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List of abbreviations

ACT	Artemisin-based combination therapy
AD	Anno Domini
AS	Artesunate
ATQ	Atovaquone
BC	Before Christ
BMM	Blood medium mixture
ca.	circa
CCM	Complete culture medium
CERMEL	Centre de Recherches Médicales en Lambaréné
CoMal	Plasmodium species co-infections in Anopheles mosquitoes: A pilot study of parasite-vector interactions that define transmission in Africa
COVID-19	Coronavirus disease 2019
CQ	Chloroquine
dcFCS	Decompartmentalized foetal calf serum
DDT	Dichlorodiphenyltrichloroethane
DMSO	Dimethylsulfoxide
DSS	Drug stock solution
EBA	Erythrocyte-binding antigen
ELISA	enzyme linked immunosorbent assay

et al.	et alii/et aliae
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
HRP-2	histidine-rich protein-2
IRS	Indoor residual spraying
ITN	Insecticide-treated mosquito net
LUM	Lumefantrine
mAb	Monoclonal antibody
PBS	Phophate buffered saline
PCR	Polymerase chain reaction
Pf	Plasmodium falciparum
pLDH	Pan-malaria lactate dehydrogenase
Pm	Plasmodium malariae
Poc	Plasmodium ovale curtisi
Pow	Plasmodium ovale wallikeri
PS	Phosphatidylserine
Pv	Plasmodium vivax
RBC	Red blood cell
SARS-CoV-2	Severe acute respiratory syndrome coronavirus type 2

SIRPα	Signal regulatory protein α
WHO	World Health Organisation
WS	Working solution

1. Introduction

The following study was carried out between October 2021 and September 2022 in the CERMEL institute of Lambaréné, Gabon.

1.1 The global impact of malaria

While rarely an issue of public discourse in the western world, malaria remains one of the most widespread diseases afflicting mankind. It is caused by single-celled parasites of *Plasmodium spp.,* which are mainly transmitted through mosquitoes of the *Anophelinae* genus. *Plasmodium spp.* is a rather large genus, with at least six different species known to be pathologic to humans. These are – in order of prevalence– *P. falciparum, P. vivax, P. malariae, P. ovale curtisi*, *P. ovale wallikeri* and *P. knowlesi*. In the sub-Saharan country of Gabon, *P. malariae* is the second most common species, with *Plasmodium knowlesi* not having been observed yet. (Woldearegai et al, 2019).

Malaria is currently endemic to 85 countries worldwide, especially in sub-Saharan Africa. In 2022, about 94% of all globally reported cases occurred in the WHO African Region. The disease is especially prevalent in the populous countries of Nigeria, Uganda, and the Democratic Republic of Congo; those three countries alone accounting for over half of the total amount of cases on the continent. In second place, at a mere 3.3% of cases, stands the WHO Eastern Mediterranean Region, with the countries of Yemen, Sudan and Somalia being most afflicted. The WHO South-East Asia Region, formerly second-place in regards of reported malaria cases, recently experienced a decline in cases despite the ongoing pandemic, now only accounting for about 2% of all cases worldwide. 66% of these were coming from India alone (WHO, 2023).

An estimated amount of 241 million people contracted malaria in 2020, of which over 627,000 died. Of those, 77% were below the age of five. It is assumed that the disruptions of public health brought about by the SARS-CoV-2 pandemic were attributable to – if not directly caused – about 47,000 otherwise preventable deaths. This may be the reason for the rise in yearly malaria deaths in 2020, contrary to the plateauing of the years before (WHO, 2021). In 2022, the number

of cases rose to an estimated 249 million, however, fortuitously, the WHO reported a decrease in mortality to 608,000 fatalities in the same year (WHO, 2023).

Despite these numbers, progress toward eradication is slow. The underlying reasons are manyfold – economic, political, environmental, or simply stemming from a lack of knowledge of the pathogen. At least in regards to the latter, this work hopes to help the global effort against malaria.

1.1.1 A brief history of malaria

Mankind's firs forays into the medical arts likely had the same motivations as the more sophisticated methods employed today – to ease their fellow's sufferings and heal them from any sicknesses that ailed them. As such, it can be reasonably assumed that man's battle with malaria is one of the most enduring in our history.

Plasmodium spp. is a very old genus of parasites, with first members of the species having been found enclosed in amber in 1993, dating back about 30 million years (Poinar, 2005). Assumedly, it co-evolved alongside non-human primates, most likely gorillas (and their predecessors), as *P. falciparum* is assumed to have emerged from precisely these primates about 10,000 years ago.

By the times of Ancient Greece, malaria, then called ελονοσία (elonosía), 'disease of the marsh', was prevalent in most parts of the human populated world (Boualam et al., 2021). The current name of malaria is derived from the Italian 'mal'aria', meaning bad air. However, this name has only found widespread use after Horace Walpole described a recurring plague in the region of Rome, the symptoms of which were described congruent with those of (albeit diverse) forms of infections with *Plasmodium spp.* (Hempelmann & Krafts, 2013).

At the latest by the 18th century, malaria was prevalent in all of Europe, some cases even being reported as far north as Finland (Neghina *et al.,* 2010). Only after the identification of the *Plasmodium spp.* as the culprit behind malaria could the superstitious theories on the disease's origin be dispelled; a feat achieved by

French military surgeon Alphonse Laveran in 1880 (Neghina *et al.,* 2010). Interestingly, he himself seemed to object to the term 'malaria', preferring 'paludisme', referring to its ancient associations with swamps and marshes (Hempelmann & Krafts, 2013). This still remains the common way to refer to the disease in francophone parts of the world.

Today, Italy is one of the most popular tourist destinations in all of Europe. However, this is only a relatively recent development. Especially the plains surrounding Rome have been a hotbed of the fittingly named 'Roman Fevers' for the longest time, a major hurdle to the tourism industry at the time. This state of affairs changed when serious eradication efforts began around the Second World War, beginning with the draining of the Pontine marshes by order of then-dictator Benito Mussolini in 1928. By 1955, after rigorous insecticide use, especially the insecticide DDT, to combat the vector species and widespread oral application of the then newly discovered quinine against the parasites themselves, Italy was deemed free of malaria at last. However, several species of *Anopheles spp.* have been observed to thrive in the climates of Corsica, raising fears of a re-emergence of the once-eradicated 'marsh fevers' (Boualam *et al.,* 2021).

Briefly, before the discovery of modern antibiotics and faced with a lack of treatment alternatives, *Plasmodium spp.,* especially *Plasmodium vivax*, enjoyed a brief career as quasi-drugs in the treatment of general paresis – a typical manifestation of neurosyphilis – in the field of psychiatry. This form of therapy, commonly known as 'malariotherapy', was first properly described by Dr. Wagner-Jauregg in 1922, later earning him a Nobel prize in human medicine in 1927. Due to fears of inducing endemic infections beyond the hospitals themselves, strict vector control rules and guidelines were created. This somewhat limited the spread of the practice (Nevin & Croft, 2016). Observed results were initially very promising, however, with the subsequent emergence of alternative, non-infectious treatments, malariotherapy was gradually faded out of use (Snounou & Pérignon, 2013). Malariotherapy has since then been discussed as a potential option in the treatment of Lyme disease (Heimlich 1990) and more so HIV (Heimlich *et al.,* 1997; Chen *et al.,* 2003). However, it has not found its way back into guidelines and treatment recommendations.

1.1.2 Existing malaria control strategies and treatment options

Methods to combat malaria have been made for a long time, probably for as long as the 'marsh-fever' was known.

1.1.2.1 Vector control

Even before treating infected individuals, it is of great importance – and, importantly, far more cost-effective – to prevent the parasite's transmission to begin with.

One of such measures is the removal of adequate breeding grounds for mosquitoes, commonly achieved by drainage or weeding. The earliest projects of eradication and disease prevention mainly focussed on the (usually large-scale) attempts at removing the believed cause, marshes, and other wetlands (Boualam *et al.,* 2021). While having achieved great successes, such as the previously mentioned draining of the Italian marshes during the 1930s, in areas where lasting drainage is not feasible, these methods have been proven too costly in manpower and time and consequently been discontinued by most sub-Saharan countries. Similarly, the use of larvicides and insecticides to combat mosquito larval growth have seen lesser use amongst increasing resistances and overreaching environmental impact (Savi, 2022).

An alternative is the use of insecticide-treated bed nets (ITNs) – widely provided to the civilian populace as part of both national and international malaria intervention efforts – as well as indoor residual spraying (IRS). For both, the main chemical used are called pyrethroid, a group of insecticides known to be harmless to mammals and long-lasting in their effects. ITNs especially have been associated with marked decreases in malaria transmissions, as they provide a chemical barrier on top of the mechanical one. For example, between the years 2005 and 2010, Ghana saw a decrease of malaria cases of up to 41%, which was largely attributed to the widespread distribution of ITNs to local households (Savi, 2022).

Indoor residual spraying itself has proven efficacious in eradication efforts, however, in recent years, its value has declined. As of 2022, only 47 countries implement IRS. Even then, only a mere 1.8% of the at-risk population are considered protected thereby; a further decline from the rather paltry 5.5% in 2010 (WHO, 2023). This may have been caused by *Anopheles spp.* increasing resistance to the utilised insecticides.

Drug resistances do not only emerge among the different strains of *Plasmodium spp.* The disease's vector has also shown increasing resistance to common insecticides and larvicides such as DDT and synthetic pyrethroids. In fact, in the WHO African and Eastern Mediterranean Regions, over 80% of observed mosquito population demonstrated resistance against pyrethroids and over 50% against organophosphates. In large parts of sub-Sahara Africa mosquito populations have been observed to exhibit resistance against all four commonly used classes of insecticides (pyrethroids, organophosphates, organochlorines & carbamates), further stymying attempts at eradication (WHO, 2023).

1.1.2.2 Commonly used antimalarial agents

Throughout history, many different drugs have been administered to treat malaria, with varying amounts of success. Herbal therapeutics, for instance, which have a long history and are at least as old as the field of medicine itself, were some of the first remedies used against malaria. Ancient Chinese cultures are known to have used certain plants as cures; the sources for both quinine (and thusly chloroquine) and artemisinin being found therein (Mohammadi *et al.,* 2020). Due to the low cost of such therapies, recently, there have been discussion on the topic of using herbal infusions as stop-gaps or even full regimens for the treatment of infected patients. The main advantage of herbal remedies can be found in its simplicity; the local cooperation and readiness to accept therapy might increase due to the 'traditional' and 'well-known' nature of the substances administered. Studies to estimate the relative efficacy have thus far proven inconclusive. In animal models, *Artemisia annua* based teas have been shown to be ineffective in the suppression of malaria (Atemnkeng *et al.,* 2019), whereas

whole plant therapy (as in: ingestion of dried leaves) has been claimed to adequately lower parasitaemia (Elfwal *et al.,* 2015). A more recent study thereof from 2020 by Munyangi *et al.* had to be retracted due to doubts regarding the veracity of the data published. Regardless, the significance of herbal cures for malaria ought not be underestimated.

Current treatment guidelines recommend the use of various drugs in the treatment of malaria, both as mono-therapy and in tandem with other compounds. While it would be impossible to list and explain all, some of the four most common synthesised drugs (artemisinin, lumefantrine, chloroquine, and atovaquone) used in antimalarial treatment – which were consequently chosen for the ex vivo drug testing for this work – are to be elucidated on in the following passages.

The currently most frequently used drug for the treatment of *falciparum*-malaria – artemisinin – was extracted from the sweet wormwood plant (*Artemisia annua*) in 1971 as part of a Chinese governmental effort to cure malaria. It (as well as its derivatives) is of further importance to medical science because of its antitumor, anti-infectious, anti-inflammatory, anti-angiogenesis, and possible immuno-modulatory activity (Liu *et al.,* 2020). Despite its widespread use and longtime service as a first-line drug, its precise mode of action is currently not fully understood. Among the possible modes of action, it is widely assumed that artemisinin and its derivatives act as pro-drugs, which release free radicals interfering with the parasite's iron metabolism (Klonis *et al.*, 2013). Lumefantrine was discovered in a similar manner, being introduced during the Vietnam War by the Chinese military, and is today used in tandem with artemisinin, giving rise to the so-called artemisinin-based combination therapies (ACTs). While, similarly to artemisinin, its precise mode of action is at the moment poorly understood, a direct schizonticidal effect could be observed. Moreover, in *P. vivax* and *P. malariae*, an additional gametocidal effect has been documented (Achan *et al.*, 2011; White *et al.*, 1999). Other therapeutics, such as pyrimethamine and sulfadoxine, as well as mefloquine, are commonly paired with artemisinin in order to more successfully cure malaria (Mohammadi *et al.,* 2020). ACTs have been recommended as first-line therapy by the WHO since 2006. While instrumental in the regress of both malaria cases and deaths from 2007 onwards, resistance

to the compound has begun to spread to Africa after initial mutations in South East Asia (WHO, 2021).

Along with artemisinin, the WHO has recommended some other drugs as first- or second-line therapeutics. Among them chloroquine, one of the oldest and longest-serving antimalarial drugs, which rose to fame in the 1930's as a proclaimed miracle cure for malaria. It was the first compound used by the WHO in its so-called Global Malaria Eradication Program of the 1950's, which failed shortly after due to increasing levels of resistance among common malaria strains (Elfawal *et al.,* 2015). Therefore, its main indication in recent years has been the treatment of uncomplicated *Plasmodium vivax* malaria. Moreover, because of the varying severity of artemisinin-based compounds' teratogenic side-effects, in pregnant women, the use of either quinine or the lincosamide clindamycin is recommended (Mohammadi *et al.,* 2020). Chloroquine exhibits its antiparasitic effect by means of inhibition of hemoglobin proteolysis in the parasite's digestive vacuole. In healthy *Plasmodium spp.*, this process would result in the formation of hemozoin, also known as malaria pigment. By stopping this crystallisation, however, toxic heme accumulates and leads to the lysis of both parasite and infected erythrocyte (Sullivan *et al.*, 1996).

Among the newer drugs administered to combat the spread of malaria is the ubiquinol analogue atovaquone, one of the main agents (alongside the pro-drug proguanil-hydrochloride) in the drug Malarone® – most often used as malaria prevention for travels to endemic regions. It specifically targets a parasite's mitochondrial electron transport chain – the cytochrome bc_1 complex – foregoing the mammalian equivalent. As such, atovaquone has been used since 1999, initially both as an antimalarial and as a fungicide in the treatment of *Pneumocystis jirovecii* pneumonia (Carter-Timofte *et al.,* 2021). Since, it has also been used to treat other parasites, such as babesiosis and toxoplasmosis (Verdaguer *et al.,* 2021). Interestingly, atovaquone has also been discussed as a potential antiviral agent, not least in the possible treatment of SARS-CoV-2 (Carter-Timofte *et al.,* 2021). However, it has been shown to be inadequate as a mono-therapeutical drug – as in; without its common partner compound proguanil-hydrochloride – with recrudescence and resistance being regularly

reported. Additionally, compared to ACTs, its high cost begets inaccessibility in most endemic regions (Verdaguer *et al.,* 2021).

1.1.2.3 Vaccine programmes

There are historic reports of vaccinations having been conducted as far back as the 19th century, oddly enough making use of an inoculation intended to combat *Salmonella typhi*. However, it is likely that these treatments ‘merely’ boosted natural immunity in non-naïve patients, as effects were only documented in those already having suffered from marsh fevers (Shanks, 2019).

More modern attempts at vaccination have been made throughout the last decades, with no lasting agent having yet been found. Various projects making use of either attenuated sporozoites, purified immunoglobulins or the most promising candidate of recent years, RTS,S/AS01E, attempt to induce long-term immunity against the disease. RTS,S/AS01E itself consists of a recombinant *P. falciparum* circumsporozoite protein conjugated to a hepatitis B surface antigen, thereby inducing immunisation against the pre-erythrocytic stages of *Plasmodium falciparum* (Frimpong *et al.*, 2018). Regardless of this novel and altogether modern approach, in clinical trials, the vaccine only showed an efficacy (after 4 doses) of up to 43.9% in patients aged 5-17 months and merely 27.8% in children 6-12 weeks old. The efficacy further waned with time and has yet to fulfil the requirements set by the WHO. Nevertheless, many other possible vaccine targets are currently undergoing clinical trials (Frimpong *et al.*, 2018).

1.1.3 Challenges to eradication

Financial restrictions, scientific challenges and sometimes politics, as well as the changing climate impact mankind’s attempts at curbing the disease adversely. *Plasmodium spp.* itself has been repeatedly shown to evolve and develop mechanisms of resistance – both in and ex vivo.

1.1.3.1 Drug resistance in Plasmodium spp.

Similarly to many other pathogens, unrestricted and uncontrolled application of antimalarials has led to numerous emerging resistances among the various different strains of malaria – going as far back as the first large scale treatment programmes back in the 1950's (Elfawal *et al.,* 2015). Chloroquine, being the first antimalarial drug to find widespread use, was also the first to face emerging resistance in circulating malaria strains (Elfawal *et al.*, 2015). The first reported case of chloroquine resistance was reported in 1961 in South America by Moore and Lanier (1961), with first the first resistant *P. falciparum* strains in Africa being found in 1979 (Fogh, Jepsen and Effersøe, 1979). While being relegated to treatment of non-*falciparum* species due to the progressive decrease in effectiveness, reports of resistance against CQ in *P. vivax* has been observed since the 1980's (Rieckmann, Davis and Hutton, 1989). While – at the time of writing – only six papers published data on drug susceptibility in *P. malariae*, there were reports of diminished chloroquine susceptibility in isolates from Indonesia (Maguire *et al.*, 2002). Consequently, the drug has been relegated to being of limited importance in the current struggle against malaria.

