Aus der

Medizinischen Universitätsklinik Tübingen

Innere Medizin VII

Institut für Tropenmedizin, Reisemedizin, Humanparasitologie

Epidemiology of Plasmodium malariae in Gabon and Cameroon

Inaugural-Dissertation
zur Erlangung des Doktorgrades
der Medizin

der Medizinischen Fakultät
der Eberhard Karls Universität
zu Tübingen

vorgelegt von

Beger, Felicitas Georgina

2026

Dekanin: Professorin Dr. S. Y. Brucker

1. Berichterstatter: Professor Dr. S. Borrmann
2. Berichterstatter: Professor Dr. N. Schmitz

Tag der Disputation: 13.05.2026

Mut steht am Anfang des Handelns, Glück am Ende.

- Demokrit

Table of Contents

List of Figures

List of Tables

List of Acronyms

ANOVA	Analysis of variance
CERMEL	Centre de Recherches Médicales de Lambaréné, Gabon
COMAL	*Plasmodium* species co-infections in *Anopheles* mosquitoes: a pilot study of parasite-vector interactions that define transmissions in Africa
Cp	Crossing point
CRID	Centre for Research in Infectious Diseases, Cameroon
Ct	Cycle threshold
DNA	Deoxyribonucleic acid
DARC	Duffy antigen receptor for chemokines
FCRM	Fondation Congolaise pour la Recherche Médicale, Congo
FORS	Fondation pour la Recherche Scientifique, Benin
IQR	Interquartile range
ITM	Institute for Tropical Medicine, Tübingen
HIV/AIDS	Human immunodeficiency virus infection/ acquired immune deficiency syndrome
P. falciparum / P.f	*Plasmodium falciparum*
P. malariae / P.m	*Plasmodium malariae*
PfEMP1	*Plasmodium falciparum* erythrocyte membrane protein one

qRT-PCR / qPCR	Quantitative Reverse Transcriptase Polymerase Chain Reaction
RBC	Red blood cell (erythrocyte)
RNA	Ribonucleic acid
mRNA	Messenger ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SSA	Sub-Saharan Africa
WP1	Work Package 1 (of the COMAL project)
WHO	World Health Organisation
X^2	Chi-Square Test

1 Introduction

According to the World Health Organization, malaria remains, after COVID-19, tuberculosis and HIV/AIDS, one of the deadliest infectious diseases in the world (WHO, 2023). In 2022 there were almost 250 million cases worldwide, compared to 230 million cases in 2015. This reflects the increase in the worlds' population, but also the stagnating fight against the disease. According to the WHO World Malaria Report 2023, 94% of all the global malaria cases occurred in the WHO Africa region in 2022. With case incidence stagnating, deaths from malaria are overall slowly decreasing, with around 608,000 cases in 2022. However, the death rate of children under the age of 5 years remains stable since 2015 (WHO, 2023). While there are five *Plasmodium* species infecting humans, strategies to eliminate malaria have so far been focused on the two most prevalent species, *P. falciparum* and *P. vivax* (*A framework for malaria elimination*, 2017).

Malaria is a disease that most severely affects pregnant women and children. Not only does it concern health, but it indirectly has an enormous social and economic impact, which can contribute to the cycle of poverty in countries with a high malaria prevalence. It is therefore imperative to find strategies for decreasing the burden of malaria, not only from a medical point of view but also for the economic and social prosperity of the affected countries.

As the elimination efforts against malaria have been stalling in recent years, it has become increasingly clear that in order to advance in the fight against these deadly pathogens, a concerted effort against all species, not only *Plasmodium falciparum* and *vivax*, is necessary (Lover *et al.*, 2018).

1.1 Introduction to *Plasmodium* parasites and their life cycle

Malaria is an infectious disease transmitted from female *Anopheles* mosquitoes to humans. During the female mosquitoes' blood meal, the asexual stages of the *Plasmodium* parasite, sporozoites, are injected into the human's blood stream. They find the liver and each sporozoite infects one liver cell, a

hepatocyte. Within the hepatocytes they replicate asexually, and, after 7-15 days (depending on the kind of *Plasmodium* species), release thousands of merozoites into the blood stream. These merozoites go on to infect red blood cells (RBCs/erythrocytes), inside which they replicate by mitosis, form schizonts and release 6 to 36 new merozoites, which then infect more red blood cells.

Clinical symptoms such as fever, headaches, chills and nausea only occur once the parasites enter the blood stream. The period before the parasites can be detected by a thick blood film microscopically is called the prepatent period.

For each malaria parasite, the time span between the red blood cell infection and the merozoite release into the blood stream is different and can take from 24 to 72 hours. For the parasite studied in this paper, *Plasmodium malariae*, the cycle is the slowest of all human *Plasmodium* species and lasts about 72 hours, explaining why it is called the 'quartan fever': a fever that relapses every 72 hours, parallel with the blood stream invasion by newly released merozoites.

Within 15 days of infection, some of the merozoites in the blood stream are reprogrammed to become sexual stages of the parasite. They enter the bone marrow of their host to mature, and once matured into either male or female gametocyte they enter the blood stream again, where they can be accessed by a feeding mosquito. Once inside of the mosquito's midgut, the male and female sexual stage of the parasite form an ookinete together which migrates through the gut of the mosquito into its hemolymph, where it becomes an oocyst. The oocyst in its turn produces sporozoites, the asexual form of the parasite. The sporozoites enter the mosquitoes' salivary glands and are ready to be reinjected into a human when the mosquito feeds, repeating the cycle of infection.

The life cycle of *Plasmodium* parasites can be seen in the illustration from Nilsson, Child, Buckee et. al (2015) in Figure 1.

