Parasiten noch nach der Vollendung von vier Zellzyklen in der Zellkultur zu beobachten, gibt Grund zu der Annahme, dass es durchaus möglich ist, *P. malariae* zu kultivieren. Aufgrund der geringen Anzahl interpretierbarer Ergebnisse und des Versäumnisses, eine langfristige *P. malariae*-Kultur zu etablieren, empfiehlt es sich allerdings dringlich, mehr *ex vivo*- beziehungsweise *in vitro*-Studien über den Erreger durchzuführen. Zusammenfassend ist zu sagen, dass diese Studie zum besseren Verständnis der Biologie und Pharmakotherapie des wissenschaftlich vernachlässigten Malariaerregers *P. malariae* beigetragen hat.

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9. Erklärung zum Eigenanteil

Ich erkläre, dass ich die der medizinischen Fakultät der Eberhard Karls Universität Tübingen zur Promotion eingereichte Dissertation mit dem Titel "*Ex vivo* drug sensitivity assay on *Plasmodium malariae* in Lambaréné" im Centre de Recherches Médicales de Lambaréné und am Tropeninstitut Tübingen mit Unterstützung der Arbeitsgruppe um Prof. Dr. med. Steffen Borrmann und Prof. Dr. med. Dr. rer. nat. Ayola Akim Adegnika ohne sonstige Hilfe durchgeführt und bei der Abfassung der Dissertation keine anderen als die aufgeführten Hilfsmittel benutzt habe.

Die Studie wurde mit Hilfe von Frau Dr. Yudi Tatiana Röckl-Pinilla konzipiert. Die Arbeit wurde von Herrn Prof. Dr. med. Steffen Borrmann und Herrn Prof. Dr. med. Dr. rer. nat. Ayola Akim Adegnika betreut. Die Zellkulturen, die drug assays und die anschließende Auswertung wurde durch mich, Anton Heinrich Wilhelm Hoffmann, durchgeführt. Die Daten der CoMal Studie wurden durch das Studienteam in Lambaréné, Gabun gesammelt. Die Literaturrecherche sowie die statistische Analyse erfolgten ausschließlich durch mich.

Bei der Abfassung der Dissertation sind Rechte Dritter nicht verletzt worden. Ich habe diese Dissertation bisher an keiner in- oder ausländischen Hochschule zur Promotion eingereicht.

Freiburg, 03. März 2021

Anton Heinrich Wilhelm Hoffmann

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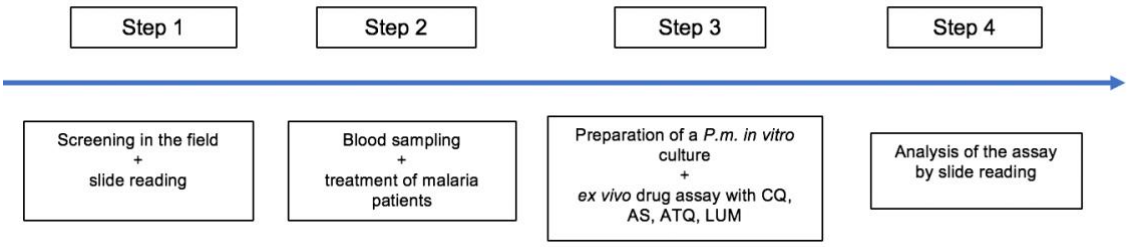
Appendices

Appendix 1

Flowchart of all study activities.



Appendix 2

Criteria for inclusion:

- Participants had to be at least three years old for blood to be collected.
- Having lived in the study area at least two weeks before the survey.
- Provision of informed consent from a parent or legal guardian.

Criteria for exclusion:

- Refusal to provide informed consent.
- Testing negative for *P. malariae.*
- Anaemia with haemoglobin level less than 6 g/dl.