Lumefantrine – in combination with Artemether – is part of the single most common contemporary ACT combination in use in Africa. Despite this commonality, artemether-lumefantrine has shown remarkable effectiveness in the treatment of *P. falciparum*. However, the infamous and – unfortunately – ever more frequent Kelch 13 mutation has also been linked to decreased efficacy. Interestingly, this very mutation was first observed in the south-east-Asian country of Cambodia. The first reports of reduced susceptibility to first chloroquine and then artemisinin have been observed in Cambodia as well (Ariey *et al.,* 2014). As of 2022, more than 150 different therapeutic efficacy studies utilising artemether-lumefantrine have reported treatment failures in *P. falciparum* strains, suggesting an increasingly worrying degree of resistance to the drugs (WHO, 2023). Studies by Dembele *et al.* (2021) showed frequent resistance to the drug in *P. malariae* as well.

Artesunate, alongside artemether, is currently one of the most commonly used antimalarial drug worldwide and a regular compound of ACTs, usually paired with mefloquine or sulfadoxine-pyrimethamine (WHO, 2023). Due to the frequency of application, various resistant strains have been reported worldwide; especially in western Cambodia (of the WHO South East Asian Region), with first molecular markers being found in 2002 (Ariey *et al.*, 2014). However (as of 2022), in comparison to the more commonly utilised artemether-lumefantrine, fewer studies have reported partial resistance to the different artesunate combination drugs as compared to artemether and its combinations (WHO, 2023).

Atovaquone, being a part of the popular drug Malarone®, finds widespread use both as a first-line therapeutic in non-endemic countries, as well as prophylaxis for travellers to endemic regions. Due to its widespread application and commonality after rising resistances to common antimalarials like artesunate and chloroquine, there have been increasing reports of reduced susceptibility in *P. falciparum* strains, especially in single agent uses (Staines *et al.*, 2017). In *P. vivax*, it unfortunately exhibits a reduced clinical cure rate, as the species' dormant hypnozoites are not affected (Staines *et al.,* 2017). In regards to *P. malariae*, there are conflicting reports, some reporting resistances (Müller-Stöver *et al.*, 2008), with others reporting widespread susceptibility to the drug (Pradines *et al.,* 2010).

1.1.3.2 The economics of malaria

While it could be successfully eradicated from Europe – the WHO European Region being officially malaria-free since 2015 (WHO, 2021) – malaria still remains a major roadblock to many countries belonging to what was formerly called the Third World. Gallop and Sachs stated in 2001 that malaria-endemic countries possessed a 70% lower per-capita GDP, with a 10% decrease to malaria exposure resulting in up to 0.26 percentage points of increase to the annual per capita growth rates. More recent analyses by Sarma *et al.* in 2019 calculated this growth increase to a similar value at 0.27 percentage points. However, in regions of sub-Saharan Africa the growth increase per decrease in

malaria exposure was estimated to be as high as 0.5 percentage points, with an assumed 15.9% growth to product purchasing power in case of malaria extermination.

Still, the impact of malaria is far from limited to the financial –its greatest burden remains its human cost. Both are closely linked; by the existing programmes alone, an estimated 2.1 billion cases and 11.7 million deaths could have been prevented between 2000 and 2022, as, the WHO's estimates, upwards of US$ 7.8 billion yearly would have been necessary to adequately limit malaria transmission and treat infected individuals by 2022. Unfortunately, only US$ 4.1 billion could be used to fund the organization's programmes, a deficit that has been increasing throughout the years, with actual funding falling way short of the targeted sum. The funding gap was calculated at 'only' US$ 2.3 billion in 2018, as opposed of the US$ 3.7 billion of 2022. Of the US$ 4.1 billion raised for the fight against malaria, about US$ 603 million were spent on malaria research. Throughout the last decade, 69% of the total funding was provided by the international community, especially the United States of America, the United Kingdom and France. Recent political changes of the United States might have a deteriorating effect on those efforts in future years. Their contributions amounted to about 48% of the total funding in 2022, with 34% being provided by malaria-endemic countries (WHO, 2023).

During the SARS-CoV-2 epidemic, already existing programmes were unfortunately experiencing major disruptions. Especially the delaying of vital deliveries of insecticide-treated mosquito nets and anti-malarial drugs to regions with low health infrastructure has had deleterious effects on local malaria eradication efforts. As such, most participating countries of the WHO were unable to fulfil the target quota of delivery in 2020 (WHO, 2021). This situation has shown to be slow to alleviate (WHO, 2023).

Still, many countries all over the world report successes in their struggles against the disease. However, countries with poor public health systems and low standards of living are struggling to follow suit – some simply being unable to

shoulder the financial burden of fighting a disease as enduring as malaria (WHO, 2023).

1.2 The Plasmodium malariae parasite

Plasmodium malariae is a rather unappreciated member of *Plasmodium spp* compared to other members of *Plasmodium spp.* Studies regarding this species of parasite remain limited as even the WHO World malaria report (WHO, 2023) rather mentions *P. knowlesi* as a threat to public health and continued eradication efforts as opposed to the far more common *P. malariae*. In fact, *P. malariae* is only mentioned as a prerequisite for achieving the certification of malaria eradication in a respective country. No further mentions of this species of parasite take place. The reasons thereof are many and might be explained in the following passages.

1.2.1 Biology of *P. malariae*

Plasmodium malariae, like any other member of *Plasmodium spp.* pathologic to humans, is a vector-borne pathogen. As such, just like other members of the genus, it possesses both an intra-vector and intra-host developmental cycle. In the mosquito, the mature gametocytes ingested during the blood meal produce sexual gametes, which start the process of fertilization. The creation of these sexual gametes is called 'exflagellation' in male gametes and 'activation' in females. The fertilized zygote differentiates into a motile ookinete, which is able to invade and cross the mosquito's midgut wall. There, surviving ookinetes differentiate into oocysts, which in turn create the next generation of sporozoites. These migrate to the mosquito's salivary glands to restart the process of infection (Delves *et al.,* 2012).

During an infected mosquito's bite, only up to 100 infectious sporozoites can be transmitted (Collins & Jeffery, 2007; Delves *et al.,* 2012). These then travel to the host's liver, infecting hepatocytes. Therein, they divide and create so-called merozoites, far smaller and more numerous forms of the parasite, able to infect

red blood cells. The merozoites, tens of thousands of which are released per infected hepatocyte, infect RBCs, transforming into ring stages – named after their distinctive shape seen with light microscopy. Inside the infected erythrocyte, the merozoites matures into a trophozoites, and develops into an asexual schizont. The schizont produces numerous new merozoites, releasing them in order to restart the erythrocytic lifecycle. Some – if few – of the merozoites develop into sexual gametocytes, which in turn are ingested by a feeding mosquito, enabling the spread to other hosts. Most of the merozoites, however, restart the erythrocytic cycle by infecting RBCs and maturing into schizonts (Collins & Jeffery, 2007).

There are some notable differences between *P. malariae* and other species of *Plasmodium*. For instance, the pre-erythrocytic stage lasts for approximately 15 days in *P. malariae* (Collins & Jeffery, 2007), whereas *P. falciparum* merely requires 2-10 days (Cowman *et al.* 2016) for the infection to develop. The intraerythrocytic cycle lasts about 48 hours in *P. falciparum and P.* vivax. In *P. malariae*, this cycle takes about 72 hours (Collins & Jeffery, 2007). Even after fully matured schizonts rupture, *P. malariae* releases only between 6 and 14 merozoites (Collins & Jeffery, 2007), far fewer than the 16-32 merozoites (Cowman *et al.,* 2016) observed in *P. falciparum* infections. These factors are speculated to lead to a more adequate host immune response, resulting in lower maximum parasitaemia and, consequently, in lesser symptoms of infection and a better predicted clinical outcome (Collins & Jeffery, 2007).

1.2.1.1 Microscopic identifiers of P. malariae

While definite diagnosis and differentiation of the different species of *Plasmodium* is by no means easy and requires a certain degree of expertise and careful observation, there are some definite differences between the different parasites that can be seen under the microscope.

In general, *P. malariae* can most often be identified by its characteristic yellow pigment, most impressively seen in its trophozoite and schizont stages. In contrast to the more common species, *P. falciparum* and *P. vivax*, infected red

blood cells usually do not appear enlarged or distorted. Rings are seen far less frequently than in other species of *Plasmodium*, and are usually thicker, denser and smaller than those of *P. falciparum*, for example. Ring stages of *P. malariae* always possess but one nucleus, seen as a characteristic red dot under the microscope (WHO, 2010).

Trophozoites contain 30-50 black granules, contrasting against the previously mentioned yellow pigment. Typical for *P. malariae* is the formation of a band-like shape in the process of trophozoite growth. Its trophozoite stages are both the most common and, likely, most easily identifiable stages of this malaria species in regards to light microscopy (WHO, 2010).

Schizonts can be identified by their appearance as a solid mass of merozoites filling the entirety of the infected cell. While *P. malariae* schizonts contain – as previously mentioned – between 6 and 14 merozoites, their number averages around 8. The typical yellow pigment is present at this stage as well, usually being found around the centre of the infected erythrocyte (WHO, 2010).

The sexual stages of *P. malariae*, its gametocytes, are usually of a round shape; distinctly different to the oblique ones of *P. falciparum.* They contain a single enlarged, deeply red nucleus surrounded by dense blueish-purple cytoplasm and permeated with yellow pigment (WHO, 2010). While both forms can be found, microgametocytes appear to be more common than macrogametocytes (Collins & Jeffery, 2007; WHO, 2010).

1.2.1.2 PCR detection of P. malariae

While microscopy remains the cheapest, most easily accessible, and thus, most common way for malaria diagnosis, its sensitivity heavily relies on both the quality of the slide and the experience of its reader. Even with a highly competent analyst, however, the minimum parasitaemia to be reliably diagnoseable by light microscopy has been stipulated to lay around 50 parasites/µl under field conditions for *P. falciparum* (Moody, 2002; Gimenez *et al.,* 2021), with an estimated 200 parasites/µl for non-*falciparum* species (Gimenez *et al.,* 2021). Therefore, it may be found lacking.

More accurate and sensitive is the diagnosis by means of polymerase chain reaction (PCR). Thanks to the amplification of parasite DNA, modern PCR is capable of detecting parasitaemia as low as 0.2-5 parasites/µl for all species of *Plasmodium*. As such, it remains the gold standard for diagnosis of sub-microscopic infections, which, due to their asymptomatic nature, would – as the name might suggest – go unnoticed if solely investigated by microscope, possibly continuing the cycle of infection and reinfection with the pathogen. However, this standard PCR remains basically a purely lab-based method of assessment, as its high cost and requirement for well-trained and well-equipped personnel make it unsuitable for widespread field use in the developing parts of the world (Gimenez *et al.,* 2021).

1.2.2 Plasmodium malariae and Gabon

As previously mentioned, malaria has afflicted mankind for a long time, especially sub-Saharan Africa. As such, efforts had been made to combat the disease all over the continent. However, success therein appears to have stagnated at least locally in Gabon in recent years, as no decline in prevalence could be observed between 2006 and 2013 (Assele *et al.,* 2015). Assumedly, this trend might have worsened during the turbulences of the recent SARS-CoV-2 epidemic, as it has in many parts of Africa (WHO, 2021). As such, of the five common *Plasmodium spp.* subspecies – *P. falciparum, P. vivax, P. malariae, P. ovale* and *P. knowlesi* – the three species found in Sub-Saharan Africa – *P. falciparum, P. malariae* and *P. ovale* – remain prevalent throughout the country and Moyen-Ogooué province.

In the most recent survey of rural Gabon in 2019, Woldearegai *et al.* found 2% of all PCR-screened patients to be mono-infected with *P. malariae*, with a further 66.4% showing the presence of the strain as part of a co-infection, most commonly in tandem with *P. falciparum*. Conspicuously, mono-infections with *P. malariae* presented a significantly higher median age than those with other species of the parasite; 60 years of age for *Pm* and 23 years of age for *Pf*. While this may sound excessive, Woldearegai's work (Woldearegai *et al.,* 2019) in general screened 74% of all its participants positive for any member of

Plasmodium spp. This number was heterologous between age groups; rising as far as 83.3% for participants between the ages of 10 and 20. Only 3.1% (2.8% of total study population) of participants showed symptoms of infections.

In contrast, another study by Manego *et al.* (2017) found microscopic evidence for malaria infection in a mere 46% (up to 77% among those between 5 and 20 years of age) of screened participants, with 23.3% of those being marked as 'non-*falciparum*'. According to the study, the low parasitaemia typical for infection with *P. malariae* and *P. ovale* prevented precise microscopical analysis of the 'non-falciparum' samples. The differences between these findings highlight the significant difference in detection sensitivity between PCR and light microscopy.

As such, the prevalence of any type of *Plasmodium spp.,* and even more so *P. malariae,* in the total population might be difficult to ascertain, as the vast majority of infections are both sub-microscopic and asymptomatic. This must, however, not lead one to assume *Pm* to be a negligeable parasite.

1.2.3 Clinical features

Infection with *P. malariae* is characterised by regular intervals of fever every 72 hours, owing to the longer life cycle of the parasite when compared to other *Plasmodium spp.* This also led to the infection being colloquially known as 'malaria quartana'. In general, this species is associated with comparatively benign symptoms and relatively low parasitaemia. This relative benignity may have contributed to quartan malaria being the least studied of all the human malarias (Collins & Jeffery, 2007).

The so-called hypnozoites, dormant parasites residing in hepatic cells, known from its relatives, *P. ovale (*and even more so *P. vivax),* have not been observed in *P. malariae* (Oriero *et al.,* 2021).

While infections with *P. malariae* are indeed regarded as minor, one must not be imprudent, as Kotepui *et al.* (2020) estimated about 3% of all cases turning severe, some of which (an estimated 0,17%) lethally so. These were especially associated with severe anaemia as the main clinical complication. While this

might contradict the findings of Woldearegai *et al.*, one must know that the former mainly included studies from non-endemic countries and thus mostly malaria-naïve patients, whereas the latter likely observed semi-immune individuals. In patients with said acquired partial immunity to the *Plasmodium* parasite, severe cases of *P. malariae* infection can be estimated to be far, far lower.

Rarely, but unique to *P. malariae,* an infection may result in the creation and deposition of immune complexes in the patient's kidneys, resulting in lowered function of the organ due to nephritis (Millner*,* 2018). Moreover, it has been observed to cause relapsing parasitaemia months or even years after the initial infections, the reasons to which are still poorly understood (Marteau *et al.,* 2021; Grande *et al.,* 2019). Kotepui *et al.* (2020) speculated this to be a result of inadequate treatment schedules which might – owing to the previously mentioned longer life cycle in *P. malariae* – be too short to entirely eradicate the parasite from the patient's bloodstream. The infection might thusly stay asymptomatic and – consequently – undetected for extended periods of time.

Nevertheless, *Plasmodium malariae*-infections most often either remain asymptomatic or are diagnosed by chance in tandem with other species of *Plasmodium,* most often *Plasmodium falciparum* and *Plasmodium ovale knowlesi* (Millner, 2018). A recent study by Boumbanda *et al.* (2020) suggests a higher prevalence of the parasite among asymptomatic carriers compared to febrile symptomatic study participants, further adding to its stereotype of being comparatively benign.

1.3 Target tropism as an explanation for *P. malariae*'s infection dynamics

Target tropism, in the context of pathogens, refers to evolutionary specificity in regards to host species, targeted tissue or cell type. Such has been observed in nigh every virus, bacterium, fungus, or parasite. *Plasmodium spp.* especially demonstrates a narrow tropism regarding host species and, in some subspecies, erythrocyte fraction (Cowman *et al.,* 2017). For *Plasmodium malariae*, a similar tropism might be present, if currently poorly studied.

1.3.1 Target tropism in *Plasmodium spp.*

A target tropism in regards to erythrocyte age has been reported in multiple species of *Plasmodium*. In humans, this has especially been documented in *P. vivax* (Malleret *et al.*, 2015). However, it is by no means exclusive to this species of the parasite – both the zoonotic *P. berghei* and *P. yoelii*, or even (while not exclusively) *P. falciparum* (Leong *et al.*, 2021) has been observed to preferentially target reticulocytes.

For *P. vivax*, this tropism goes even further – apart from the reliance on the Duffy receptor exhibited by the RBC's membrane and a nigh-exclusive preference for reticulocytes, it additionally exhibits a strong preference for $CD71^+$ reticulocytes in the bone marrow as opposed to those in peripheral circulation (Malleret *et al.*, 2015). Additionally, the parasite's tropism for the Duffy receptor precludes it from infection of populations with a mutated receptor types – as is the case for most of sub-Saharan Africa – thus limiting its global spread and impact. However, increasing reports of *P. vivax* infection in Duffy negative individuals have led to concerns over adaptations to this previous limitation (Kanjee *et al.,* 2018).

While, as previously stated, *Plasmodium spp.* usually exhibits strict host species adherence, *P. falciparum* has been documented infecting New World monkeys such as bonobos and, inversely, the otherwise zoonotic *P. knowlesi* has been shown to be transmissible from macaques to humans. Worryingly, while exhibiting a nominal preference for reticulocytes, both species have demonstrated an ability to expand their host niche in erythrocyte populations, leading to increased disease severity (Cowman *et al.,* 2017).