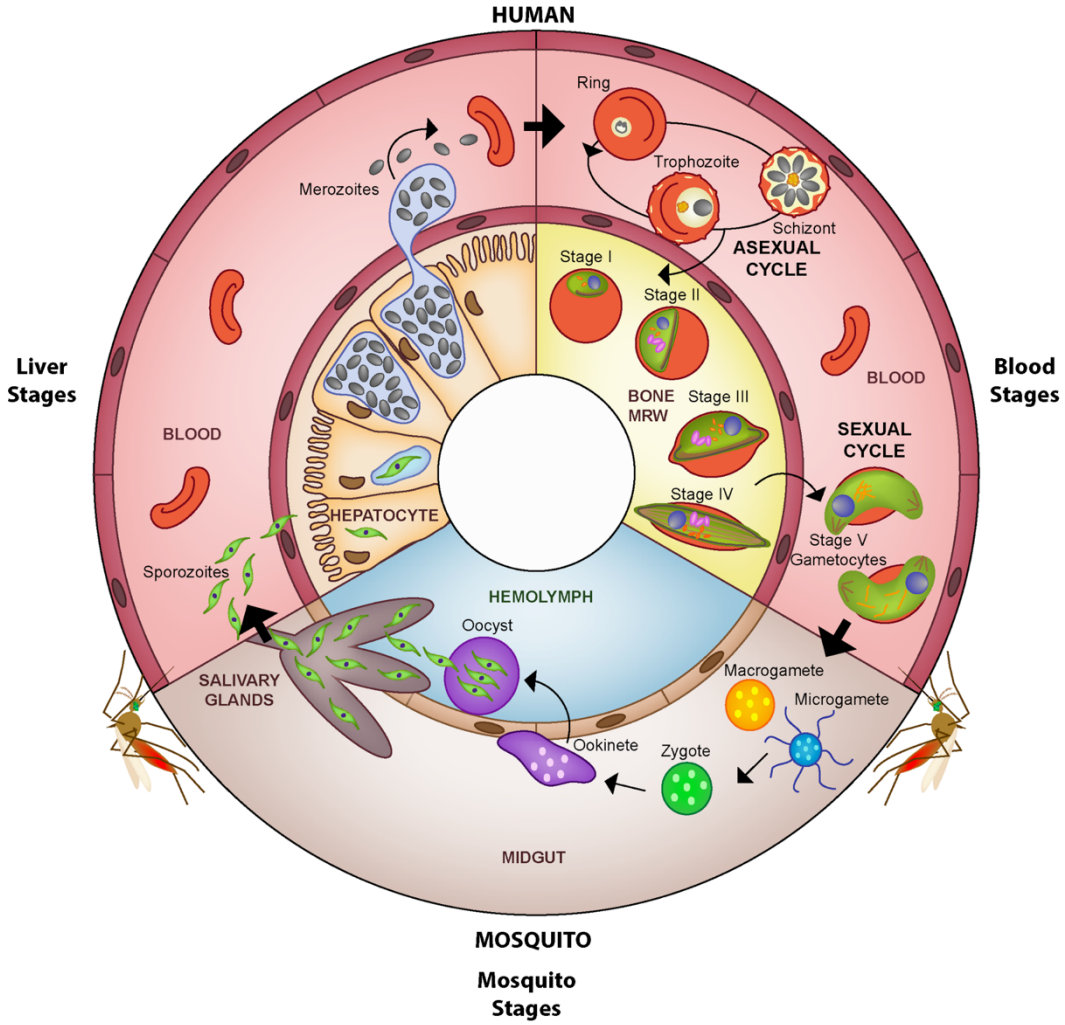

Figure 1 – Life cycle of the Plasmodium parasite, Figure created by (Nilsson et al.*, 2015) (open access)*

There are five main *Plasmodium* parasite species that cause infections in humans: *P. falciparum*, *P. vivax*, *P. ovale* spp. (*P. ovale wallikeri* and *curtisi*, in the following referred to as *P. ovale*), *P. malariae* and *P. knowlesi*.

Plasmodium falciparum is known to be the deadliest species, causing the severe so-called ‘malaria tropica’. Most research, treatment and eradication efforts in the last decades have focused on *Plasmodium falciparum* infections. It is prevalent in tropical and subtropical countries all over the world, with a heavy focus on the African continent (Weiss *et al.*, 2019).

The second most researched and common malaria parasite is *Plasmodium vivax*. It occurs mainly outside of Africa, in South America, in the Western Pacific regions and South-East-Asia (Battle *et al.*, 2019). Like the mostly in Africa and Asia occurring *Plasmodium ovale*, it does not only cause an active malaria infection but also dormant liver stages, so called hypnozoites, that can cause a relapse years after the primary infection. *Plasmodium vivax* and *ovale* both have a lifecycle that leads to fever peaks every 48 hours if left untreated, therefore having been called ‘tertian malaria’.

Plasmodium knowlesi is a parasite infecting primarily macaques. It is mainly present in South-East Asia and infections occur as a zoonosis (primate to mosquito to human). Human (to mosquito) to human transmission has not been recorded so far, while it has been shown to be biologically possible in experimental studies (Ruiz Cuenca *et al.*, 2022).

Finally, *Plasmodium malariae* causes what is known as the ‘quartan fever’, a fever every third day, and has as such been “recognized since the Greek and Roman civilizations over 2,000 years ago”(Collins and Jeffery, 2007), notably with the wording ‘quartana te teneat’ (quartan fever is tenacious). It was the first of the *Plasmodium* parasites that was microscopically detected and described as the causative agent for malaria, by the French military physician Charles Louis Alphonse Laveran in 1880 (Cox, 2010). Causing infections that can become chronic, and, as some researchers speculate, even last a lifetime, it has among others been used in the mid 1900s as a cure for neurosyphilis.

Plasmodium malariae has been known for a very long time, but there is a surprising number of facts that are still unknown about this parasite.

For instance, the prepatent period for *Plasmodium malariae* has been found to be very variable in *P. malariae* strains around the globe. Collins and Jeffery describe different findings from multiple studies that range from 16 to 59 days (Collins and Jeffery, 2007). It also remains unclear which mechanism the parasites use to enter their host cells, namely hepatocytes and red blood cells.

The parasitemia of *P. malariae* often does not reach the high levels that *P. falciparum* or *P. vivax* reach and therefore does not often induce severe episodes of malaria, but rather the typically recurring ‘quartan fever’. This supposedly “benign” level of pathology has led to the past underestimation of *Plasmodium malariae* (Snounou, Pinheiro, *et al.*, 1993; Zhou *et al.*, 1998; McKenzie, Jeffery and Collins, 2001). It has been shown however, that *P. malariae* can lead to substantial levels of chronic anemia, splenomegaly and damage to the infected hosts kidneys, inducing nephrotic syndrome (Chim CS *et al.*, 2004; Collins and Jeffery, 2007; Langford *et al.*, 2015).

Over the last years it has become increasingly clear that there is indeed a clinical and epidemiological relevance to *Plasmodium malariae*, and that it is much more prevalent than it was previously believed to be (Mueller, Zimmerman and Reeder, 2007; Sutherland, 2016; Culleton, Pain and Snounou, 2022; Mbama Ntabi et al., 2022; Nguiffo-Nguete et al., 2023).

Due to its frequently low parasitemia, it is often not detected microscopically and has been missed by many epidemiological studies in the past (Snounou, Pinheiro, *et al.*, 1993).

One of the most pressing remaining questions about *Plasmodium malariae* is its actual prevalence, especially in countries with a high burden of malaria.

1.2 COMAL project

The COMAL project (*Plasmodium* species co-infections in *Anopheles* mosquitoes: a pilot study of parasite-vector interactions that define transmissions in Africa (COMAL, study ID number: DFG BO 2494/3-1)) is a research project started in 2019 with the aim of “investigating the cryptic burden of *P. malariae*, a neglected human malaria parasite” (Recker *et al.*, 2019). Specifically, the project focuses on studying the epidemiology and transmission dynamics of *Plasmodium malariae*, as well as its vectors. In the final steps, it aims to find out about vector and parasite genetic factors that are crucial to transmission.

Under the “COMAL-umbrella” there are seven different work packages, all revolving around research targeting *Plasmodium malariae*. Work package 1 focuses on studying the epidemiology of *Plasmodium malariae*, which has not been studied comprehensively up to this point.

In June 2024, when researching for papers about *Plasmodium falciparum* on PubMed over the last 112 years, the search engine finds 46,523 research papers. In the same time span only 2,003 papers have been published on *Plasmodium malariae* on PubMed.

The difference of 44,520 papers makes it clear that *Plasmodium malariae* remains a neglected malaria parasite. To further advance in the fight against malaria it is paramount to understand not only *Plasmodium falciparum* and *vivax*, but also the other *Plasmodium* species and their epidemiology. Understanding the impact that these neglected malariae parasites have on humans will help researchers and policy makers around the world to find better strategies for the elimination of the deadly disease which is malaria.

1.2.1 Aim of this study

The current study focuses on data collected within the framework of the COMAL work package 1. This work package aims to lay the groundwork for the COMAL project and studies the epidemiology and transmission of *Plasmodium malariae* in Gabon, Cameroon, Benin and Congo.