The concept of *P. malariae* having a tropism for 'older' erythrocytes was initially hypothesised in a work by Kitchen (1939), wherein the senescent erythrocytes were identified by means of light microscopy. Since then, it has been cited numerous times and been a central part to the current understanding of the parasite. This study undertook to verify these claims by modern flowcytometric analyses.

1.3.2 Significance of surface proteins as markers for erythrocyte age

Besides physiologic and visual changes to the erythrocyte, surface proteins can also be used to approximate a red blood cell's (RBCs) maturity. The markers hereafter were chosen as appropriate for the planned staining assays, however, many other possible markers exist and could lend themselves to further study.

1.3.2.1 CD71

CD71 is a marker commonly expressed by reticulocytes. In this study, it was chosen to mark these very same cells in the RBC population.

CD71, also known as transferrin receptor I, is a known sign of immaturity in RBCs, as it is mainly expressed throughout the erythropoietic process, especially on erythroblasts and reticulocytes. Its main purpose is the supply of iron needed for haemoglobin synthesis (Aisen, 2004). The transferrin receptor I sees its peak of expression during the erythropoietic stages of basophilic/polychromatophilic erythroblasts and decreases thereafter. On mature RBCs, it is entirely absent (Grzywa et al., 2021).

In adult individuals, $CD71^+$ cells are nigh exclusively found in the bone marrow; with only about 0.1-1% of peripheral RBCs exhibiting the marker. However, it may be found on erythrocytes of the peripheral blood in times of heightened erythropoiesis. For example, up to 10% of RBCs in anaemic individuals and as high as 20% in pregnant women (Grzywa *et al.,* 2021). Rapidly proliferating cells, such as the ones found in malignancies, are also known to express CD71 in great quantities (Aisen, 2004). Interestingly, $CD71^+$ appear to be more common in females; the reason for which is speculated to be the greater frequency of anaemia due to menstrual bleeding (Mashhouri *et al.*, 2021). However, while anaemia is associated with increased expression, hypoxia was found to be linked to the loss of CD71 from the surface of the erythrocytes (Bapat *et al.*, 2021).

Its significance for immunologic processes within a human's body is currently subject of intensive research, as findings suggest that increased levels of $CD71^+$ cells directly contribute to suppression of the individual's immune response by

means of production of reactive oxygen species and a causal decrease in T-cell proliferation. These apparent perks are of high interest in the physiology of SARS-CoV-2, HIV, leukaemia, and many other prominent diseases (Grzywa *et al.,* 2021). In murine models, a negative effect on disease resilience could be demonstrated, which was even greater after previous induction of anaemia (Mashhouri *et al.,* 2021)

1.3.2.2 CD235a

CD235a represents a ubiquitous, if unspecific marker commonly expressed my many cells in peripheral blood. It was used as a broad-spectrum marker for successful staining of erythrocytes in this study. It was intended to serve as a tool to ascertain the total number of erythrocytes in each sample investigated.

CD235a, commonly referred to as glycophorin A, is one of the four human glycophorins (the others being glycophorin B, C and D, respectively), which themselves are a class of transmembrane glycoproteins (Jaskiewicz *et al.,* 2019). They are present throughout both the erythropoietic as well as the mature life cycle of RBCs. However, different from CD71, expression of CD235a increases steadily throughout the cell's maturation (Grzywa *et al.,* 2021) as well as during hypoxic conditions (Bapat *et al.*, 2021).

Glycophorin A is especially important in the context of erythrocytic invasion by the *Plasmodium* parasite. It has been observed that the *Plasmodium falciparum* parasite's erythrocyte-binding antigen 175 (EBA175), which in turn requires the presence of CD235a, is of key importance for parasite adhesion during invasion. This is currently being investigated as a possible target for future antimalarial treatment. Moreover, it has been observed that the CD235a/EBA175 interaction contributes to post-infection erythrocyte clustering and – therefore – to the propagation of infection (Jaskiewicz *et al.* 2019).

This significance has contributed to genetic polymorphisms of the protein within individuals living in highly endemic areas, which may lessen their susceptibility to the parasite. For example, a glycophorin A-B hybrid – known as the Dantu phenotype – has shown protective qualities in *in vitro* studies. However,

glycophorin A is not uniquely necessary for successful RBC invasion by *Plasmodium falciparum* (Jaskiewicz *et al.,* 2019).

1.3.2.3 Annexin V and Phosphatidylserine

Annexin V was to serve as a maker for aging, senescent cells specifically, in this study. Annexin V – being part of the larger Annexin family of proteins – is a Ca^{2+}-dependant phospholipid binding protein with high affinity towards negatively charged phospholipids. In an haematologic context, it is most commonly associated with its strong binding to phosphatidylserine (PS), which is commonly exhibited during apoptotic and/or necrotic processes within human cells (Reutelingsperger & van Heerde, 1997).

Phosphatidylserine is, in healthy cells, asymmetrically expressed on cell membranes facing the cytosol – the inside of the cell. This asymmetry is actively upheld by cells by the use of numerous proteins and channels. However, most human cells have been shown to possess mechanisms to specifically invert this state; being able to expose large quantities of PS within a short timeframe after exposure to adequate stimulants – such as thrombin and collagen. This plays a major role in both controlled cell suicide as well as inflammatory responses and blood clotting (Reutelingsperger & van Heerde, 1997).

Ageing (senescence) of erythrocytes is linked to an increase to external PS accumulation. This externalisation is presumed to induce targeted removal of – as previously described – potentially procoagulant and possibly damaged erythrocytes. Phagocytes have been shown to react to PS-linked signals by targeted removal of associated cells. Therefore, phosphatidylserine likely serves as a specific recognition molecule for preventative removal of potentially proinflammatory or otherwise less potent cells. Interestingly, a downregulation of PS externalisation has been observed in malignant cells, likely being a mechanism of immune evasion (Reutelingsperger & van Heerde, 1997).

1.3.2.4 CD47

Similarly to CD71, CD47 was chosen to mark younger erythrocytes and especially reticulocytes; due to the rarity of expression in the former cell type, it was intended to further augment the age specification of the RBC population.

CD47, alongside CD31, is one of the major inhibitors of phagocytosis of human cells. While the latter can be found on platelets, lymphocytes, and endothelial linings, CD47 is mainly expressed on macrophages, dendritic cells, and erythrocytes, among others. By its interaction with the signal regulatory protein alpha (SRPα) it delivers a strong “do not eat me” signal to phagocytes, thereby preventing unwanted removal of otherwise healthy cells. The expression of the marker is heterologous throughout a cell’s life cycle, being highly expressed in young cells and waning with cell age (Thaker *et al.*, 2022; Torrez Dulgeroff *et al.*, 2021).

The CD47/SRPα interaction is also a vantage point for immune avoidance in malignant growths, which, by means of unnatural overexpression of the marker downregulate macrophage activity and thereby the immunologic response to the cancer. Consequently, the marker is a well-known target for oncologic therapy regimes (Thaker *et al.,* 2022). It has also been observed to play a major part in infection with *Plasmodium spp.* in murine models; as it is assumed to be critical in the erythrocyte invasion of – at the very least – the zoonotic *Plasmodium yoelii* (Torrez Dulgeroff *et al.,* 2021).

Torrez Dulgeroff *et al.* (2021) found anti-CD47 treatment to be highly effective in the prevention of *P. falciparum* cerebral malaria in mice, as 80% of all cerebral cases could successfully be prevented with targeted therapy, without an unwanted induction of anaemia. A beneficial effect on parasitaemia could also be observed. As such, anti-CD47 drugs have since been discussed as possible compounds for therapy of severe cerebral malaria in humans, but have not yet entered regimes.

1.4 Rationale and Study Design

1.4.1 The CoMal project

This thesis is an ancillary that was carried out as part of 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 multicentric study with participating members from Benin, Cameroon, Congo, and Gabon. The study was originally planned to run from 2018 to 2021, however, due to the COVID-19 pandemic, it was prolonged. The aim of the CoMal project is to gain further insight into *P. malariae* transmission and methods thereof. Interactions between the parasite and its host, as well as its vector, are studied to further the understanding of the means of parasite transmission and development.

CoMal 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.

One of the long-term goals of CoMal is to contribute to the establishment of a standardized experimental human blood-stage model for *P. malariae,* which has thus far have proven unsuccessful in other experiments. Such models would be instrumental in the study of *P. malariae,* allowing a controlled environment for an extensive study of the hitherto comparatively neglected parasite.

1.4.2 Thesis's objectives and design

This work aims to investigate the lesser-known *P. malariae* parasite in the sub-Saharan African country of Gabon in concordance with the previously mentioned CoMal study. In doing so, it continues previous projects in the area; such as Dr. Anton Hoffmann's thesis (2021), which had to be prematurely stopped due to the initial SARS-CoV-19 outbreak, and consequently continues parasite surveillance in the area.

The primary objective was an analysis of *P. malariae* prevalence, co-infection rates and its clinical features in infected individuals. Following CoMal's primary objective, the exiting data on *P. malariae*'s role in local malaria disease burden was augmented and any changes to preexisting assumptions noted.

The secondary objective was an experimental evaluation of the ex vivo susceptibility of *P. malariae* isolates to chloroquine, artesunate, atovaquone and lumefantrine.

The goal was to screen for emerging reduced drug susceptibility to commonly used antimalarials in *P. malariae* in the region around Lambaréné. Moreover, it was evaluated if such resistances could be cause of the parasite's common recrudescence and sub-clinical manifestation – thus aiding in CoMal's third objective.

An additional tertiary objective aimed to establish staining protocols for future flowcytometric investigation of erythrocyte-age-based target tropisms in *P. malariae.* These would further CoMal's secondary and tertiary objectives by both further characterising *P. malariae*'s life cycle and possibly enabling the parasite's cultivation in vitro.

The study protocol was approved by the institutional review board of CERMEL (CEI-014/2018) and the ethics committee of the medical faculty and the university clinics of the University of Tübingen (010/2018BO2).

2. Materials and methods

2.1 Study site

The study was conducted at the Centre de Recherches Médicales de Lambaréné (CERMEL), located in the Moyen-Ogooué province of Gabon. Its Medical Entomology Lab – this study's place of origin – can be seen pictured in **Figure 1**. Like most of the country, the area can be characterised by abundant rainforests, tropical climates, and a plethora of fauna, owing to its vicinity to the equator. The province covers an area of 18,535 km^2 and is home to about 69,000 people, of which an estimated 35,000 live in its provincial capital, Lambaréné, a town situated by the Ogooué river, about 250km to the south-east of Gabon´s capital, Libreville. It also acts as the economic centre of the region and is pivotal for the infrastructural connection of the province to the rest of the country. The transmission of malaria in the region has been observed to be perennial (Sylla, Kun and Kremsner, 2000; Grobusch *et al.*, 2007).

Figure 1: The Medical Entomology Lab of CERMEL[1]

CERMEL originated as the "Medical Research Unit" of the Hôpital du Albert Schweitzer, the successor of the famous working grounds of renowned German

[1] Author

philanthropist, musician, doctor, and Nobel-prize-winner Albert Schweitzer, and has developed into an independent non-profit organization with close ties to the Deutsche Forschungsgesellschaft, the University of Tübingen and many other universities all over Africa. Research is focussed on infectious diseases such as malaria, tuberculosis, helminths and – more recently – SARS-CoV-2. The organization was somewhat famous in recent history in its involvement with the RTS,S/AS01E malaria vaccine trials.

2.2 Field Screening and sample collection

Blood samples were collected by the personnel of CoMal study group from October 2021 to September 2022. Due to the rareness of the parasite subtype, it was decided to involve as many isolates as possible in the study. A P. falciparum culture (NF54) that had previously been shown to be well adapted to clinical observations was used as positive control for both the drug assays as well as the testing of the staining protocols.

Blood samples for the experiments were collected from assenting and/or consenting individuals (enrolled within the cross-sectional CoMal study) meeting the inclusion criteria. Twice or thrice a week, a team consisting of two field workers screened up to 60 people for malaria after obtaining informed consent, signed in writing or by thumbprint. For individuals below the legal age, signed assent by a legal guardian or parent was required and acquired instead. Basic demographic datapoints, such as weight, age, sex, and body temperature were taken. Two thick and thin smears were made (with one of each on the same slide) for each study participant and analysed by means of light microscopy. 10 µl of blood taken from finger prick lesions was used for each thick smear, whereas 5 µl were used for each thin smear. Any individual was assigned a specific study identification number (CMLXXX) which was noted on the corresponding slide alongside time and date of making. The slides were stained and read once by qualified readers of CoMal study team. After slide reading, all participants deemed to be infected with *Plasmodium spp.* were treated with Coartem®. If individuals were identified as carrying either *P. malariae* infections or *P.*

falciparum gametocytes, they were requested to provide blood samples to be used in experimentation prior to the administering of treatment.

2.3 Material

2.3.1 Lab equipment

Table 1: Lab equipment with corresponding manufacturer

Name	Manufacturer
Autoclave	Melag, Euroclav 29 VS, France
Biological Safety Cabinet	Thermo Scientific, Hera Safe KSP, USA
Centrifuge	Thermo Scientific, Heraeus Megafuge 8, USA
CO_2-Incubator	Mytrom, Germany
FACSymphony™ A1 Flow Cytometer	BD Biosciences, USA
Falcon tubes, 15 ml and 50 ml	BD Falcon™, USA
Filter papers	Macherey-Nagel, Germany
LightCycler 480 II	Roche, Switzerland
Heparin tubes	Becton Dickinson, 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
Polystyrene round-bottom tubes	BD Falcon™, USA
QIAAmp® Blood Mini Kit	Qiagen, Germany
Rapid test for the antigen detection of malaria (RDT)	Access Bio, USA
Reagent Reservoir	VWR, PA, 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
Tissue Culture Plate, 96 well	Corning Incorporated, USA
Uno^{96} thermal cycler	VWR, PA, USA
Vortex mixer	neoLab, Germany
Water bath	Thermo Scientific, Haake, SC 150, USA

2.3.2 Software

Table 2: Software with corresponding manufacturer

Name	Manufacturer
Graph Pad Prism 8.0	Graph Pad Software Inc., CA, USA
IBM SPSS Statistics 28	IBM, NY, USA

ICEstimator	(Le Nagard *et al.*, 2011)
FACSDiva	Becton Dickinson, OR, USA
FlowJo	Becton Dickinson, OR, USA
LightCycler® 480 Software, Version 1.5	Roche, Switzerland
Microsoft Excel 2016	Microsoft, USA
Microsoft Word 2016	Microsoft, USA

2.3.3 Reagents and chemicals

Table 3: Reagents and chemicals and their respective manufacturer

Name	Manufacturer
Albumax II Lipid-Rich BSA	Gibco Invitrogen, New Zealand
Alexa Fluor® 647 Annexin V	BioLegend, USA
Annexin V Binding Buffer	BioLegend, USA
Artesunate	Sigma Aldrich, USA
Atovaquone	Sigma Aldrich, USA
Chloroquine diphosphate salt	Sigma Aldrich, USA
CD47 Antibody, anti-human, PE, REAfinity, Clone REA220	Miltenyi Biotec, Germany

CD71 Antibody, anti-human, PE-Vio® 615, REAfinity, Clone REA902	Miltenyi Biotec, Germany
CD235a (Glycophorin A) Antibody, anti-human, APC-Vio® 770, REAfinity, Clone REA175	Miltenyi Biotec, Germany
Decompartmentalized foetal calf serum (dcFCS)	Universitätsklinikum Tübingen
Dimethyl sulfoxide (DMSO)	Sigma Aldrich, USA
Dulbecco's Phosphate Buffered Saline (PBS)	Sigma Aldrich, USA
Gentamicin 50 mg / ml	Sigma Aldrich, USA
Giemsa R solution	RAL Diagnostics, France
Glutaraldehyde	Universitätsklinikum Tübingen
HEPES 1M H0887	Sigma Aldrich, USA
Human AB serum	Universitätsklinikum Heidelberg, Germany
Hoechts 33342	Thermo Fisher Scientifc
L-Glutamine 200 mM	Sigma Aldrich, USA
Lumefantrine	Sigma Aldrich, USA
MACS Comp Bead, anti-human Igκ Kit	Miltenyi Biotec, Germany
Merck Phosphate buffer tablets	Sigma Aldrich, USA

Methanol	VWR chemicals, France
Paraformaldehyde	Universitätsklinikum Tübingen
RPMI 1640 without L-Glutamine R0883	Sigma Aldrich, USA
Sodiumazide	Universitätsklinikum Tübingen

2.3.3.1 Complete Culture Medium (CCM)

For adequate parasite growth, a culture medium was prepared. For this, 42.2ml of RPMI were mixed with 5ml of Albumax II solution; 1200µl of 1M HEPES; 1000µl of human AB serum; 600µl of 200mM L-glutamine and 50µl of 50mg/ml gentamicin solution. The medium was stored at 4°C and heated to 37°C prior to usage.

2.3.3.2 70% Ethanol

For surface disinfection, 70% ethanol solution was prepared. In a 1 l bottle, 700 ml of pure ethanol were mixed with 300ml of distilled water. The solution was stored at room temperature and used to disinfect surfaces under the laminar hood before and after experimentation.

2.3.3.3 FACS buffer solution

The FACS buffer used in the experiments was a solution consisting of PBS deprived of its Ca^{2+} and Mg^{2+} content, colloquially known as Dulbecco's PBS. To this, sodium azide (NaN_3) and decompartmentalized foetal calf serum (dcFCS) were added. The final mixture contained 0.05% of NaN_3 and 1% FCS. The buffer was stored at 4°C.

2.3.3.4 Phosphate buffer

As per supplier's instruction, one tablet of phosphate buffered saline was dissolved in 1 l of distilled water (pH 7.2 – 7.6). The buffer was stored at 4°C.

2.3.3.5 10% Giemsa stain solution

For adequate staining free of clutter, pure Giemsa was transferred in aliquots through a filter paper to 50 ml falcon tubes. Then, this filtrate was diluted in a 1:9 ratio with Merck Phosphate Buffer solution, depending on the amount needed. The staining solution was mixed specifically before each usage and discarded thereafter.

2.4 Methods

2.4.1 Blood Processing

From patients aged 5 years and above, 10 ml of blood were drawn into heparin tubes. Thin and thick smears were made and labelled according to their origin. The slides were dried, fixed with methanol, and stained with 10% Giemsa solution for 15 minutes.

Blood taken from study participants was first centrifuged for 5 minutes at 2000 g. After removal of the supernatant serum, the remaining pellet was resuspended with RPMI 1640 in a 2:1 dilution – for example, 3 ml of pellet were resuspended with 6 ml of RPMI 1640. Then, it was washed twice more with RPMI 1640 for 5' at 2000 g. After the final removal of the RPMI supernatant, the blood pellet was ready for induction.

2.4.2 Determination of parasite density during microscopy

Parasite density for the screening of study participants was determined according to the Lambaréné method, as described by Mischlinger *et al.* (2018). Therein, up to 100 fields were counted, after which a slide could be determined to be negative, provided no parasite was seen. Slide reading was halted earlier if more

than 5 parasites were seen in 30 fields, or more than 50 parasites in 20 fields, respectively. Parasitaemia was expressed as the number of asexual parasites per microlitre of blood and calculated according to the following formula:

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

2.4.3 Drug stock solutions

All the drugs used in the *ex-vivo* assay were provided in powder form. To create drug stock solutions (DSS), AS, ATQ and LUM were dissolved in DMSO, whereas CQ was dissolved in distilled water. To create working solutions (WS), the DSS were further diluted with RPMI 1640 and stored at 4°C.

Table 4: Preparation of drug solutions used in the ex-vivo assays

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	200	40	160

2.4.4 Drug sensitivity testing

The *ex vivo* drug susceptibility testing was based on the schizont maturation test established by the WHO (Wernsdorfer, 1980). Chosen were artesunate, chloroquine, lumefantrine and atovaquone. For each compound, a triplicate serial dilution was performed on a 96-well plate. Values from previous experiments of CoMal study group, especially those of Hoffmann (2021), were taken to estimate the maximum concentration needed for full inhibition.

2.4.4.1 Blood preparation

Blood medium mixture (BMM) was prepared at 4% haematocrit. 400 µl of washed RBC-pellet was mixed with 600 µl of 20 % human AB-serum. This mixture was diluted in a VWR reagent reservoir with 9 ml of CCM.

2.4.4.2 Dosage of the 96-well plates

In previous studies, a certain effect on parasite growth pertaining to varying levels of evaporation based on distance from the lid of the plate was observed (Walzl *et al.,* 2012). Due to the inhomogeneous loss of liquid within the wells during incubation there would have been a risk of preventable growth disparities. To prevent this, the outermost wells were left empty. Recent works, such as Hoffmann's thesis (2021) showed this to be an effective measure.

The plates were incubated for half of the expected period of the parasite's life cycle; 36 hours in *P. malariae* and 24 hours in the *P. falciparum* control assay.

To dose the 96-well plate, to each well (barring those at the edges; as previously mentioned) 50 µl of CCM were added. To the first assay column – the plate's second column due to sparing the edges and thus its first – 50 µl of a drugs WS were added. Serial dilution was performed by means of multi-channel pipetting 50 µl of the previous well into the next. After mixing the pipette's content on the last well (ninth of the assay, tenth row of the plate), 50 µl of the well's content were discarded. The eleventh column on the plate was not dosed to act as a negative control without drug exposure. All drug dilutions were performed in triplicates. After preparing the plate thusly, 50 µl of BMM was added to each well previously dosed. 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. Incubation was stopped relative to the observed isolate's expected life cycle.

2.4.4.3 WHO schizont maturation test

The schizont maturation test was developed by the World Health Organisation in order to allow for the assessment of possible drug resistances even under primitive and complicated field conditions. The test compares the number of schizonts in the respective test well to the number of schizonts in the control wells.

The 96-well-plates were incubated for 36 hours. After incubation, the experiment was stopped. 80 µl of the supernatant medium was discarded and 10 µl of the remaining BMM was transferred on a microscopic slide to create thick smears as pictured in **Figure 2**. The thick smears were stained with 10% Giemsa dye for 15 minutes.

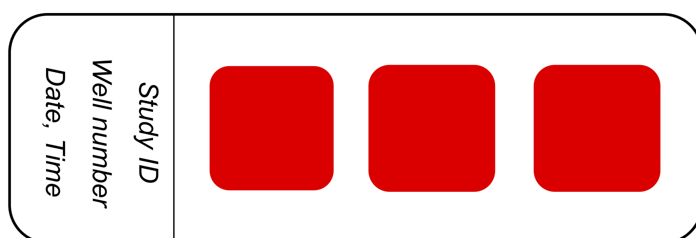

Figure 2: Layout of study slides

A drop of immersion oil was put on the slide to enable proper functionality of the 100x magnification scope. The number of schizonts seen in each well as well as the control well was noted on a sheet. For non-control wells, the mean was calculated. Using the formula hereafter, the proportion of schizonts in the drug dilution was calculated:

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

The percentage indicated the average amount of surviving schizonts in the drug wells compared to the non-dosed controls. Using these numbers, the IC_{50}-values for each drug were calculated.

2.4.6 Flow cytometric sample staining

Isolates were stained as described below. In this study, the monoclonal antibodies used were as listed in **Table 5**:

Table 5: Dyes used in flowcytometric staining

Marker	Monoclonal antibody	Filter	Laser line
CD235a	anti-human, APC-Vio® 770, REAfinity, Clone REA175	780/60	637 nm
CD71	CD71 Antibody, anti-human, PE-Vio® 615, REAfinity, Clone REA902	610/20	561 nm
Annexin V	Alexa Fluor® 647 Annexin V	670/20	637 nm
Hoechst 33342	n/a	450/40	405 nm
CD47	CD47 Antibody, anti-human, PE, REAfinity, Clone REA220	585/42	561 nm

2.4.6.1 First Iteration

In order to stain the collected sample, either 5 µl of RBC pellet or 10 µl of whole blood was taken and placed into a 2 ml Eppendorf tube. It was washed with 200 µl of FACS buffer and centrifuged for 5 minutes at 2000g.

After removing the supernatant, the pellet was resuspended with 200µl of solution consisting of 191 µl of FACS buffer mixed with 4 µl of CD235a- and CD71-monoclonal antibodies (mAb), as well as 1 µl of Hoechst 33342 solution. The tube was wrapped in aluminium foil and placed in a dark environment and let to incubate for 20 minutes. After that, the sample was washed twice.

After removing the buffer supernatant, 5 µl of fluorochrome-conjugated Annexin V was added alongside 200µl of Annexin V buffer. The tube was again wrapped in aluminium foil and let to incubate for 15 minutes. After washing the sample once more with 200 µl FACS buffer, the remaining pellet (suspended, again, in 200 µl of FACS buffer solution) was ready for analysis.

Should it have been impossible to conduct the analysis on the same day, the samples were additionally fixed with 20 µl of a solution consisting of 4% paraformaldehyde and 0.0075% glutaraldehyde. After 10 minutes of incubation, the sample was washed one last time with FACS buffer and set aside, wrapped in aluminium foil, for future analysis by means of flow cytometry within two weeks.

This staining method was only used on the very first samples; CML0012, CML0027, CML0039, CML0040, CML0042 and CML0056.

2.4.6.2 Second Iteration

From thereon, it was decided to stain each marker separately. To do so, either 5 µl of RBC pellet or 10 µl of whole blood was taken and placed into three 2 ml Eppendorf tubes. They were washed with 1000 µl of FACS buffer and centrifuged for 5 minutes at 2000g.

After removing the supernatant, the pellet was resuspended with 200µl of solution consisting of 195 µl of FACS buffer mixed with either 4 µl of CD235a- or CD71-mAb, as well as 1 µl of Hoechst 33342 solution. The tube was wrapped in aluminium foil and placed in a dark environment and let to incubate for 30 minutes. After that, 800 µl of FACS buffer were added so the sample could be washed twice. To a third Eppendorf tube, 5 µl of fluorochrome-conjugated Annexin V and 1 µl of Hoechst 33342 solution was added alongside 200µl of Annexin V buffer. The tube was wrapped in aluminium foil and let to incubate for 20-30 minutes. After washing the sample once with 800 µl FACS buffer, the remaining pellets (suspended, again, in 1000 µl of FACS buffer solution) were ready for analysis. This way, each assay consisted of three separate stains: CD71/Hoechst, CD235/Hoechst and Annexin V/Hoechst.

Should it have been impossible to conduct the analysis on the same day, the samples were additionally fixed with 20 µl of a solution consisting of 4% paraformaldehyde and 0.0075% glutaraldehyde. After 10 minutes of incubation, the sample was washed one last time with FACS buffer and set aside, wrapped in aluminium foil, for future analysis by means of flow cytometry within two weeks.

2.4.6.3 Third Iteration

To stain the collected sample, either 5 µl of red blood cell pellet or 10 µl of whole blood was taken and placed into a 2 ml Eppendorf tube. It was washed with 1000 µl of FACS buffer and centrifuged for 5 minutes at 2000g.

After removing the supernatant, the pellet was resuspended with 200µl of solution consisting of 187 µl of FACS buffer mixed with 4 µl of CD235a-, CD47- and CD71-mAb, as well as 1 µl of Hoechst 33342 solution. The tube was wrapped in aluminium foil and placed in a dark environment and let to incubate for 30 minutes. After that, 800 µl of FACS buffer were added so the sample could be washed twice.

After removing the buffer supernatant, 5 µl of fluorochrome-conjugated Annexin V was added alongside 200µl of Annexin V buffer. The tube was again wrapped in aluminium foil and let to incubate for 20-30 minutes. After washing the sample once more with FACS buffer, the remaining pellet (suspended, again, in 1000 µl of FACS buffer solution) was ready for analysis.

Should it have been impossible to conduct the analysis on the same day, the samples were additionally fixed with 20 µl of a solution consisting of 4% paraformaldehyde and 0.0075% glutaraldehyde. After 10 minutes of incubation, the sample was washed one last time with FACS buffer and set aside, wrapped in aluminium foil, for future analysis by means of flow cytometry within two weeks.

This method was only used for CML0514.

2.4.7 Flow cytometric compensation controls

The following compensation setup was made: for CD235a, CD71 and CD47 the MACS Comp Bead, anti-human Igκ Kit was used. As the beads do not bind to Hoechst 33342 (a nuclear stain), nor Annexin V (binding to phosphatidylserine), a heavily infected *Pf*-sample was used as a single stain control instead. The same sample was used as the unstained control. The compensation values were automatically calculated by the FACSymphony™ A1's own FACS Diva software.

2.4.8 PCR

2.4.8.1 DNA extraction

For DNA extraction, the QIAamp Blood Mini Kit was used according to manufacturer's instructions.

In an Eppendorf tube, 20 µl of protease K were added to 200 µl of sample blood. This mixture was thoroughly mixed with 200µl of lysis buffer by vortexing. It was then incubated at 56°C for 10 min. Afterwards, 200 µl of >96% ethanol was added. After vortexing the sample again, the tube's content was transferred to a mini spin column placed above a 2 ml collection tube. This column was then centrifuged at 6000 g for 1 min.

After discarding the flowthrough and changing of the collection tube, 500 µl of washing buffer was added. The tube was again centrifuged at 6000 g for 1 min. After changing of the collection tube, 500 µl of washing buffer were added to the spin column. It was then centrifuged at 20,000 g for 3 min.

For elution of the extracted DNA, the spin column was placed above a 1.5 ml microcentrifuge tube. 200 µl of elution buffer were added and it was incubated for one minute at room temperature. Finally, the sample was centrifuged again at 6000 g for 1 min; the final flowthrough containing the eluted DNA. The eluted DNA was stored at -20°C until usage.

2.4.8.2 Pre-amplification

To increase yield, a pre-amplification of the samples' DNA was performed. For this, the VWR Uno 96 thermal cycler was used.

To amplify the extracted DNA, pan-*Plasmodium* primers were used:

- rPLU5: 5'-CCT GTT GTT GCC TTA AAC TTC-3'
- rPLU6: 5'- TTA AAA TTG TTG CAG TTA AAA CG-3'

The pre-amplification PCR mix as well as the cycler program can be inferred from **Table 6** and **Table 7**.

Table 6: Pre-amplification PCR mix

Stock concentration	Component	Volume (per 50 µl reaction)	Final Concentration
10x	PCR Buffer	5 µl	1x
25 nM	dNTPs	0,5 µl	250 nM
10 µM	rPLU5	1,5 µl	300 nM
10 µM	rPLU6	1,5 µl	300 nM
5 U	Taq DNA Polymerase	0,2 µl	1 U
	Template	5 µl	
	Nuclease Free Water	36,3 µl	

Table 7: Pre-amplification cycler program

Step	Temperature	Duration	Nr. of cycles
Initial denaturation	95°C	5 min	1
Denaturation	95°C	30 sec	20
Annealing	58°C	30 sec	
Extension	72°C	1 min 20 sec	
Final Extension	72°C	5 min	1
Cooling	15°C	indefinite	

2.4.8.3 Speciation PCR

A real-time PCR (qPCR) was performed using the Roche Light Cycler 480 II. Two assays were made:

a. Detection of *P. falciparum* or *P. ovale.*
b. Detection of *P. malariae*.

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

- Pf-1 Primer: 5'- ATT GCT TTT GAG AGG TTT TGT TAC TTT-3'
- Pf-2 Primer: 5'- GCT GTA GTA TTC AAA CAC AAT GAA CTC AA-3'
- Po for Primer: 5'- ATT TCA AAG AGT CAT GGC GTT TCT G-3'
- Poc rev Primer: 5'- TTG TAA AGG AGA CAC TTT CTT GAA ATC G-3'
- Pow rev Primer: 5'- TGT AAA GGA GAC AAC TTT CTT GGA GCTA-3'
- Pf Probe: [FAM] 5'- CAT AAC AGA CGG GTA GTC ATC-3' [MGBEQ]
- Povm Probe: [Cy5] 5'-CTG AAT ACA AAT GCC-3' [BHQ-2]

As positive control, a *P. falciparum,* and a *P. ovale* plasmid was used.

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]

As positive control, a *P. malariae* plasmid was used. The negative control for both assays was nuclease-free water.

Twenty-five samples were run in duplicates with two positive and two negative controls (one each). **Table 8** shows the reagents and corresponding volumes that were used to prepare the master mix for assay (a) and assay (b).

Table 8: Reagents and corresponding volumes for the master mix of assay (a) and (b).

For assay (a)			For assay (b)		
Component	**Final Concentration**	**Volume (per 10 µl reaction)**	**Component**	**Final Concentration**	**Volume (per 10 µl reaction)**
2X SensiFast One-Step Mix	1x	5 µl	2X SensiFast One-Step Mix	1x	5 µl
10µM Taqman probe Pf (Fam-MGB-BHQ1)	150 nM	0,15 µl	10 µM Taqman probe Pm (Hex-MGB-BHQ1)	200 nM	0,2 µl
10µM Taqman probe Povm (Cy5-MGB-BHQ2)	150 nM	0,15 µl	10 µM Pm-fwd	400 nM	0,4 µl
10 µM Pf-for	400 nM	0,4 µl	10 µM Pm-rev	400 nM	0,4 µl
10 µM Pf-rev	400 nM	0,4 µl	Nuclease-free water		3 µl
10 µM Po-for	400 nM	0,4 µl	Template		1 µl
10 µM Poc-rev	400 nM	0,4 µl			
10 µM Pow-rev	400 nM	0,4 µl			
Nuclease-free water		1,7 µl			
Template		1 µl			

The dosed light cycler plates were then analysed using the program shown in **Table 9**.

Table 9: qPCR cycler program

<table>
<tr><th>Step</th><th>Temperature</th><th>Duration</th><th>Nr. of cycles</th></tr>
<tr><td>Transcription</td><td>45°C</td><td>20 min</td><td>1</td></tr>
<tr><td>Hot start activation</td><td>95°C</td><td>3 min</td><td>1</td></tr>
<tr><td>Denaturation</td><td>95°C</td><td>15 sec</td><td rowspan="2">9
(Without signal detection)</td></tr>
<tr><td>Annealing</td><td>60°C</td><td>60 sec</td></tr>
<tr><td>Denaturation</td><td>95°C</td><td>15 sec</td><td rowspan="2">41
(With signal detection)</td></tr>
<tr><td>Annealing</td><td>60°C</td><td>60 sec</td></tr>
<tr><td>Cooling</td><td>40°C</td><td></td><td></td></tr>
</table>

2.4.9 Statistical analysis

2.4.9.1 Ex-vivo drug assays

The relative inhibitory effect of the respective drug dilution was calculated as follows:

The maximum inhibition was determined to be the complete inhibition of schizont growth in the analysed wells. The minimum inhibition was determined to be present in the control wells bereft of drug exposure. The differing IC-values for each drug were calculated using the free online software “ICEstimator” (available at http://www.antimalarial-icestimator.net/). The system uses a regression to approximate an inhibitory sigmoid E_{max} model, validated and analysed in detail by La Nagard *et al.* (2011). To initiate calculation, one was requested to enter the drug concentration used in the experiment (x-axis) and their respective observed relative effect on schizont growth (y-axis). The relative effect and – consequently

the IC-values – were calculated automatically. Furthermore, all values were given in their respective confidence intervals and under consideration of the standard error.