Besides taking a closer look at the epidemiology of *P. malariae*, this dissertation will also try to answer a question about seasonality that results from findings from a big malaria field study in Nigeria in the late 1990s, the Garki project.

In the Garki project, which took a closer look at the epidemiology of the different malaria parasites present in Nigeria at the time, the findings were surprising: in the rainy season the collected data showed many cases of *Plasmodium falciparum* while in the dry season the picture changed, with most infections being caused by *Plasmodium malariae*. With almost no overlap, it almost

seemed like *Plasmodium falciparum* was suppressing the other parasite during the rainy season (Molineaux et al., 1980).

This study, as a small part of the COMAL framework, aims to tackle the question of the prevalence of subclinical *Plasmodium malariae* infections in two sub-Saharan countries, Gabon and Cameroon, and give some insights into this so far unknown terrain of research. Furthermore, using the resulting epidemiological data, it aims to take a closer look at the question of possible interactions of the two malaria parasites *P. malariae* and *P. falciparum* in human mixed infections.

This new knowledge could in the future be helpful for a targeted elimination approach against malaria.

2 Materials & Methodology

2.1 Materials

The products indicated below are the ones that are important to make the experiments reproducible. Standard laboratory equipment has not been listed.

2.1.1 Field sampling

Table 1 – Products used for field sampling

Products	Product Number	Manufacturer
PaxGene RNA collection tubes	762165	BD Biosciences
EDTA sampling tubes, e.g. S-Monovette®	SAR-041901	Sarstedt
Adapter for BD blood collection tubes, e.g. Safety-Multifly®	85.1638.235, 14.1207	Sarstedt

2.1.2 RNA extraction

Table 2 – Products used for RNA extraction

Product	Product Number	Manufacturer
Mag-Bind® PX Blood RNA 96 Kit	M7763-00	Omega Bio-tek
1.5 mL safe-lock microcentrifuge tubes	0030120086	Eppendorf
50ml Falcon® tubes	352070	Corning
20 µL, 200 µL, and 1000 µL pipettes	3120000909	Eppendorf
Aerosol resistant tips for 20 µL, 200 µL, and 1000 µL pipettes	24-404, 24-412, 24-430	Genesee Scientific
KingFisher™ plates: 96 deep well plates, 96 normal well plates, 96 tip combs for deep-well magnets	95040450, 97002540, 97002534	ThermoFisher Scientific
Isopropanol	1015-10	Linker
Nuclease Free Water	AM9939	Fisher Scientific

2.1.3 qPCR

Table 3 – Products used for performing qPCR

Product	Product Number	Manufacturer
RNase AWAY™	7003	Fisher Scientific
1.5 mL safe-lock microcentrifuge tubes	0030120086	Eppendorf
2.5 µL, 20 µL, 200 µL, and 1000 µL pipettes	3120000909	Eppendorf
Aerosol resistant tips for 2.5 µL, 20 µL, 200 µL, and 1000 µL pipettes	24-401, 24-404, 24-412, 24-430	Genesee Scientific
LightCycler® 480 Multiwell Plate 384, white including sealing foils	04729749001	Roche
Nuclease Free Water	AM9939	Fisher Scientific
Specific Primers P.f and P.m		Eurofins Genomics
Specific Probes P.f and P.m		Eurofins Genomics
SensiFAST™ Probe No-ROX One-Step Kit	BIO-76001	Bioline
LiChrosolv® Water	1.15333	Merck
Roche LightCycler® 480 II incl Roche 348 well cooling system		Roche

2.1.4 Data & statistical analysis

Table 4 – Software used for data presentation & statistical analysis

Product	Manufacturer
LightCycler® 480 Software 1.5.1	Roche
RStudio 2023.03.0-386	Posit
R 4.3.0 binary for macOS 11	R Foundation for Statistical Computing
QGIS 3.30.3 "s-Hertogenbosch'	Open-Source Geospatial Foundation (OSGeo)

2.2 Sample collection

2.2.1 Overview of the WP1 of the COMAL project

As part of the COMAL project, work package one focuses on household based cross-sectional studies across four African countries: Gabon, Cameroon, Benin, and Congo.

These cross-sectional surveys were executed two times per year, in both peak and low malaria seasons. The present study focuses on the samples collected in Gabon and half of the samples collected in Cameroon from February 2021 to February 2022.

During the cross-sectional surveys, both blood samples from the members of the participating households, as well as mosquitoes within and around the surveyed households were collected. Participants of all ages were included. Before sampling, all participants were informed about the goal and process of the study. All participants signed a consent form. Parents consented as legal guardians for their children under 18 years old.

Performing venipuncture, two venous blood samples were collected per participant: 2.5ml of blood were collected into PaxGene® tubes, and 1ml was collected into an EDTA tube.

The blood stored in the EDTA tube was used for the microscopic diagnosis of parasite infection and determining parasitemia, as well as measuring hemoglobin concentration within the participants' red blood cells, measured in g/dL.

To solidify the dataset with regards to anthropometric data, the surveyed participants' age, sex, weight, height, and tympanic temperature were measured and recorded.

The sampling process can be seen in Figure 2.