The values derived from this software were entered into an Excel spreadsheet (Microsoft, USA) and were statistically analysed using SPSS 28 (IBM, NY, USA). The normality of the data was validated using the Shapiro-Wilk test. The observed number of schizonts in the test wells were compared to those in the control wells using ANOVA, Kruskal-Wallis, and paired t-tests. P-values of 0.05 were determined as being statistically significant.

2.4.9.1 Flow cytometric analysis

Data captured by the flow cytometer was automatically fed to the machine's own FACSDiva software (Becton Dickinson, OR, USA). The FCS files produced were further analysed using the FlowJo software (Becton Dickinson, OR, USA). Gating was done by hand and data derived therefrom was statistically analysed using SPSS 28 (IBM, NY, USA).

2.4.9.2 PCR

The output of ultrasensitive q-RT-PCR reactions was analysed by visual inspection and 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. Since all samples were analysed in doublets, only those samples with both wells indicating positive reactions were classified according to the PCR's results.

3. Results

3.1 Organisation and results of the field screening

Individuals living in the rural areas around the city of Lambaréné were offered participation in CoMal study as part of local malaria screening efforts and source of antimalarial treatment. Usually, the arrival of CoMal’s team of field workers was previously arranged with the village’s elder or an agrarian foreman days before. Blood sampling for malaria screening is a commonplace sight in Gabon and consequently, many readily agreed to provide blood. Subjectively, many mothers or similar female relatives brought the village’s children for testing, especially if episodes of fever were recent. This also applied to family members of personnel of Albert Schweitzer hospital. Adults seemed more reticent to participate, some only approaching if ordered or feeling ill.

After returning to the Medical Entomology Lab, the thick and thin smears obtained were fixed, stained, and read by light microscopy. The CoMal field group received the ensuing screening results and returned to the sampled area in the afternoon to communicate results, administer treatment, and, if applicable, request blood samples for further analysis. Upon returning, the gathered blood samples were used for experimentation by both this study and CoMal study group.

748 (349 male and 399 female) individuals were enrolled in the CoMal study between April and August 2022. **Table 10** shows the basic demographic make-up of the pool of participants.

Table 10: Basic demographic data of study population

		Age (y)	Weight (kg)	Temperature (°C)
N (=748)	Valid	747	743	746
	Missing Values	1	5	2
Average		24.09	39.99	36.70
Median		15	41	36.70
Minimum		1	4	36.00
Maximum		97	125	40.00

Of the enrolled participants, a total of 31.15% (233 positive screening slides out of 748 participants) were determined to be positive for *Plasmodium spp.* Of those 233 positive cases, 12.02% (28 out of 233) were deemed to be (co-)infected with *Plasmodium malariae*, with 7.30% (17 out of 233) being classified as mono-infected. No other species of *Plasmodium* other than *P. falciparum* and *P. malariae* were observed within the timeframe of this study. **Table 11** and **Figure 3** show the screening results of the positive slides.

Table 11: Results of microscopic analysis of screening slides (N=748)

Plasmodium spp.		**233/748 (31,15%)**
Plasmodium falciparum		205/233 (87,93%)
Parasitaemia (parasites/µl)	Average	7.818,59
	Median	580
	Minimum Maximum	20 628.700
Plasmodium malariae		17/233 (7,30%)
Parasitaemia (parasites/µl)	Average	954,12
	Median	677
	Minimum Maximum	75 2.960
P. falciparum + P. malariae		11/233 (4,72%)
Parasitaemia (parasites/µl)	Average	2.010,4
	Median	922
	Minimum Maximum	232 8.620

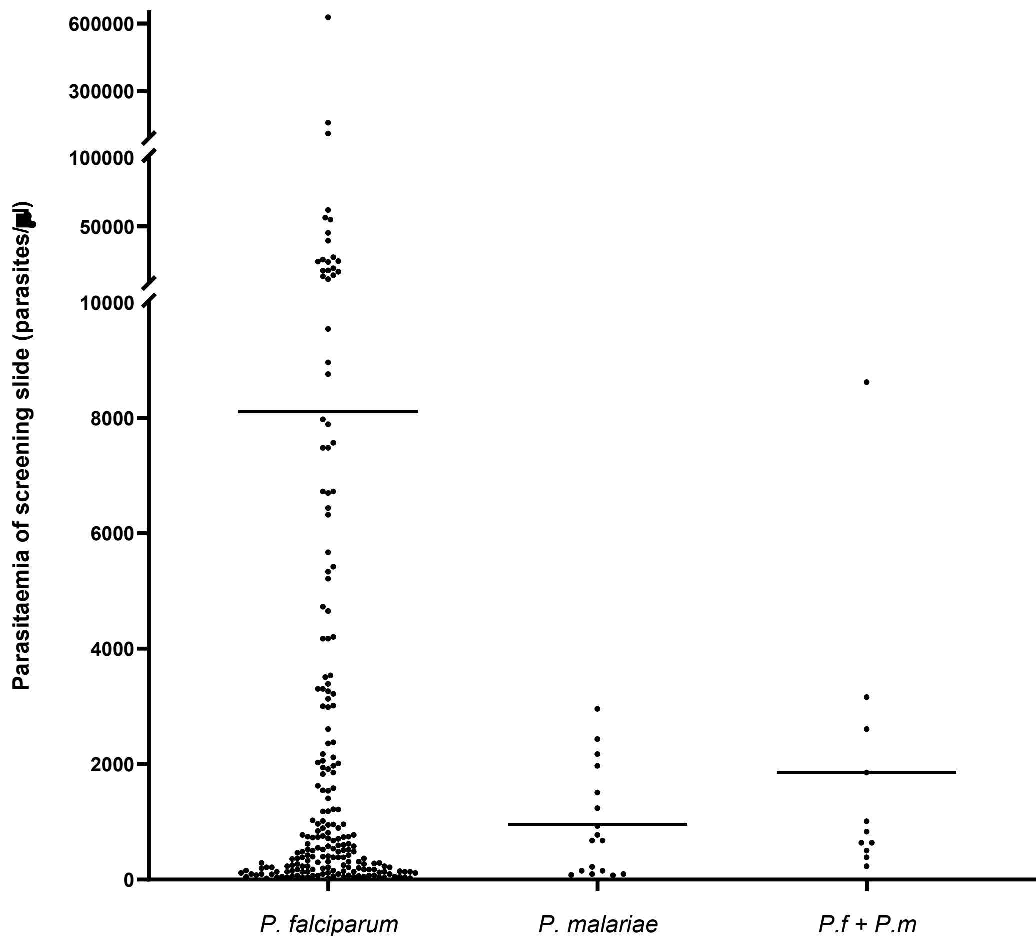

Figure 3: Scatterplot of slide parasitaemia respective to species

Scaled up to the total study population, 3.74% of all study participants were determined to be infected with *Plasmodium malariae*, with 2.27% being uniquely infected with this species.

Both also serve to illustrate the characteristic lower average parasitaemia for infections with *P. malariae* as compared to *P. falciparum.* Especially the lack of high-parasitaemia cases (or even hyperparasitaemia) showcases the previously observed relative benignity of *P. malariae.* In contrast, 18 of the *P. falciparum*-infected participants showed a parasitaemia >10.000/µl; 3 being counted >100.000/µl and one of them >200.000/µl, whereas the highest parasitaemia observed in *P. malariae* constituted a mere 2.960/µl. As such, the average parasitaemia determined for each slide has shown to differ significantly (p=0.037)

respective to the parasite species. Interestingly, the median parasitaemia is lower in *P. falciparum* infections, highlighting the far broader span of possible infection dynamics.

No study participant was hospitalised during their participation and no fatalities were recorded. Only two study participants infected with *P. malariae* – CML39 and CML570 – presented with a fever. Of those, one was an infant and as such predisposed to a more severe reaction to the infection. Interestingly, both participants had a rather low parasitaemia – with 928/µl and 1.972/µl, respectively. While 7.1% (2 out of 28) of study participants diagnosed with *P. malariae* presented as febrile, for those infected with *P. falciparum* the rate was about twice as high at 14.6% (30 out of 205).

The distribution of study participants' age groupings relative to species can be inferred from **Table 12** and **Figure 4**.

Table 12: Frequency of infection relative to age group

Age in years (range)	Total number of participants	Positive for *Plasmodium* spp. (%)	Positive for *P. falciparum* (%)	Positive for *P. malariae* (%)	Positive for *P. falciparum* and *P. malariae* (%)
1 - 5	139	39 (28,06)	37 (26,62)	1 (0,72)	1 (0,72)
6 - 10	144	60 (41,67)	55 (38,19)	1 (0,69)	4 (2,78)
11 - 15	93	49 (52,69)	40 (43,01)	4 (4,30)	5 (5,38)
16 - 20	51	19 (37,25)	16 (31,37)	3 (5,88)	-
21 - 89	320	66 (20,63)	57 (17,81)	8 (2,50)	1 (0,31)

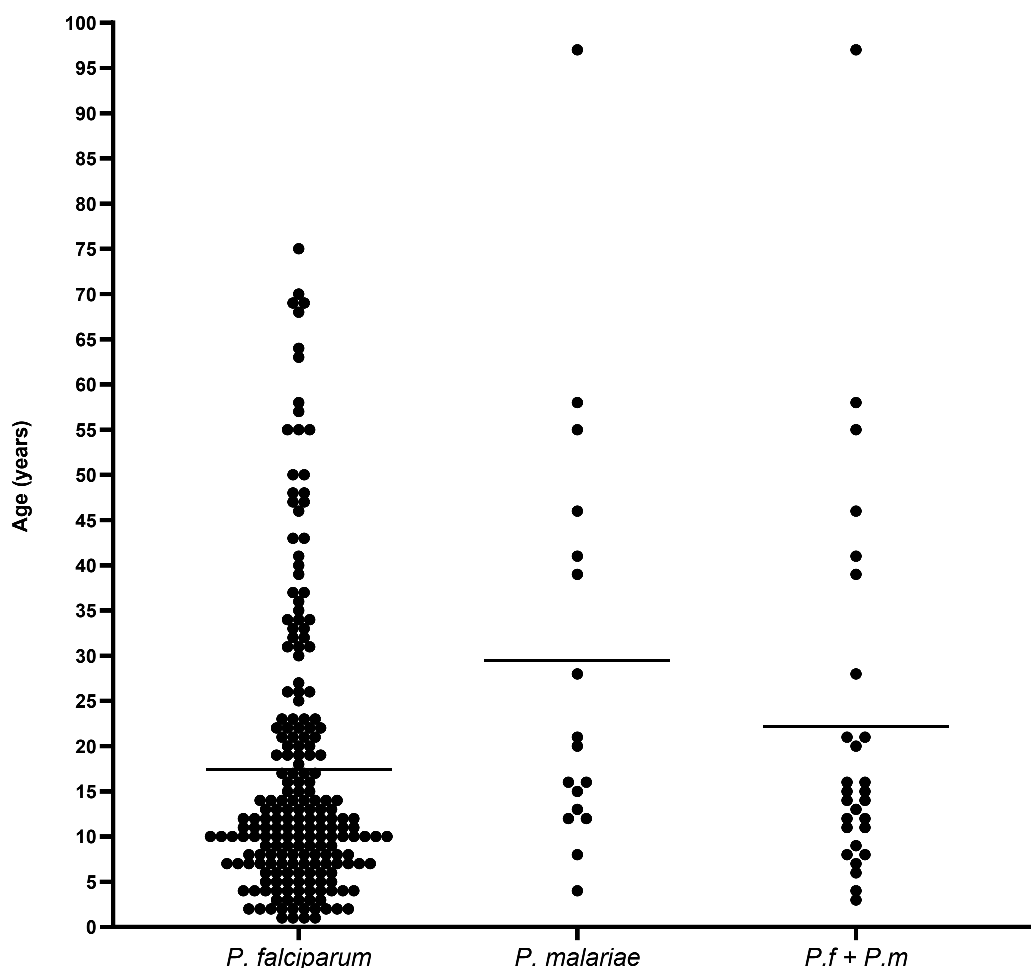

Figure 4: Scatterplot of age distribution respective to species

As can be seen, both on average and by median, study participant diagnosed with a *P. falciparum* infection were younger than those infected with *P. malariae*: 17.45 years (95% CI: 15.23 – 19.67) for *P. falciparum* and 29.47 years (95% CI: 17.11 – 41.83) for *P. malariae*. Nevertheless, the majority of all *P. falciparum* infected individuals were aged 15 years and younger (64.39% of all *P. falciparum* cases), whereas the majority of all *P. malariae* cases was found to be older than 15 years (64.71% of all *P. malariae* cases). In co-infections of both strains of the parasite, the screened individuals were even younger, being 10.82 years (95% CI: 7.45 – 14.19) old on average. This difference in average age has been shown to be significant compared to both *P. malariae* (p=0.006) and *P. falciparum* (p=0.002) mono-infections.

Of the 28 participants at least partially infected with *Plasmodium malariae*, 18 were included in this analysis. Among the 10 excluded individuals, two (CML0194 and CML0570) were too young for sampling, one (CML0480) failed to provide signed assent and the other 7's (CML0215, CML0318, CML0402, CML0436, CML0468, CML0548 and CML0748) parasitaemia was deemed to be too low for successful study – based on experiences made during the first month, a parasitaemia above 500/µl was considered sufficient. The samples for the 18 experiments conducted in this study were collected from 10 males and 8 females aged between 7 and 46 years, with a median of 16 years of age. A more detailed overview thereof can be found in **Table 13**.

Table 13: Characteristics of study participants enrolled in experiments

Nr.	CML-ID	Sex	Age (years)	Temperature (°C)	Parasitaemia (parasites/µl)	Species (Microscopy)
1	12	F	21	36,0	153	*P. malariae*
2	27	M	11	36,0	2.610	*P.f + P.m*
3	39	M	20	38,7	928	*P. malariae*
4	40	F	8	36,4	1.508	*P. malariae*
5	56	M	12	37,4	221	*P. malariae*
6	148	F	7	36,4	8.620	*P.f + P.m*
7	149	M	8	37,0	1.013	*P.f + P.m*
8	167	M	12	36,4	667	*P. malariae*
9	268	M	14	36,0	831	*P.f + P.m*
10	269	F	21	36,8	503	*P.f + P.m*
11	325	M	15	36,9	1.237	*P. malariae*
12	326	F	41	36,9	773	*P. malariae*
13	392	F	16	37,0	2.960	*P. malariae*
14	548	F	28	37,0	154	*P. malariae*
15	589	M	39	36,0	677	*P. malariae*
16	627	F	46	unknown	2.175	*P. malariae*
17	678	M	11	37,3	1.856	*P.f + P.m*
18	742	M	16	36,9	2.436	*P. malariae*

3.3 Molecular confirmation of screening results

Due to the relative inaccuracy of speciation in *Plasmodium spp.* by light microscopy, it was decided to confirm the results from the slide readings by performing PCR analysis on all isolates included in the assays.

Unfortunately, the PCR analyses set up to determine the parasite species only worked in part. While it was possible to prove presence of *P. malariae* DNA in all samples (see **Figure 5**) that were claimed to be infected with it, the *P. falciparum* and *P. ovale* detection assay (assay (a)) failed to yield interpretable results (see **Figure 6**).

As can be seen in the *P. malariae* detection assays, the cycler ought to be able to capture an increasing intensity of probe signals in positive samples as it binds to more and more of the DNA replicated throughout the PCR. Instead, in the non-*malariae* assays, no such signal was captured, not even in the control. This could be a consequence of a multitude of causes, but likely either faulty reagents or unsuccessful DNA extraction were to blame. Unfortunately, due to constraints of assay material it was not possible to repeat the experiments.

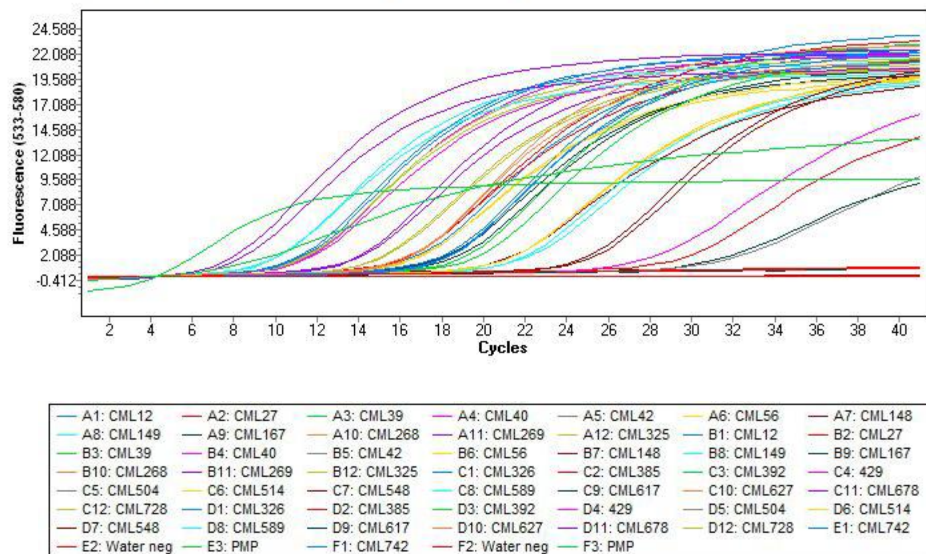

Figure 5: Results of the *P. malariae* speciation assay: Note the significant signal responses from all samples microscopically identified as *P. malariae*

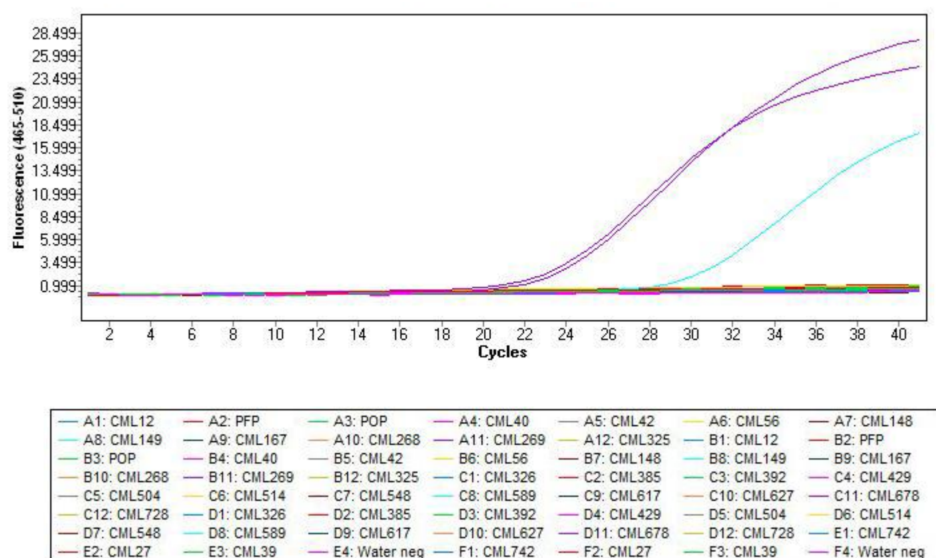

Figure 6: Results of the (failed) *P. falciparum* speciation assay: Note the distinct lack of significant responses, especially from the *P. falciparum* control wells (red lines). The responses from some of the non-control wells can therefore be discarded.

As is, while all isolates examined by PCR could be confirmed to contain *P. malariae*, it was not possible to accurately identify co-infections with other species of *Plasmodium.*

3.3 *Ex-vivo* drug assays

Blood samples taken from study participants screened as positive for *P.* malariae infection were prepared as described and used for dosage of the 96-well-plates used for the drug assays. By comparing the relative growth of schizonts in the different wells as compared to the drug-free control, it was possible to estimate the inhibitory effect of the drugs on the parasite's growth. By statistical analysis of schizont count throughout a drug's dilution series, an inhibitory value for each drug was calculated and compared to those of the NF54 control known to be susceptible to all agents utilised. If a calculated IC-value were to be significantly higher in a study isolate compared to the control, this would have indicated

reduced susceptibility. A complete lack of growth inhibition would have indicated total resistance.

Of the 28 study participants screened as infected with *P.* malariae, 19 study participants met the inclusion criteria. From those, 18 blood samples were collected and used for assays set up to investigate the drug susceptibility. 15 showed successful inhibition of schizont maturation. Using the WHO schizont maturation assay, the *ex-vivo* IC_{50}-values for the used drugs; LUM, AS, ATQ and CQ were calculated. **Figure 7** shows an overview of the study progress.

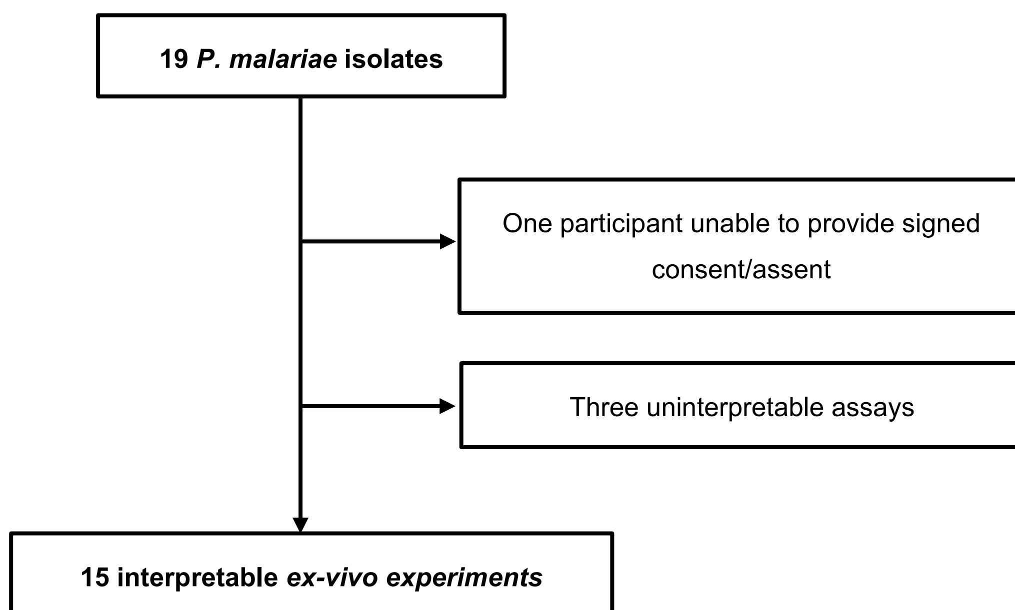

Figure 7: Flowchart of the P. malariae isolates used in the ex-vivo assays

The geometric mean of the IC_{50}-values was 8,67 nM, with a median of 8,33 nM in CQ; 3,74 nM with a median of 3,21 nM in AS; 4,90 nM with a median of 4,05 nM in ATQ and 8,56 nM with a median of 9,53 nM in LUM. Except for LUM, these values were similar to those calculated for the NF54 control at 7.42 nM, 3.46 nM, 4.9 nM, and 4.69 nM, respectively. For LUM, the mean IC_{50}-value was about twice as high as for the NF54 control. All isolates except CML627 were shown to be fully susceptible to the drugs tested. In CML627, hints towards reduced susceptibility to LUM could be observed, its calculated IC_{50}-value of 26.58 nM being more than quintuple of the NF54 control's. As the wells showing no

parasite maturation also showed extensive signs of RBC lysis, this could indicate total resistance in the isolate. This was observed throughout the experiment at LUM concentrations up to 50 nM. As such, LUM could have shown itself to be inadequate to treat CML627's infection *in-vivo*. Also, in CML742, the AS dilution series could not be interpreted due to a contamination of the working solution. Are more detailed overview can be found in **Table 14**.

An overview of the individual calculated IC_{50}-values for each drug can be found in **Figure 8** and **Table 15**, respectively.

Table 14: Calculated IC values for each drug tested (in nM) – while 15 interpretable assays were created, one had to forego its AS series due to a suspect contamination of the WS (marked with '*')

		CQ	**AS**	**ATQ**	**LUM**
Valid Values		15	14*	15	15
IC_{50} (nM)	**Mean** (95% CI)	**8,67** (6,4 – 12,51)	**3,74** (2,83 – 5,64)	**4,68** (2,92 – 8,37)	**8,56** (6,53 – 13,38)
	Median	8,33	3,21	4,05	9,53
IC_{90} (nM)	**Mean** (95% CI)	**47,48** (37,19 – 69,14)	**18,97** (13,74 – 30,36)	**22,82** (18,24 – 30,65)	**39,26** (33,99 – 49,49)
	Median	44,23	18,04	20,77	40,69
IC_{99} (nM)	**Mean** (95% CI)	**303,70** (170,64 – 669,50)	**111,74** (71,89 – 217,76)	**128,73** (99,20 – 198,06)	**206,98** (160,77 – 320,42)
	Median	270,05	129,02	119,96	206,54

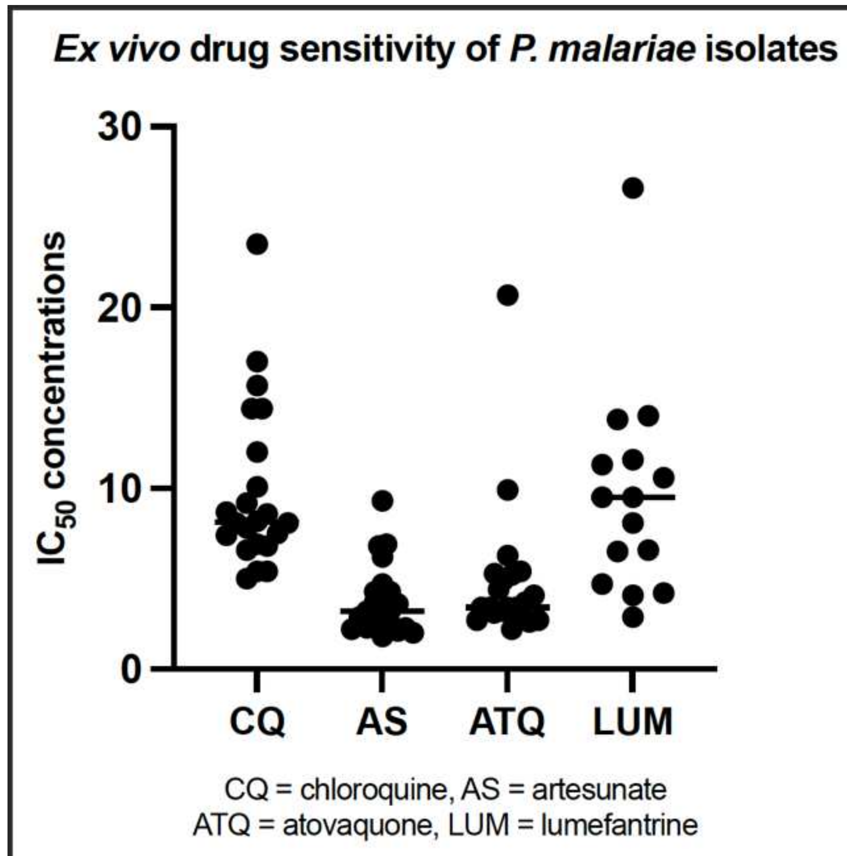

Figure 8: Spread of calculated IC_{50}-values (in nM)

Table 15: Calculated IC_{50}-values (in nM) of each isolate as well as the NF54 control

CML-ID	CQ	AS	ATQ	LUM
12	6,91	2,13	20,67	4,07
27	5,39	2,23	3,66	2,89
148	8,59	6,77	5,28	8,05
149	17,01	3,69	3,42	4,24
167	6,58	6,24	4,43	6,58
268	9,19	6,94	5,37	9,51
269	5,04	2,92	3,21	9,54
325	7,50	2,97	2,56	10,60
326	10,07	2,00	3,34	6,54
392	5,43	4,25	2,71	11,30
589	23,54	3,21	6,30	14,02
627	8,07	2,45	3,05	26,58
678	12,02	9,30	9,87	13,83
742	8,73	n/a	5,21	11,64
NF54 (control)	***7,41***	***3,62***	***4,90***	***4,69***

3.4 Flow cytometry

To investigate possible erythrocyte age tropisms in *P. malariae*, it was attempted to establish flowcytometric staining assays. Blood samples taken and induced into the ex vivo drug assays were further stained as described and used for flowcytometric analysis. Due to various difficulties in analysis, to be further

discusses in chapter 4.4, different approaches to staining had to be employed. It was continually attempted to optimise staining procedures primarily by means of changing the number of simultaneously analysed dyes and clarifying signal analysis.

3.4.1 First iteration assays

Due to the spectral overlap between the dyes and the rareness some of the markers observed – especially Annexin V – the samples derived from the first staining protocol did not provide conclusive results. Rather, the data derived from the first attempts had too much interference between the dyes to be considered interpretable by any of the standards of the following assays (example, see **Figure 9**).

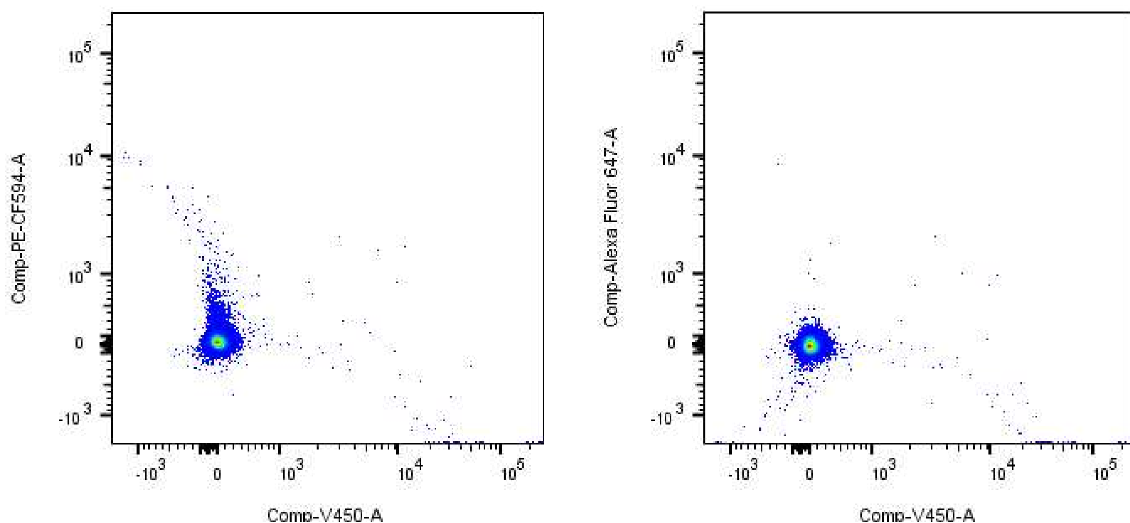

Figure 9: Example for inadequate compensation of samples acquired during first staining iteration – CML0039 CD71/Hoechst 33342 (left) and Annexin V/Hoechst 33342 (right)

In a successful assay, there would be a distinct grouping of signals allowing differentiation between captured cells positive for either or both stained markers. However, due to the aforementioned spectral overlap from the multitude of fluorescent dyes, similarly discussed in chapter 4.4, it was not possible to

separate – as pictured above – the signals captured, leading to an indistinct mix of data points.

3.4.2 Second iteration assays

To remedy this, it was decided to separate the dyes with the greatest signal overlap and to analyse them independently. Instead, they were combined only with the Hoechst 33342 stain to mark infected erythrocytes. The low overlap between Hoechst 33342 and the other dyes allowed for far more precise analysis of the *Plasmodium spp.* isolates.

Each of the observed markers was run against Hoechst 33342 individually; CD71-positive signals were identified as young erythrocytes, Annexin V-positive ones as senescent erythrocytes and CD235-positive signals were used for comparative purposes as erythrocytes of unspecified age. These were then subsequently categorised as infected (Hoechst 33342-positive) or uninfected (Hoechst 33342-negative). Afterwards, the isolate's respective populations of infected erythrocytes were compared based on their frequency in the three age groups – to estimate if the greatest number of infected cells could be found in young erythrocytes, senescent ones, or those of indeterminate age.

By comparison of marker prevalence among infected cells (i.e.: those positive for Hoechst 33342) it was attempted to infer possible preferences for age groups. If a significant tropism for a certain erythrocyte age group were to be present, it would be expected to exhibit isolated high marker positivity among infected cells. In the flowcytometric analysis this would be represented by a comparatively higher percentage of infected marker positive cells to infected erythrocytes of other age groups (i.e.: marker negative). **Table 16** Shows an overview of the invasion parameters observed.

Table 16: Overview on the staining results of assays derived from the separate staining protocol ("-" meaning that no positive signal was captured; mono-infections in bold, P. falciparum in grey undertone)

CML-ID	Percentage of marker (+) cells among infected cells (%)		
	CD71	**Annexin V**	**CD235a**
148	2.74	-	4.00
149	5.88	0.72	39.62
167	**78.46**	**8.49**	**71.07**
268	23.31	11.42	28.98
269	46.88	16.45	10.00
325	**0.65**	**2.84**	**2.38**
326	**1.37**	**10.72**	**-**
385	25.66	4.66	49.48
392	**10.84**	**6.07**	**0.71**

For CD71, on average, 21.26% of infected cells were positive for this marker. In mono-infections, a rather similar 22.83% of infected cells showed marker positivity. However, both in co- and mono-infections, the values were not statistically significant – with p=0.073 and p=0.155, respectively.

Annexin V, while exhibited by a comparably low number of infected erythrocytes, at 7.03% and 9.53% in mono- and co-infected isolates, respectively, appears statistically significant for overall infections with *P. malariae* (p=0.004) and, interestingly, mono-infections (p=0.013). It is, however, not statistically significant for co-infected isolates (p=0.08). The percentage of infected cells exhibiting Annexin V was far lower in the *P. falciparum* control – almost half as low at 4.66%.