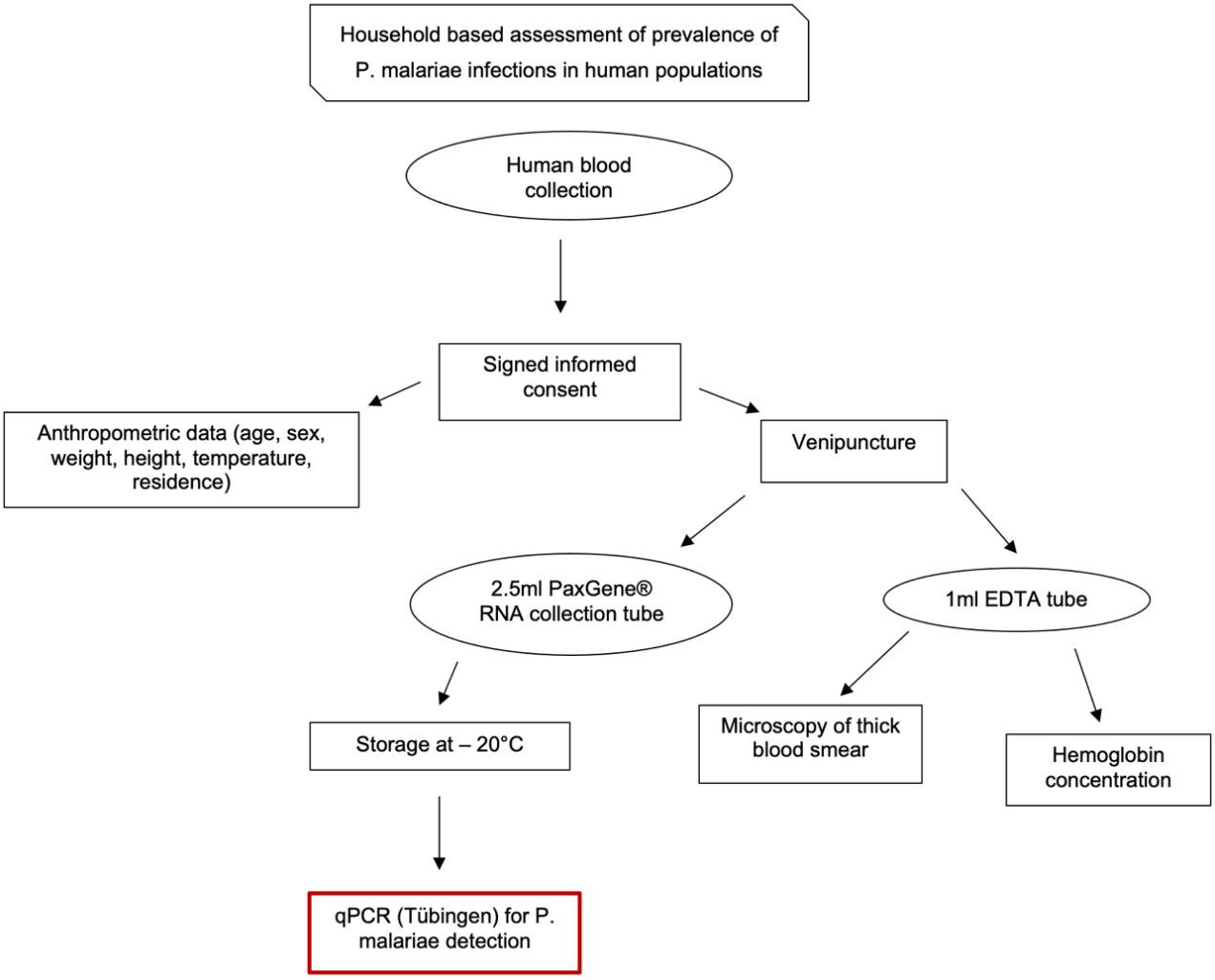

Figure 2 – Sampling process for the household-based assessment of prevalence of P. malariae infections in human populations in Gabon and Cameroon

In Gabon, samples were collected in the rural areas on the roads between Fougamou and Libreville, including Lambaréné town (Figure 3). In Cameroon the field research was focused on the communities of Mbotto, Mbougam, Mibellong, Moinkoing and Ngatti (Figure 4).

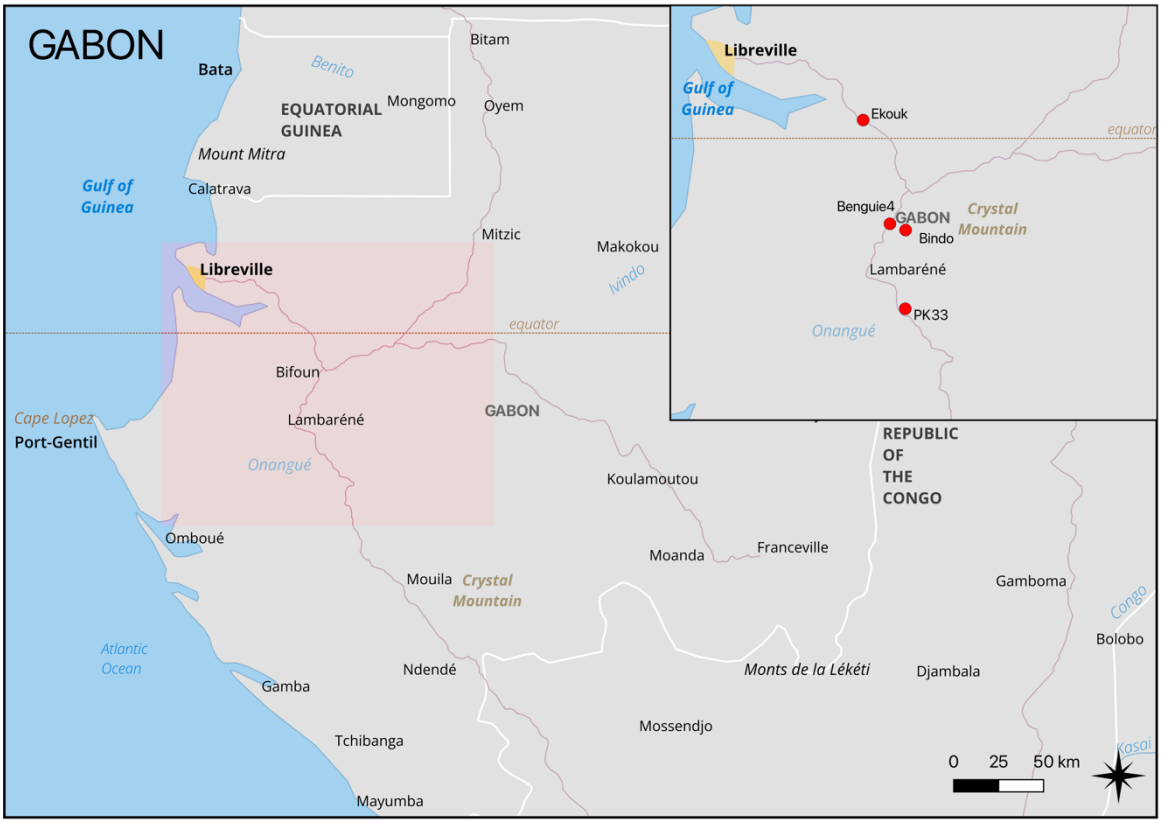

Figure 3 – Sampling sites in Gabon (map created with QGIS)

The current study, as part of the COMAL work package 1, focuses on the processing of the human blood samples collected in Gabon as well as the first 500 blood samples collected in Cameroon.

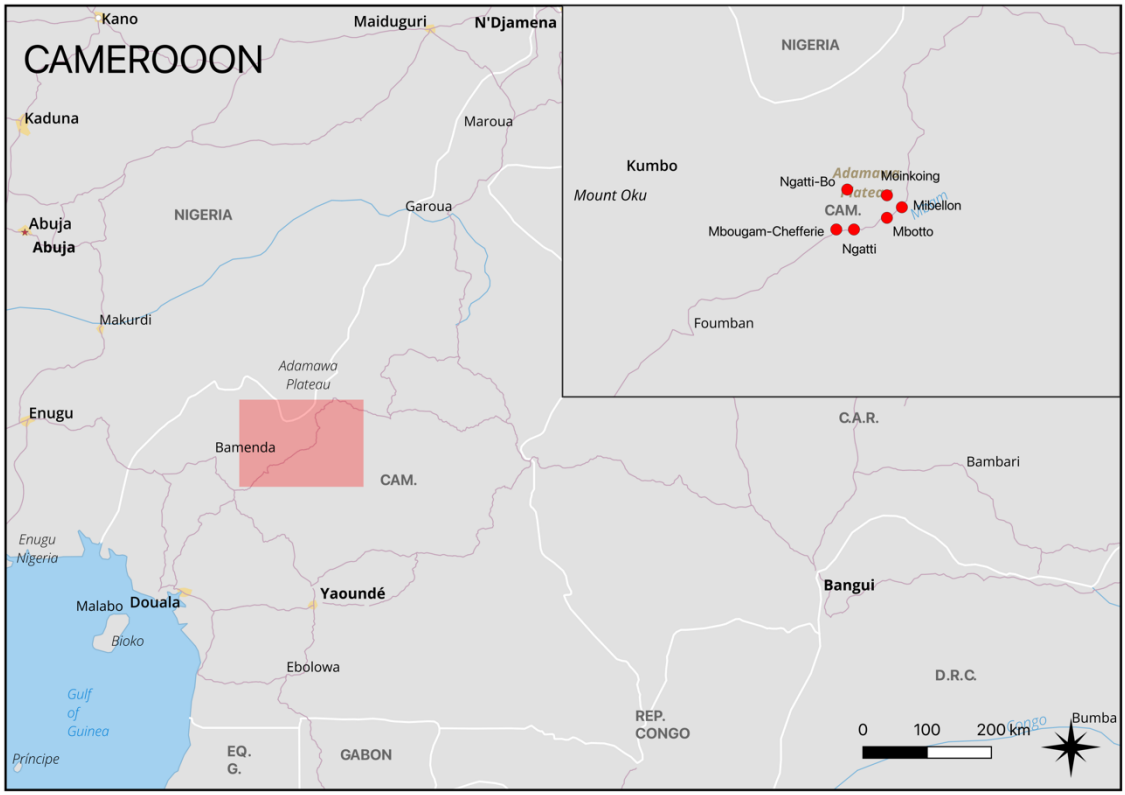

Figure 4 – Sampling sites in Cameroon (map created with QGIS)