While the percentages of infected cells exhibiting CD235a-positivity were comparable in mono- and co-infection at 24.72% and 20.65%, CD235a-positivity was shown to be statistically significant for co-infections ($p=0.044$). No statistical significance could be found for mono-infections, however ($p=0.199$). It bears mentioning that in the *P. falciparum* control, the percentage of CD235a^{+} infected cells was more than twice the average of the *P. malariae* infected isolates. Unfortunately, CD235a's main use in the assay was to serve as positive control; as such, the very low percentage of CD235-positivity is indicative of the poor interpretability of the acquired data.

In mono-infections with the parasite, Annexin V shows a weak correlation to parasitaemia ($r_{Annexin}=0.362$), with a stronger (if negative) correlation of parasitaemia to CD235 ($r_{CD235}= -0.691$). CD71 has been calculated to show a low negative correlation of $r_{CD71}= -0.447$. In co-infections, no positive and no significant correlation could be observed ($r_{CD71}= -0.144$; $r_{CD235}= -0.217$ and $r_{Annexin}= -0.374$). It must be noted that none of the calculated correlation values – regardless of mono- or co-infections – could be considered statistically significant.

However, the results of the second staining iteration were very inhomogeneous. Three assays outperformed their peers entirely (CML148, CML149 and CML167) and had some interesting findings; showing a significantly higher percentage of Hoechst 33342/CD71 double-positive signals in the *P. malariae*-monoinfected sample as opposed to both P. *falciparum*/*P. malariae* – coinfections (0,0058% and 0,0041% versus 0,051%). A visual comparison thereof can be found in **Figure 10**. These differences were even more pronounced when comparing the relative number of CD71-positive cells to the total number of Hoechst 33342 signals: 0,03 and 0,06 versus 3,64. This meant that while double-positives were a rarity in co-infections, they were the majority in the observed mono-infection. However, these findings, unfortunately, could not be replicated reliably, and are, thus, not statistically significant. Nonetheless, they might indicate a trend of increased CD71-positivity among *P. malariae*-infected erythrocytes, which could be further investigated in more optimised experiments.

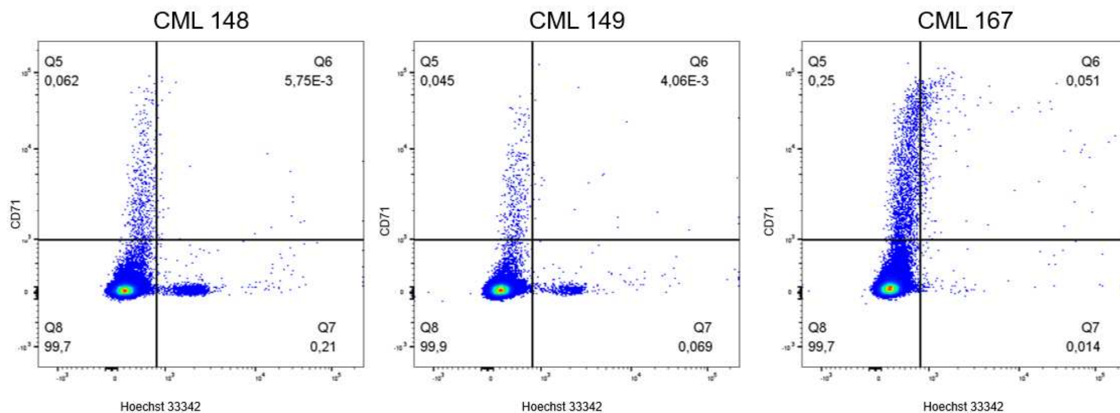

Figure 10: The three most successful assays of the second iteration: CML148, CML149, CML167. Note the significantly higher percentage of double signals in CML 167.

3.4.3 Third iteration assays

Based on the experiences made during the first experiments, it was decided to add a marker to enhance signals captured from healthy blood cells. Due to the commonality of the marker, CD47 was chosen. To exclude false positive Hoechst signals coming from remaining leucocytes in the blood pellet, it was discussed to include CD45, however, due to supply issues, the corresponding mAb never arrived and could not be used. Due to the availability of compensation beads for the creation of a proper matrix, it was decided to combine the markers again.

Thanks to the availability of proper compensation controls for the third iteration of the staining protocol, it was attempted to combine all stains once more. However, again, due to the rarity of some of the markers analysed – especially CD71 and Annexin V – relative to the fluorescence of their respective mAb – PE-Vio 615 and Alexa Fluor 647 being quite dim compared to PE and APC-Vio 770 – no viable compensation could be achieved. Regrettably, no data could be gained from the assays performed.

4. Discussion

4.1. Disease severity

Malaria remains a constant and important health hazard in Gabon. A whole 31.5% of study participants were diagnosed as infected with *Plasmodium spp.* – 92,65% of those with *P. falciparum.* These numbers are comparable to the 46% reported by Manego *et al.* (2017), but fall far short in comparison to the 83.3% claimed by Woldearegai *et al.* (2019). This difference may be explained by the latter's use of more sensitive PCR analysis – future screenings in the regions might consider using such. In regards to the respective species' frequencies of detection, this study's finding are far more biased towards *P. falciparum* as opposed to the findings published my Manego *et al.* (2017) – whereas this study classified 12.02% of study participants as non-*falciparum*, Manego *et al.* observed 23.2%. Again, both percentages fall far short of the 43.7% non-*falciparum* plasmodial DNA detected by PCR in Gabonese individuals as published by Woldearegai *et al.* (2019). As argued by Manego *et al.*, the difference could be explained by the difficulty to detect and differentiate *P. malariae* from other subspecies of *Plasmodium spp.* Therefore, it is possible that some participants of this study were mistakenly identified as uninfected or *P. falciparum*-mono-infected, especially due to *P. malariae*'s proclivity for comparatively low parasitaemia.

Of the only two individuals infected with *P. malariae* to exhibit fever, none did so while simultaneously presenting with high parasitaemia. Previous study similarly observed little correlation between parasitaemia and pyrogenicity (Roucher *et al.,* 2014). In contrast, in *P. falciparum*, there were far more cases with very high parasitaemia or fever – both in absolute numbers and relative to their respective infected population. While somewhat unsurprising, this study's findings once more characterise *P. malariae* as a more benign counterpart to *P. falciparum.*

Woldearegai *et al.* claimed that parasitaemia in *P. malariae* infections decreases with age (Woldearegai *et al.,* 2019). While our study did not provide any evidence to support this claim, the limitations posed by our rather low sample size and use

of comparatively inaccurate analysis by means of light microscopy (as opposed to the PCR method employed by Woldearegai *et al.*) need to be kept in mind.

P. malariae is commonly found in co-infections, most frequently with *P. falciparum* (Roucher *et al.,* 2014; Millner, 2018), a fact that could be supported by our findings. This ought not be too surprising, as by the mere frequency of the different species of *Plasmodium*, a co-infection with a non-*falciparum*-species would be statistically unlikely. In fact, throughout this study, no other members of *Plasmodium spp.* were observed. While our PCR unfortunately was unable to analyse the prevalence of non-*malariae* species in our samples, even by microscopy, 39.3% of our *P. malariae* isolates were found to be co-infections – all of them with *P. falciparum.* Due to the higher sensitivity of PCR concerning parasite detection, it can be assumed that this number might have been greater (Gimenez *et al.,* 2021).

A recent hospital-based surveillance study from Indonesia by Langfort *et al.* (2015) observed a median age of 22 years in *P. malariae* infected individuals. In our study, the median age was lower at 14.5 years. However, in mono-infected study participants, the median age was very similar at 20 years. The lower median age in co-infected individuals may be explained by *P. falciparum*'s tendency to primarily affect infants and children (Woldearegai *et al.,* 2019; WHO, 2023). As most reported cases of malaria are from this age group this might be indicative of a high number of clinically inapparent infections with *P. malariae* in infants and young children. Most of these study participants were brought to our field workers by their mothers out of concern for their health – seemingly healthy children with asymptomatic *P. malariae* infections might not have been screened. The same can be readily assumed from the adult population. There is little reason to assume an age tropism of the host themselves, as the spread amongst the different age groups is rather homogenous.

In summation, while benign compared to the more common *P. falciparum, P. malariae* remains an altogether common threat to public health in Gabon. While its clinical presentation throughout this study does not give cause to immediate

and overwhelming concern, it is still a disease demanding intervention. For this, further surveillance and study will be required.

4.2 Drug susceptibility

This study aimed to evaluate ex-vivo resistance of *P. malariae* to four of the common antimalarial drugs currently employed – artesunate, chloroquine, lumefantrine and atovaquone. This study's mean IC_{50}-values of 3.74 nM, 8.67 nM, 8.56 nM, and 4.68 nM, respectively, do not suggest widespread resistance to these drugs in rural Gabon – if any.

While for ex-vivo drug assays on *P. malariae* are sparse, there is a bevy of data on *P. falciparum*. Recent studies in the nation's capital of Libreville reported IC_{50}-values for artesunate of 2.9 nM (Pradines *et al.*, 2001); whereas studies from Lambaréné reported values of 2.1 nM (Kurth *et al.*, 2009). Both are similar to this work's calculated values of 3.7 nM. Therefore, similar, and continued susceptibility to AS in both *P. malariae* and *P. falciparum* in Gabon can be assumed.

Due to widespread resistance in *P. falciparum,* chloroquine's main contemporary use is in the treatment of uncomplicated non-*falciparum* malaria. While – at the time of writing – only six papers published data on drug susceptibility in *P. malariae*, there were reports of diminished chloroquine susceptibility in isolates from Indonesia (Maguire *et al.*, 2002). However, no such resistance, partial or otherwise, could be determined in this study's isolates. Chloroquine remains an efficacious compound for the treatment of – at least the most common – non-*falciparum* malaria in Gabon. Considering the commonality of CQ-resistance in *P. falciparum* and observed co-infection rates of the two species of *Plasmodium*, these findings may also imply a lack of cross-species transferrance of resistances.

Despite numerous reports of decreasing susceptibility of *Plasmodium spp.* to lumefantrine, it remains among the primary antimalarial drugs employed today. Previous studies by Dembele *et al.* (2021) showed frequent resistance to the drug in *P. malariae*, showing a median IC_{50} of 681.3 nM. While these findings do

indeed prove worrying for their respective country of Mali, this work's findings do not corroborate them for Gabon. However, it bears mentioning that in one isolate, CML627, the calculated IC_{50}-value was disproportionately higher than average at 26.58 nM. The reason for the lysis in the first dilutions of the LUM assay is most likely to be found within the high concentration of DMSO in the WS – in the first well it reached 5% – necessitated by the low solubility of lumefantrine. Due to this deficit in the drug assay's dosing – the high DMSO concentration necessitated by the low solubility of lumefantrine's powdered form preventing schizont growth possibly more so than the drug itself during the first two to three wells of each dilution series – this very isolate may indeed have been resistant to LUM in vivo. Consequently, while resistance to lumefantrine does not appear to be common in *P. malariae* in Gabon, some few strains may already have adapted to this drug. Additionally, as – even excluding CML627 as an outlier – the mean *P. malariae* IC_{50}-value of 7.84 nM was about half again as high as the NF54 control's value of 4,69 nM, continued surveillance of emerging reduced susceptibility to lumefantrine is necessary.

Atovaquone might be of greatest importance to western travellers and scientists alike, as most malaria-naïve individuals are recommended to take ATQ as chemical prophylaxis during their stays to endemic countries. For *P.* falciparum, some strains have previously been reported to exhibit reduced sensibility (Staines *et al.*, 2017). However, in most cases, it still retains its effectiveness both in the WHO African and South-East Asian Regions (Staines *et al.*, 2017; Lek *et al.,* 2022). While there have been reports of burgeoning resistance to ATQ for *P. malariae* (Müller-Stöver *et al.*, 2008), the IC_{50}-values calculated based on our findings were consistent with those published by Pradines *et al.* (2001) – indicating similar susceptibility to the drug in both *P. falciparum* and *P. malariae*.

All in all, all four compounds appear efficacious in the treatment of *P. malariae*-malaria, with no reason to assume reduced effects of current treatment guidelines. However, close monitoring remains necessary.

4.3 Limitations of susceptibility assay's results

Both *ex vivo* and *in vitro* assays are a common, tested, and reliable method for analysis of all kinds of pathogens without the possibility for endangerment of sentient life as in *in vivo* studies. Both can and are regularly used to investigate and improve on clinical findings, such as screening for possible drug resistances. However, some limitations inherent to the method itself cannot be avoided and need to be addressed.

For one, the WHO method is both time intensive and highly dependent on the readers skill and acuity, which limited the number of assays that could be set up per identified *P. malariae* isolate. As opposed to microscopic analysis of parasite growth, a more sensitive and objective method – like histidine-rich protein-2 enzyme linked immunosorbent assays (HRP-2 ELISA) or SYBR green I fluorescence which are both used in detection of *P. falciparum* – might have produced more precise results.

Wilson *et al.* found that sensitivity of *Plasmodium spp.* against various antimalarial agents depended on the individual parasite's stage in its life cycle (Wilson *et al.,* 2013). Since the parasites in this study were not synchronised – out of fear that such procedures might inhibit the already suboptimal growth rate of *P. malariae* – many different stages of the parasite were present in the patient's blood. Due to the slow-growing nature of *P. malariae*, this would largely be comprising of merozoites and schizonts – as opposed to ring stages being dominantly seen in *P. falciparum* infections (Collins & Jeffery, 2007). While all blood samples were directly placed into a (mobile) incubator after being taken, not all samples spent the same amount of time in said incubator. As such, even among the different *P. malariae* isolates, the "average" cell cycle observed might have differed. Interestingly, this could have been alleviated by the previously mentioned HRP-2- and SYBR-1-like assays, as those permit immediate *ex vivo* analysis, foregoing the loss of parasite populations due to culture adaptation (Chaorattanakawee *et al.,* 2013).

Furthermore, at the moment, no reliable medium for proper *P. malariae* cultivation is known (Collins & Jeffery, 2007). Therefore, the schizonticidic properties of the

drugs administered might not be entirely due to the inhibitory effect of the agents themselves, but rather due to improper growth conditions. While this is part of the nature of *ex vivo* analyses, it is of even greater importance in fickle and more sensitive species, such as *P. malariae*. This was further exacerbated by the very high DMSO concentration in the LUM dilution series; with obvious signs of lysis being seen in some wells.

Lastly, the number of *P. malariae* isolates studied is still quite low, a consequence of both the parasite's relative frequency and the limited timeframe of sample acquisition.

4.4 Challenges of flowcytometric analyses for investigation of cell tropisms

Throughout this work, the assays conducted suffered from some core flaws; foremost a suboptimal fluorophore panel design. Of the five dyes used, only three (CD71, CD235a, and CD47) could be used for generation of precise compensation controls. This was rather problematic, as especially CD235a was a far more common marker and any spillover could have significantly impaired analysis of the rarer, and less optimally compensated Annexin V and Hoechst 33342 signals.

This was additionally compounded by the fact that Hoechst 33342 is best excited as part of a 355 nm laser line, a line that the FACSymphony™ A1 used in this work simply did not possess. As such, it is likely that many parasites marked by this dye were not represented in the ensuing signal detection. Worse yet, since leucocytes were not depleted out of fear of losing the already rare parasites, Hoechst 33342-positive signals could also been attributed to nuclei of white blood cells, which, at the time of writing, are not known viable targets for invasion by *Plasmodium spp.*

Annexin V primarily binds to phosphatidylserine, which is externalised both prior to orderly removal by the immune system. However, being a molecule that usually resides on the inner part of a cell's membrane, it is also an available target when said membrane is fractured in its entirety. Consequently, the Annexin V used in

the assay might have primarily bound to cell detritus or similarly dead cells and thus been excluded from analysis by the (forward) side scatter threshold.

Moreover, *P. malariae*'s infection dynamics further complicate analysis by flow cytometry. Due to the aforementioned difficulty of leucocyte depletion in *P. malariae* isolates, the percentage of stained parasite nuclei among the corresponding signal would be rather low, as – due to the low average parasitaemia – most signals would be attributable to white blood cells. As a calculation example: in this study, the highest recorded parasitaemia was 2.960/µl (CML392). As 10 µl of whole blood were used for staining – assuming consistent parasitaemia at time of sampling – this would have provided the sample with a maximum of about 29.600 parasites to be analysed. This also only holds true further assuming a complete staining of all available parasites (which, unfortunately, is neither guaranteed nor likely). Assuming the average leucocyte count for sub-Saharan men of 5.3 x 10^9/L (Smit *et al.*, 2019), these would contend with about 53.000 leucocytes for staining and signal detection. This also assumes a lack of elevated leucocyte levels as a reaction to infection. Moreover, in general signal detection, again assuming average RBC count of 4.5 x 10^{12}/L for sub-Saharan males (Smit *et al.*, 2019), these signals would share a sample with 45 million erythrocytes. If one were to assume even further that all available parasites had successfully invaded available erythrocytes, at best, 0.00066% of RBCs would be infected. As, on average, about 1 million events were captured during flow cytometry, only at most 658 parasites could possibly be observed under optimum conditions. Considering the mean parasitaemia for *P. malariae* infections observed in this study was 954.12/µl, this number would be even lower in most isolates. Due to the presence of leucocytes, any detected nuclear stain would additionally have a 55.84% chance of being a stained leucocyte. In *P. falciparum,* this could be remedied both by leucocyte depletion and ex vivo enrichment of parasite count through culturing, however, these methods are not available for *P. malariae*.