2.3 Sample material

In the initial phase of the COMAL study, blood sampling was planned to happen in the form of collecting finger-prick blood samples on filter papers and then, after RNA extraction, running a multiplex qPCR on those samples to detect different *Plasmodium* species in a single PCR well. However, the low blood volume collected by finger-prick would allow only for a tiny quantity of RNA to be purified. Additionally, in the first trial using multiplex qPCR reactions, the COMAL team showed that the often much lower signal of *Plasmodium malariae* can get blocked by the massively present *Plasmodium falciparum* (see Figure 5, derived from unpublished data, by Borrmann and Recker, 2021).

Figure 5 shows that *Plasmodium malariae* can only be detected by multiplex qPCR if its ratio to *Plasmodium falciparum* parasitemia does not exceed 1:100. Consequently, in co-infected samples in which there is a high presence of *P.*

falciparum and a low presence of *P. malariae*, only a singleplex qPCR can sensitively detect *Plasmodium malariae*.

As the goal of this study was to not only very sensitively detect *Plasmodium malariae*, but also specifically detect mixed infections, the methodology for sampling was switched to venous blood sampling. This method allows to get a higher amount of purified RNA, and to perform singleplex qPCR instead of multiplex, to better detect *P. malariae*.

This approach was approved by the ethics committees of the medical faculty of the university of Tübingen as well as the ethics committee of the university hospital of Tübingen (project number 010/218BO2).

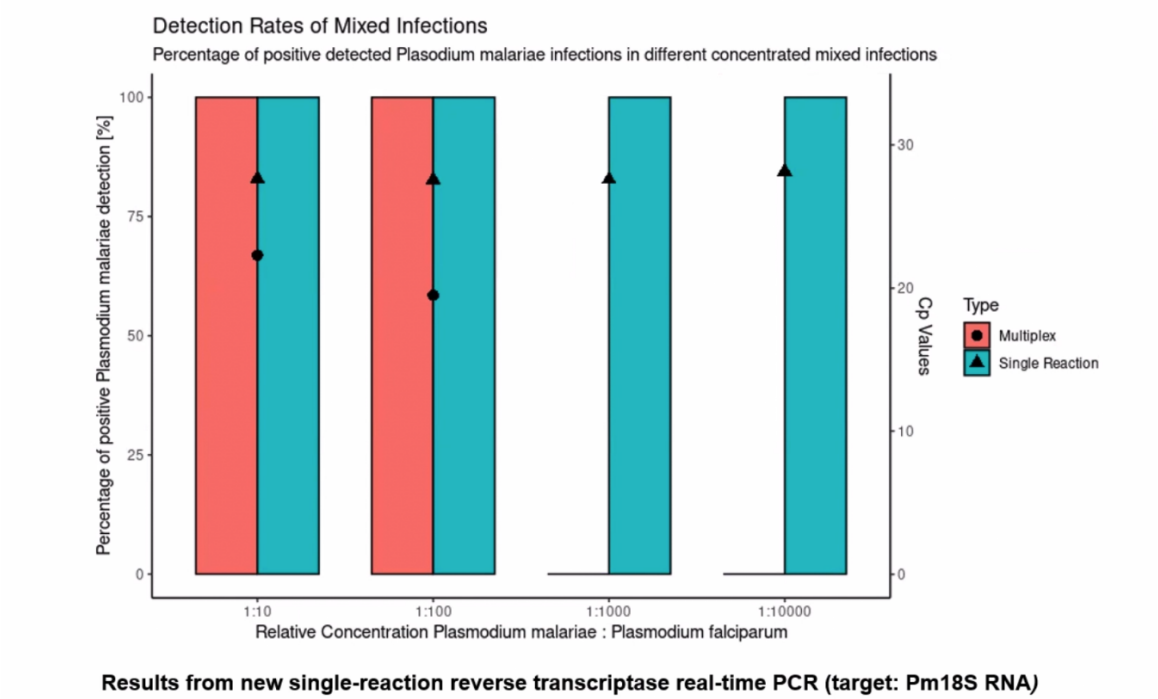

Figure 5 – Ultrasensitive detection of human P. malariae infections by qRT-PCR – showing the advantage of using a single reaction because of the difficulty in detection of P. malariae when its concentration is < 1:100 in relation to P. falciparum (unpublished data, Borrmann & Recker, 2021)

For the purpose of drawing blood to extract parasite RNA, the human blood was collected into PaxGene® tubes.

PaxGene® tubes contain 6.9ml of a reagent that stabilizes RNA and allows for the samples to be transported back to the laboratory at room temperature, at which intracellular RNA stays stable inside the tubes for up to five days (Chai *et al.*, 2005).

The samples from the cross-sectional studies in Gabon and Cameroon were drawn in the specific study sites described above and then transported back on the same day to Lambaréné and Yaoundé respectively, where they were frozen and stored at -20°C.

Subsequently, the shipment at -20°C to the Institute of Tropical Medicine (ITM) in Tübingen was organized.

A stabilizing agent such as the one used in PaxGene® tubes is necessary if the collected samples cannot be processed immediately in a lab. Indeed, in a regular blood collection system, both transportation delays and storage conditions, for example changes in temperature, highly affect RNA quality (Malentacchi *et al.*, 2016; Huang *et al.*, 2017).

It has been shown that using PaxGene® technology for blood sampling in tropical regions stabilizes RNA samples even under varying tropical temperatures and longer transportation periods. This is true especially for *18S* ribosomal RNA (Sarathkumara *et al.*, 2022) which is the target for the qPCR in this study.

2.4 Extraction of RNA using a magnetic-beads-based approach

After freezing and shipping samples to Tübingen at -20°C, the RNA extraction took place.

The extraction of the Plasmodium RNA from the collected blood samples was performed in the laboratory of the ITM in Tübingen, using a well-established, magnetic-beads-based approach, with the Omega Bio-tek Mag-Bind® PX Blood RNA 96 Kit.

The extraction protocol was supplied by the company and adapted to the local infrastructure (COMAL SOP003_V2.0, AG Borrmann, ITM Tübingen).

On the following page, a quick description of the extraction process is given.

2.4.1 Extraction procedure

After thawing the samples stood at room temperature for at least two hours, to allow the RNA stabilizer to react with the RNA contained within the samples. Then, the samples were centrifuged at 3000 g for 10 minutes, and the supernatant discarded. After adding nuclease-free water, the samples were vortexed, and thus the previously formed pellet resuspended. The samples then had to be centrifuged again. To complete the washing cycle, the supernatant was again discarded, and the remaining pellet dried upside down for 3 minutes.