Despite these difficulties, a more optimised dye panel would have helped to better investigate *P. malariae*'s supposed tropism. For example, to remedy Hoechst 33342's unviability for the utilised flow cytometer, SYBR Green I could have been

used for parasite detection. It has previously been successfully used in culture enumeration for *P. falciparum* (Jang *et al.*, 2014) and, exhibiting its excitation maximum by a 488 nm laser line, would be viable for the FACSymphony™ A1. Additionally, the use of CD45 for exclusion of leucocytes was discussed, but, due to the then-ongoing SARS-CoV19 epidemic it was logistically impossible to conduct such changes within the given timeframe.

4.5 Age tropism of *P. malariae*

The tropism towards senescent erythrocytes, as described by Kitchen (1939), appears rather unlikely. In their work, Kitchen made their assumptions on the grounds on age specification of erythrocytes by light microscopy, and included a mere three *P. malariae* isolates. Even in comparison to reticulocytes, it is rather difficult to correctly determine an erythrocyte's age by light microscopy alone. Furthermore, senescence is a rather ill-definable state for a RBC to be in, as its respective life cycle is dependent on a multitude of factors, amongst which external stress – as could be caused by a parasitic infection, for example – makes up a major one. Furthermore, exhibition of phosphatidylserine functions as a 'eat me' signal for the host's immune system, vastly limiting the erythrocyte's remaining life span. The longer-than-usual life cycle of the *P. malariae* parasite appears to contradict this, as the pathogen would be far more limited in its replication than the 72 hours observed.

This study's observation of a positive correlation between Annexin V positivity and parasitaemia is not likely to be indicative of a true tropism for senescent erythrocytes – the higher the parasitaemia, the greater the stress on the host's body and circulatory system. Even if this correlation would have been found to be statistically significant, Annexin V has shown to be an unreliable marker for erythrocyte senescence and should be seriously reconsidered as a marker for senescence. Since it is more associated with cell death in general, it is of little surprise that the number of erythrocytes entering senescence and cell death increases with disease burden, even with a comparatively benign pathogen as *P. malariae*. In fact, in the few more successful assays, a trend towards CD71-

positive cells could be interpreted. A tropism for reticulocytes would – in part – explain the lower disease burden observed in *P.* malariae, as the parasite would be limited in its available cell pool. It would also be somewhat common in *Plasmodium spp.*, since many species show such preferences.

The low CD235a-positivity is, unfortunately, indicative of the poor quality of the assays performed, as nigh every erythrocyte should exhibit this marker. Additionally, as the blood was not cleared of leucocytes, which also exhibit CD235a, false negatives could have occurred.

Regardless, this work's attempts to establish appropriate staining protocols for flowcytometric analysis have proven to be only partially successful; only the most basic groundwork could be laid and no truly reliable data for either side of the issue could be gleaned.

4.4 Outlook

Irrespective of the previously stated continued susceptibility of *P. malariae* to common antimalarials and its relatively benign clinical presentation, current knowledge on the parasite remains limited, especially in comparison to its more infamous cousins; *P. falciparum* and *P. vivax*.

Attempts at gained reliable data on *P. malariae* encounter various challenges. Foremost amongst these is the current inability to adapt the parasite to ex vivo cultures. As a result, essential processes for accurate analysis of the parasite's life cycle and physiological peculiarities – like enrichment, cycle synchronisation, or experimental transmissions – are made nigh impossible. The identification of a strict tropism as observed in *P. vivax* may prove to be a key in unravelling the mystery of *P. malariae*'s reticence towards culturing.

Another reason for the neglect shown towards *P. malariae* might be its difficulty of identification. As previously stated, speciation by light microscopy is highly dependent on the analyst's skill, experience, and equipment. Consequently, especially considering the finding of Woldearegai *et al.* (2019), a far higher prevalence of *P. malariae* than previously though can be assumed. To remedy

this, the use of an adequate rapid detection kit like those in use against *P. falciparum* would be highly beneficial. While *P. malariae* is not known to express HRP-2 – the main target for *P. falciparum* rapid detection kits – other, reliable methods allow sensitive detection of non-*falciparum* parasites. Gendrot *et al.* (2022) reported that detection kits utilising pan-malaria lactate dehydrogenase (pLDH) were capable of accurately detecting *P. malariae* infection in up to 95.2% of cases. Kho *et al.* (2022) also showed that in multiplex PCR, both *Pf*-LDH and pLDH show high sensitivity and specificity (96.2% sensitivity, 97.1% specificity; and 90.2% sensitivity, 99.0% specificity; respectively) in detection of *P. malariae* from whole blood or even solely plasma samples. Therefore, this protein might be a suitable target for future assays.

Contrary to the previously made points, one could also entertain the thought of reviving malariotherapy utilising *P. malariae* as a possible agent, especially in regards to vaccination efforts. Cross-species immunologic resistance to the various species of *Plasmodium spp.* remains poorly understood, more so for *P. malariae*. However, should infection with *P. malariae* imbue the host with partial resistance to *P. falciparum*, its relatively benign effect on even naïve individuals may make it an optimal candidate for controlled infections, as it could be somewhat accurately – if populistically – described as a pre-attenuated version of *P. falciparum.* Current attempts at inducing resistance to malaria have been largely unsuccessful; RTS,S/AS01's limited efficacy having been linked to a vaccine-induced retardation of the acquisition of natural immunity by the children's developing immune system (RTS,S Clinical Trial Partnership, 2015), for example. By deliberately triggering the children's immune system by means of *P. malariae* infection, this could be overcome. Similar to the ideas stated by the Hygiene hypothesis, controlled childhood or even neonatal exposure to *Plasmodium spp.* might possibly have a positive influence on the development of long-lasting and most importantly broadly applicable partial malaria immunity and consequently an easy, 'natural', and most importantly, affordable solution towards the goal of widespread vaccination against malaria.

While, as previously described, investigations regarding possible existing or emerging drug resistances in *P. malariae* have yielded promising results (this

work's findings amongst those), the topic of eradication avoidance is, unfortunately, a dynamic one with numerous developments, many of whom are, again, unfortunately, unforeseeable. Therefore, the impact of *P. malariae* on affected population needs to be closely monitored and regularly updated. This necessitates that studies like this one be repeated and – if possible – expanded.

All in all, the author hopes to have made apparent the urgent need for further and more in-depth study of a hitherto less popular, but in no way less fascinating species of *Plasmodium.*

5. Summary

5.1 English

Background: Malaria is a potentially deadly infectious disease which predominantly plagues developing countries. Of the five most common species of malaria infectious to humans, *P. malariae* is by far the least well studied. Considering its status as the most common non-*falciparum* species in Gabon, it most definitely should be part of any malaria eradication efforts, both local and international. However, current knowledge about its epidemiology, sensitivity to common antimalarials, and its methods of erythrocyte invasion is rather limited.

Methods: As part of the CoMal study, willing participants from the region of Lambaréné, Gabon, were screened for infection with *Plasmodium spp.* Those determined to be infected with the parasite were requested to provide a blood sample which would be used in subsequent experimentation. *Ex vivo*-assays, based on the WHO schizont maturation assay, were used to investigate the inhibition of schizont growth by chloroquine, artesunate, atovaquone and lumefantrine. Moreover, specific surface markers of the erythrocytes were stained by means of monoclonal antibodies to assess the suspected tropism of *P. malariae* by means of flowcytometry. All samples were initially identified by means of light microscopy and later confirmed by PCR.

Results: 748 individuals were enrolled in this study between April and August 2022. Of those, 233 (31.5%) were screened positive for infection with *Plasmodium spp.* In total, 28 (12.02.%) were identified as being infected with *P. malariae*, 17 (7.30%) of which were mono-infections. No hospitalisations or fatalities were recorded. Of those infected with *P. malariae*, only two exhibited fevers.

Of the 28 infections with *P.* malariae, 18 could be included in further experiments. 15 ex vivo drug assays were successfully concluded. Ex vivo drug susceptibility testing demonstrated no reduced susceptibility to chloroquine, artesunate, or atovaquone. One sample demonstrated reduced susceptibility to lumefantrine. PCR was able to confirm *P. malariae* presence in all 15 samples used for ex vivo drug assays.

Of the 15 isolates used in ex vivo testing, nine produced interpretable data for investigation of possible erythrocyte age tropism. Flowcytometric staining was unable to produce reliable results, with only a significant association between erythrocyte invasion and Annexin V exhibition. Singular observations suggest high percentages of $CD71^{+}$ infected cells. Further optimisation of methodology will be required prior to definitive, statistically significant, results.

Conclusion: *Plasmodium malariae* is a commonly observed pathogen and public health hazard in rural areas of Gabon. The IC_{50}-values derived from this work indicate a significant effect of the observed compounds on the growth of the parasite. Thus, they appear to be applicable in the treatment of *P. malariae* infections.

The drug's efficacy has been shown in both this and previous works. Therefore, it seems prudent to recommend the continuation of the assays; both to further the data pool and to mitigate the statistical effect of the relatively low sample size.

A tropism of *P. malariae* could not be unequivocally proven, however, the investigation of the unsuccessful assays has led to the hypothesis of a target tropism for younger, nascent erythrocytes. Final confirmation for such a perk would have to be realised in subsequent works.

In summation, this work hopes to have furthered the knowledge of the parasite's biology and life cycle and – perchance – contributed to the mitigation of the disparity in awareness between the different species of *Plasmodium spp.*

Maxim Viehweg Prof. Dr. med. Steffen Borrmann

5.2 Deutsch

Hintergrund: Malaria ist eine potentiell tödliche verlaufende Infektionskrankheit, welche vor allem im globalen Süden präsent ist. Von den fünf häufigsten humanpathogenen Spezies des Malariaparasiten ist *P. malariae* mit Abstand der am wenigsten untersuchte. Dennoch sollten auch seltenere Spezies des Krankheitserregers Teil der Eliminierungsstrategien sein, insbesondere angesichts der Tatsache, dass *P. malariae* die häufigste nicht-*falciparum* Malariaspezies in Gabun ist. Jedoch ist über die Epidemiologie, Sensitivität von *P. malariae* gegenüber den üblichen verwendeten Schizontiziden und seine Mechanismen der Erythrozyteninvasion ist bisher wenig bekannt.

Methoden: Im Rahmen der CoMal-Studie wurden willige Probanden aus der Region Lambaréné in Gabun auf Malaria untersucht. Bei positivem Screening-Ergebnis wurden sie um die Abgabe einer Blutprobe gebeten, welche im Rahmen dieser Arbeit weiter untersucht wurden. Mittels *ex vivo*-Assays, abgeleitet von den Schizonten-Reifungstests der WHO, wurde die Empfindlichkeit des *P. malariae* Parasiten gegen Chloroquin, Artesunat, Lumefantrin und Atovaquon getestet. Ferner wurde immunologische Färbung spezifischer Oberflächen-marker am infizierten Erythrozyten verwendet, um etwaige Invasionspräferenzen mittels Flowcytometrie zu eruieren. Alle Proben wurden zunächst vorrangig mittels Mikroskopie untersucht und klassifiziert; eine eindeutige Identifikation der Parasitenspezies erfolgte mittels PCR.

Ergebnisse: 748 Personen wurde zwischen April und August 2022 in die Studie aufgenommen. Von diesen wurden 233 (31.5%) positiv auf Infektion mit *Plasmodium spp.* gescreent. Ingesamt 28 (12.02%) waren mit *P. malariae* infiziert, 17 (7.30%) waren Monoinfektionen. Hospitalisierungen oder Todesfälle wurden nicht beobachtet. Von den mit *P. malariae* infizierten Individuen präsentierten sich zwei mit Fieber.

Von den 28 Infektionen mit *P.* malariae konnten 18 erfolgreich in weiteren Experimenten verwendet werden. 15 ex vivo drug assays konnten erfolgreich durchgeführt werden. Ex vivo Testungen auf herabgesetzte Empfindlichkeit gegenüber Chloroquin, Artesunat oder Atovaquon zeigten keine Hinweise

hierauf. Ein Isolat zeigte eine herabgesetzte Empfindlichkeit gegenüber Lumefantrin. Die Präsenz von *P. malariae* konnte mittels PCR in allen verwendeten Isolaten nachgewiesen werden.

Von den 15 Proben, die in der ex vivo Testung Verwendung fanden, konnten 9 für die Untersuchung eines möglichen Erythrozytenalterstropismus von *P. malariae* verwendet werden. Flowcytometrische Analysen ergaben keine statistisch signifikanten Ergebnisse, einzig eine Assoziation zwischen Annexin V-Exprimation und Infektion konnte beobachtet werden. Einzelne Färbungen weisen auf hohe Anteile von $CD71^{+}$ Zellen unter den infizierten hin. Zur Gewinnung endgültig belastbarer Ergebnisse bedarf es weiterer Optimierungen der Methodik.

Zusammenfassung: *Plasmodium malariae* ist ein häufiger Erreger und damit eine Gefahr für die öffentliche Gesundheit Gabuns. Die IC_{50}-Werte dieser Arbeit suggerieren eine adäquate Wirkung der verwendeten Substrate auf den Parasiten. Sie scheinen für die Therapie der Malaria quartana geeignet.
Die Wirksamkeit der untersuchten Medikamente wurde bereits zuvor in Untersuchungen bestätigt; aufgrund der niedrigen Probenanzahl und möglicher evolutionärer Dynamiken sollten ähnliche Versuche dennoch in der Region weitergeführt werden.
Ein eindeutiger Tropismus von *P. malariae* konnte nicht abschließend bewiesen werden. Allerdings lassen einzelne Färbungen den Verdacht einer Invasionspräferenz für junge Erythrozyten aufkommen. Zur endgültigen Feststellung müsste die Fragestellung in nachfolgenden Arbeiten weiter untersucht werden.
Insgesamt hofft der Autor, Interesse an diesem eher seltenen Erreger geweckt und womöglich auch einen kleinen Teil zur Überbrückung des Wissensdefizits gegenüber den anderen Vertretern von *Plasmodium spp.* Beigetragen zu haben.

Maxim Viehweg Prof. Dr. med. Steffen Borrmann

6. Publications

The results of this study are part of the following publication:

Pinilla, Y.T.*; Hoffmann, A.*; Viehweg, M.*; Saison, N.; Sambe, S. T. B.; Ndalembouly, A. G. D.; Ngossanga, B.; Awamu, F.; Adegnika, A. A.; Borrmann, S. (2025) 'Ex Vivo Drug Susceptibility of *Plasmodium malariae* Isolates to Antimalarial Drugs in Gabon.' *Pathogens*; 14(5):453.

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10. Erklärung zum Eigenanteil

Die Arbeit wurde am Centre de Recherches Médicales du Lambaréné (CERMEL) mit Unterstützung durch Abteilung VII Tropenmedizin des Universitätsklinikums Tübingen unter Betreuung von Prof. Dr. Steffen Borrmann und Dr. Florence Awamu durchgeführt.

Die Konzeption der Studie erfolgte durch Prof. Dr. Steffen Borrmann, Prof. Dr. Ayola Akim Adegnika und Dr. Florence Awamu.

Sämtliche in dem Zeitraum der Studie entnommenen Proben der übergeordneten CoMal-Studie (n=748) wurden durch mich fixiert, gefärbt und lichtmikroskopisch ausgewertet.

Sämtliche Versuche wurden nach Einarbeitung durch Dr. Florence Awamu von mir mit freundlicher Unterstützung durch die obig Genannte durchgeführt. Dies betrifft die ex vivo drug assays (n=19), die immunologische Färbung und Analyse mittels FACS (n=16), die PCR-Analyse (n=94) und die Unterhaltung der NF54-Kontrollkulturen (n=1).

Die statistische Auswertung erfolgte eigenständig durch mich.

Ich versichere, das Manuskript selbständig verfasst zu haben und keine weiteren als die von mir angegebenen Quellen verwendet zu haben.

Tübingen,

____________________ __________________________

Datum Unterschrift

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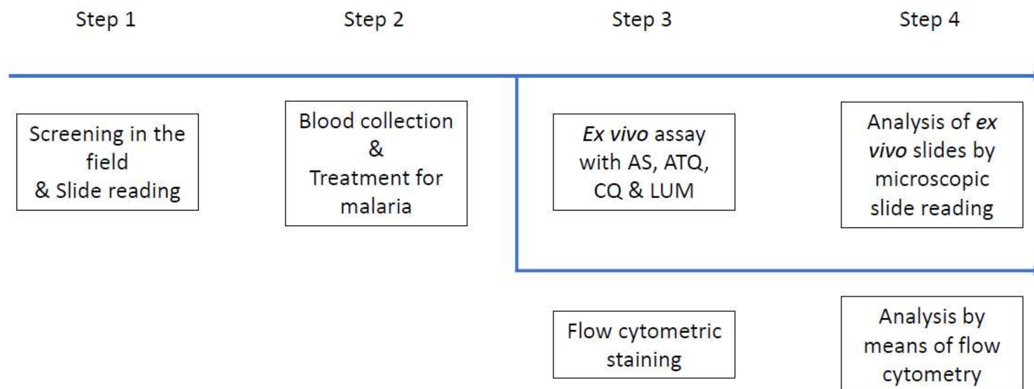
Appendices

Appendix 1

Flowchart with all study activities.



Appendix 2

Criteria for inclusion:

- Participants need to be aged five years and above for the blood collection.
- Informed consent from a parent or legal guardian.
- (For *ex vivo* assays) parasitaemia >500/µl

Criteria for exclusion:

- Refusal of informed consent.
- Negative for *P. malariae.*
- Anaemia with haemoglobin level less than 6 g/dl.