After these washing steps, proteinase was added to the samples to start the process of protein lysis. After vortexing, a lysis buffer was added to the samples. Following this step, the lysate was transferred to small 1 mL Eppendorf tubes and incubated for 10 minutes at 55°C on a shaking plate.

The following steps describe the filtration of the samples.

For filtration, to filter out remaining DNA, the samples were placed in a filter-plate sitting on top of a deep-well plate and then centrifuged at 3000g. After filtration, again some lysis buffer was added to the samples. As a final step a mixture of magnetic beads and isopropanol was added.

For the automatic RNA extraction process within the KingFisher™ Flex further plates containing different washing buffers were prepared.

Finally, all plates were inserted into the KingFisher™ Flex with the machine proceeding to wash out the RNA from the samples using the aforementioned magnetic beads.

After approximately 45 minutes, the eluate was removed. This eluate contained a high concentration of purified RNA. 20µl were transferred to 96-well plates to be used for qPCR later. The remaining eluate was transferred to the previously

prepared Eppendorf tubes and stored at -20 degrees for further analysis in the future.

The extraction of the samples that are included in this study started in September 2021 with some first trials. The final adapted protocol was applied from late January 2022 onwards. The large majority of the samples that are included in this study was extracted from January 2022 to August 2022.

All extracted samples were stored at -20°C after extraction.

2.5 Detection of *P. malariae* and *P. falciparum* by qRT-PCR

For a long time, the most used technique to sensitively detect *Plasmodium* spp. has been the polymerase chain reaction (PCR), which targets specific genes and amplifies them, so they become detectable. The most commonly used genomic material to detect *Plasmodium falciparum* is the 18S ribosomal DNA (Snounou, Viriyakosol, Jarra, *et al.*, 1993; Swan *et al.*, 2005).

This rDNA contains the codes for the structural composition of the small part of the cytoplasmic ribosome of eukaryotic cells which plays an important part in phylogenetic studies of all species. The matching rRNA is a stable form of RNA and is ubiquitous (Sorof Uddin and Cheng, 2015). In previous studies “a gene encoding the small subunit rRNA of *P. malariae* was sequenced and shown to contain unique regions that could be used as diagnostic probes” (Goman, Mons and Scaife, 1991; Collins and Jeffery, 2007).

In recent years, a so called quantitative reverse transcriptase PCR (qRT-PCR, in short qPCR) has been developed, which can detect genetic material even more sensitively and in ‘real-time’, detecting the fluorescence of a probe that binds to the genetic material that is to be amplified. This PCR has the special quality of being able to also work with RNA and not DNA, thanks to the reverse transcriptase. RNA has a much higher copy number per mL of blood than DNA. Indeed, “rRNA is 1000 – 10,000 times more abundant than rDNA in intact parasites” (Murphy *et al.*, 2012, 2018).

It can thus be detected more sensitively which has been shown in 2015 for *P. falciparum* and *P. vivax* detection by Adams (Adams *et al.*, 2015). In 2011 a study revealed that “analysis of clinical samples showed that detection of 18S rRNA genes from total nucleic acids increased the analytical sensitivity of the assay by more than 1 log unit compared to DNA only” (Kamau *et al.*, 2011).

This increased analytical sensitivity was the reason qPCR was performed on extracted RNA, not DNA, in this study. The present study works with a qPCR protocol that aims to amplify 18S ribosomal RNA (in the following named 18S rRNA).

To detect infections with *Plasmodium malariae* and *Plasmodium falciparum*, a protocol for a highly sensitive, singleplex quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), amplifying *Plasmodium* rRNA, was already set up in 2019 by the COMAL research team. For this qRT-PCR, both the *P. malariae* and the *P. falciparum* assay were derived from assays that previously worked well to detect these parasites in extracted DNA. Both qPCR probes bind to the previously described 18S ribosomal RNA.

In initial assays, it was shown that this single reaction qRT-PCR can detect a parasitemia of as low as 5-10 *P. malariae* parasites per mL of blood (unpublished data, Borrmann 2021), which is significantly lower than the generally reported detection limit of >1000 parasites/mL for PCR performed on DNA extracted from dried blood spots on filter papers (Ashley and White, 2014). Detection sensitivity for malaria microscopy, performed by experienced microscopists, lies at around 20,000 parasites/mL (Berzosa *et al.*, 2018). It has, however, been reported to be even lower when performed by routine diagnostic laboratories, at around 500,000 parasites/mL (Moody, 2002).

2.5.1 Primers and probes

Previously in the COMAL project, primers and probes were tested to detect *Plasmodium falciparum* and *Plasmodium malariae* on RNA instead of DNA.

Through this work, the methods used for the qRT-PCR in the present study were already established.

For the *Plasmodium falciparum* qRT-PCR, the primers and probe that were used were previously applied to detect *P. falciparum* in *Anopheles* mosquitoes in Cameroon and Gabon (Fadel *et al.*, 2019; Boussougou-Sambe *et al.*, 2022).

These were the primers PlasF (5′-GCT TAG TTA CGA TTA ATA GGA GTA GCT TG-3′) and PlasR (5′-GAA AAT CTA AGA ATT TCA CCT CTG ACA-3′) together with the *P. falciparum* probe labelled with fluorophore [FAM] TCT GAA TAC GAA TGT C [MGBEQ].

To detect *Plasmodium malariae* RNA, the primers PmT for (5'-GGT GTT GGA TGA TAG AGT AA-3‘) and PmT rev (5'-CCC AAA GAC TTT GAT TTC TC-3') were used together with the *P. malariae* probe labelled with fluorophore [HEX] AGG AAG CTA TCT AAA AGA AAC ACT CAT [MGBEQ]. These primers and probes were derived from another project of the Institute of Tropical Medicine in Tübingen, led by A. Lalremruata.

2.5.2 The qRT-PCR

The qRT-PCR was conducted using the Roche Light Cycler 480 II including the Roche 348 well cooling system.

For reagent preparation, two Master mixes were prepared according to the SOP 002 for qPCR in COMAL, one for *Plasmodium malariae* detection, one for *Plasmodium falciparum*. The Master mixes were distributed under a PCR working hood onto a 348 well plate, *P. falciparum* samples on the left, *P. malariae* samples on the right. 1µl of each sample was added to both the *P. falciparum* and the *P. malariae* sections in a symmetrical layout.

For both the *Plasmodium falciparum* and *malariae* assays, at least three non-template controls were added. As a control for *P. falciparum* samples, *P. falciparum*-RNA from cell culture (“SCT 1:100”), currently generated in

Tübingen, was used. For *P. malariae*, a confirmed *P. malariae* mono-infected sample from Gabon (“106 1:10”) was used as a control.

Before starting the qPCR assay, the prepared plate was centrifuged at >1,500g.

After centrifugation it was added into the Roche LightCyler480. For the qRT-PCR run, the LightCycler480 Software 1.5.1.62 was used with the run template „Dual Color Hydrolysis Probe” with a block size of 384, a reaction volume of 10µl and dynamic detection formats for FAM (465-510) and HEX (533-580). The mode of analysis was quantitative, with a single annealing step per cycle.

Reverse transcription from RNA to DNA took place for the first 20 minutes at a temperature of 45°C. Afterwards 40 cycles of DNA amplification followed suit. Each cycle started with the hot start to activate the polymerase, which was set at 5 minutes, at 95°C. Denaturation lasted for 15 seconds at 95°C, with annealing taking 60 seconds at 60°C. After 40 of these cycles, the samples were cooled for 10 seconds at 40°C and subsequently removed from the machine.

2.6 Data analysis

Several kinds of data analysis were conducted.

For the data analysis of the qPCR results, the “2nd derivative max” method within the LightCycler480 Software was used, adding color compensation from a database. The individual sample curves were manually compared to positive controls and non-template controls to verify the numeric results which were received in the form of Cp values. Cp (crossing point) values describe the point at which an amplification curve crosses the vertical threshold line to become detectable. Cp can be regarded as an equivalent to the more widely used Ct (cycle threshold) values. From this sentence on, the term Ct values will be used, as it is the more common form of describing these values in medical literature.

After manually verifying the qPCR results, they were added into an Excel database, together with the anthropometric data received from Gabon and Cameroon.

Data analysis was subsequently performed using RStudio (Posit, PBC, Boston, Massachusetts, USA) version 2023.03.0-386, with R version 4.3.0 binary for macOS 11 (R Foundation for Statistical Computing, Vienna, Austria).

The statistical tests and analyses performed were Welsh's t-test, Pearson's chi-squared test, one way analysis of variance (ANOVA), Pearson's correlation tests and linear regression models.

The level of significance was set at $p < 0.05$.

For all analyses parametric tests were used, as the distribution of the collected data is normal (see distribution curves in the results section).

Welsh's t-test was used several times to assess whether there is a difference between the means of two independent variables. Welsh's t-test does not assume that variance in both groups is equal, but when the variance is equal, it gives the same results as the more widely known Student's t-test. In scientific literature, it has been recommended to always use Welsh's t-test, as it "provides a better control of Type 1 error rates when the assumption of homogeneity of variance is not met, and it loses little robustness compared to Student's t-test when the assumptions are met" (Delacre, Lakens and Leys, 2017).

For graphical purposes the data was treated using R to be displayed in bar charts, density plots, boxplots, frequency tables, demographic tables, demographic pyramids, pie charts, forest plots and scatter plots.

3 Results

3.1 Inclusion criteria of samples

For this study, 1080 human blood samples from Gabon and 504 from Cameroon were analyzed by qPCR.

To be included, the result in form of the Ct value, if positive, was only accepted if the corresponding PCR curve was clearly positive, always in comparison to the control sample. In a number of samples, the *Plasmodium falciparum* qPCR curves were, albeit initially rising and giving a positive result, staying very low to the x axis. This issue will be further explained in the Discussion section, as it led to a change of protocol for future sample analysis.

78 samples from Gabon had to be repeated due to this issue being so advanced, that the curves were not interpretable. Samples that were clearly positive even though the curve was slightly flattened were accepted into the data set. After the second qPCR run, 12 samples, whose results were still not interpretable, had to be excluded from the study.

In total 1068 samples were included from Gabon.

For the samples from Cameroon, 39 had to be rerun because of the same issues mentioned above. Of these, 15 were excluded for the final data analysis, due to non-interpretable results.

In total, 489 samples from Cameroon were included.

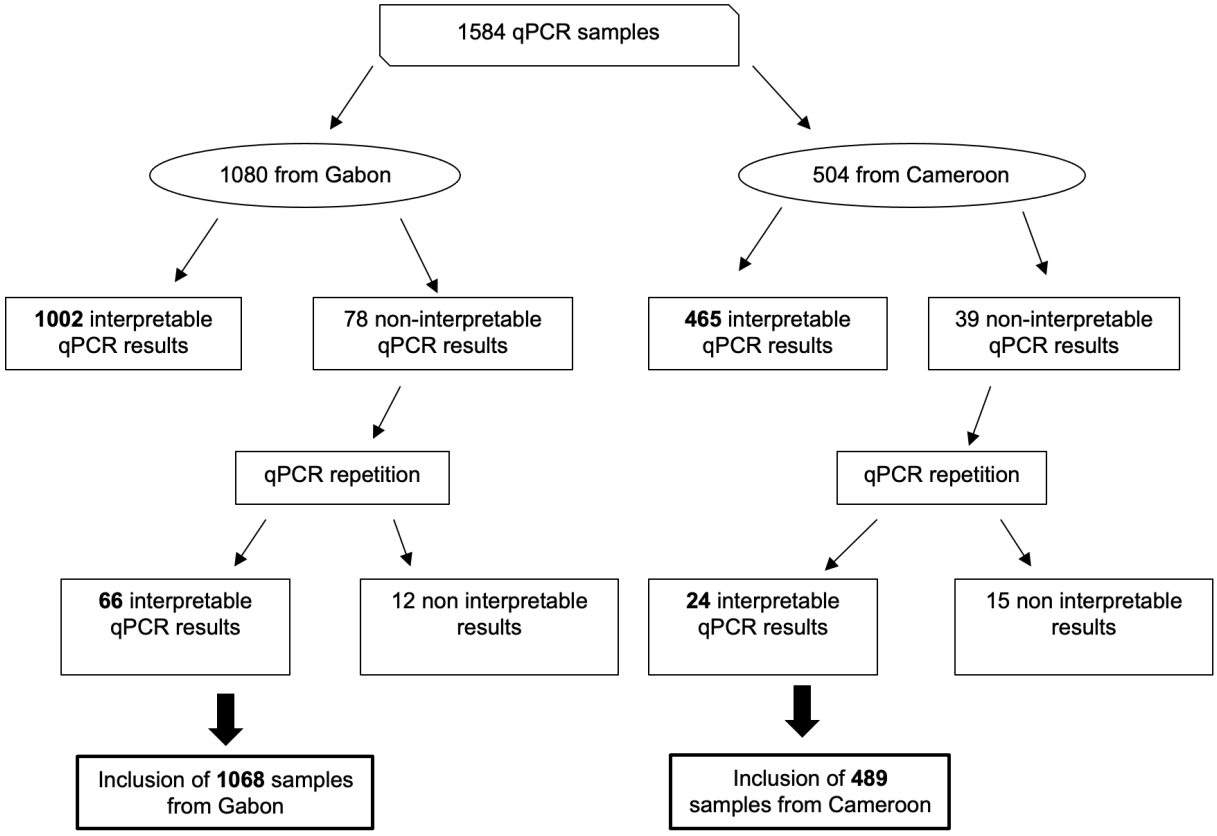

Figure 6 – Inclusion and exclusion of samples for this study

3.2 Data distribution

To be able to statistically analyze data, the distribution of this data has to be checked beforehand.

If it is normally distributed, in the sense of following a normal Gaussian curve, parametric tests can be used to analyze the data. If the data does not correspond to the distribution of a normal Gaussian curve, non-parametric tests have to be applied.

Figures 7 and 8 display the data distribution for Gabon and Cameroon, both for mono- and coinfections.

For both countries, the data is overall bell shaped and therefore normally distributed, making it possible to apply parametric tests.

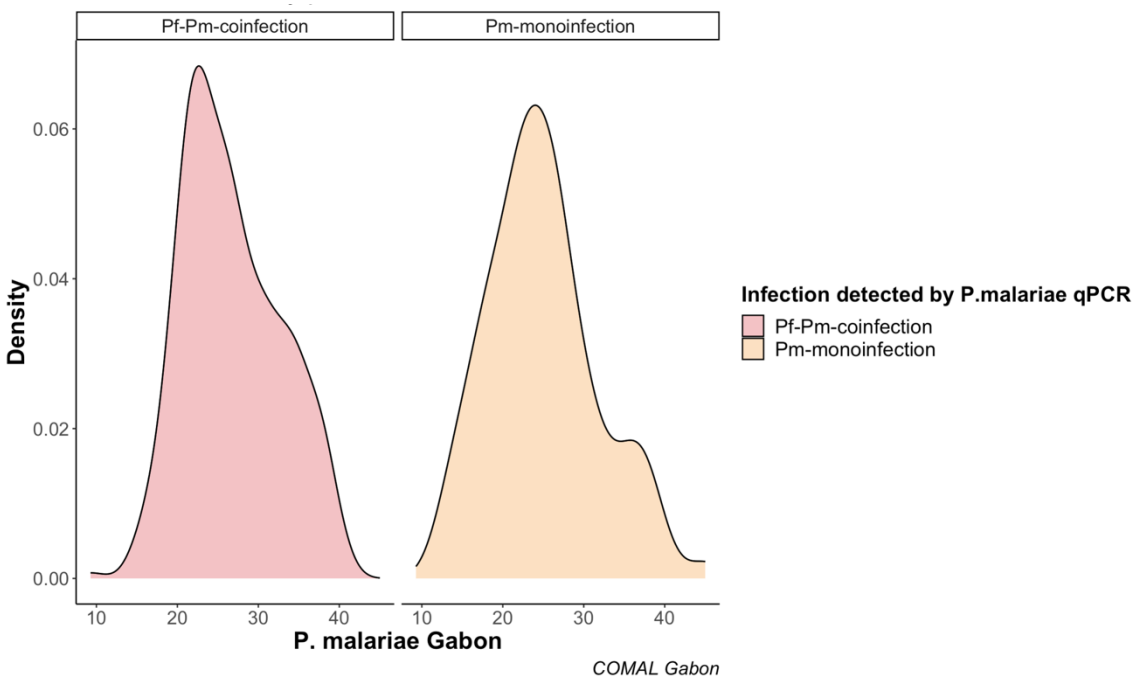

Figure 7 – Density plots for data from Gabon, showing a bell-shaped distribution

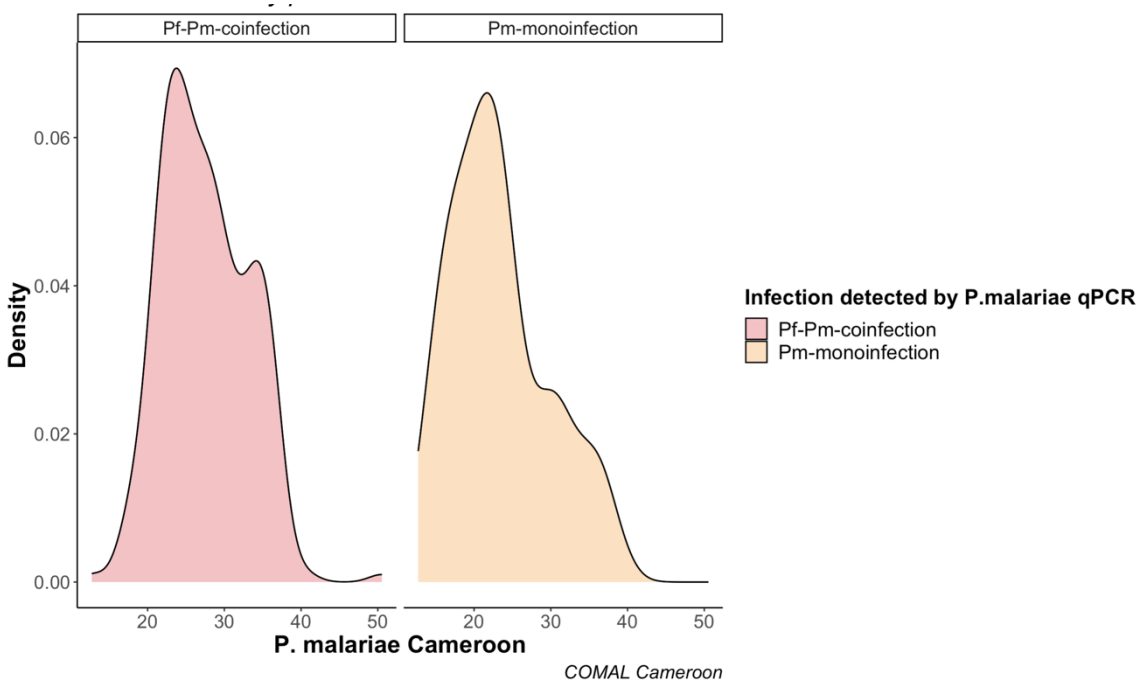

Figure 8 - Density plots for data from Cameroon, showing a bell-shaped distribution

3.3 Demographic overview of the study population

For the cross-sectional study, several anthropometric measures were collected.

Most importantly age, gender, weight, and temperature. Additionally, a point-of-care hemoglobin test was administered on site. These measurements allow for a closer look at the study population, which will be detailed in the following sections.

3.3.1 Demographics of participants in Gabon

Table 5 - Sociodemographic characteristics of the participants of the cross-sectional study in Gabon

	PK33		Bindo		Benguie4		Ekouk		Overall	
Baseline characteristic	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
Season[a]										
Rainy	156	46%	227	47,5%	98	48%	30	100%	511	49%
Dry	184	54%	251	52,5%	107	52%	0		542	51%
Gender										
Female	193	57%	270	55%	82	40%	11	37%	556	52%
Male	147	43%	223	45%	123	60%	19	63%	512	48%
Age										
1-4	38	11%	53	11%	30	15%	1	3%	122	11%
5-11	74	22%	122	25%	57	28%	0		253	24%
12-19	39	12%	88	18%	27	13%	1	3%	155	14,5%
20-60	151	44%	185	37%	85	41%	18	60%	439	41%
> 60	38	11%	45	9%	6	3%	10	33%	99	9,5%
Fever										
Yes (>38°C)	4	1%	8	2%	4	2%	0		16	1,5%
No (<38°C)	336	99%	485	98%	201	98%	30	100%	1052	98,5%

BMI[b] (adults)										
N (adults)	188	100%	229	100%	91	100%	28	100%	536	100%
Low	24	13%	20	9%	8	9%	9	32%	61	11%
Normal	108	57%	138	60%	53	58%	15	54%	314	59%
Overweight	34	18%	49	21%	22	24%	4	14%	109	20%
Obese	22	12%	22	10%	8	9%	0		52	10%
Anemia[c]										
no	190	56%	242	49%	107	52%	19	63%	558	52%
mild	72	21%	116	24%	39	19%	7	23%	234	22%
moderate	65	19%	93	19%	40	20%	4	13%	202	19%
severe	6	2%	6	1%	4	2%	0		16	2%
not assessed	7	2%	36	7%	15	7%	0		58	5%
Total	340		493		205		30		1068	

Note. N = 1068

[a] Seasons: DEC-JAN (small dry), MAY-SEP (long dry), OCT-NOV (small rainy), FEB-APR (long rainy)

[b] BMI (starting at age 20): low= <18.5, normal= 18.5-24.9, overweight= 25-29.9, obese= >30

[c] Hemoglobin cut-offs as defined by the WHO

Of the 1068 participants from Gabon, 52% were female, and 48% male. Ages from age 1 to 106 were recorded. The age pyramid (Figure 9), with a large bottom and a narrowing top, shows the typical distribution of a predominantly young population, with a high number of children and adolescents. This is indicative of an expansive population with high birth rates.

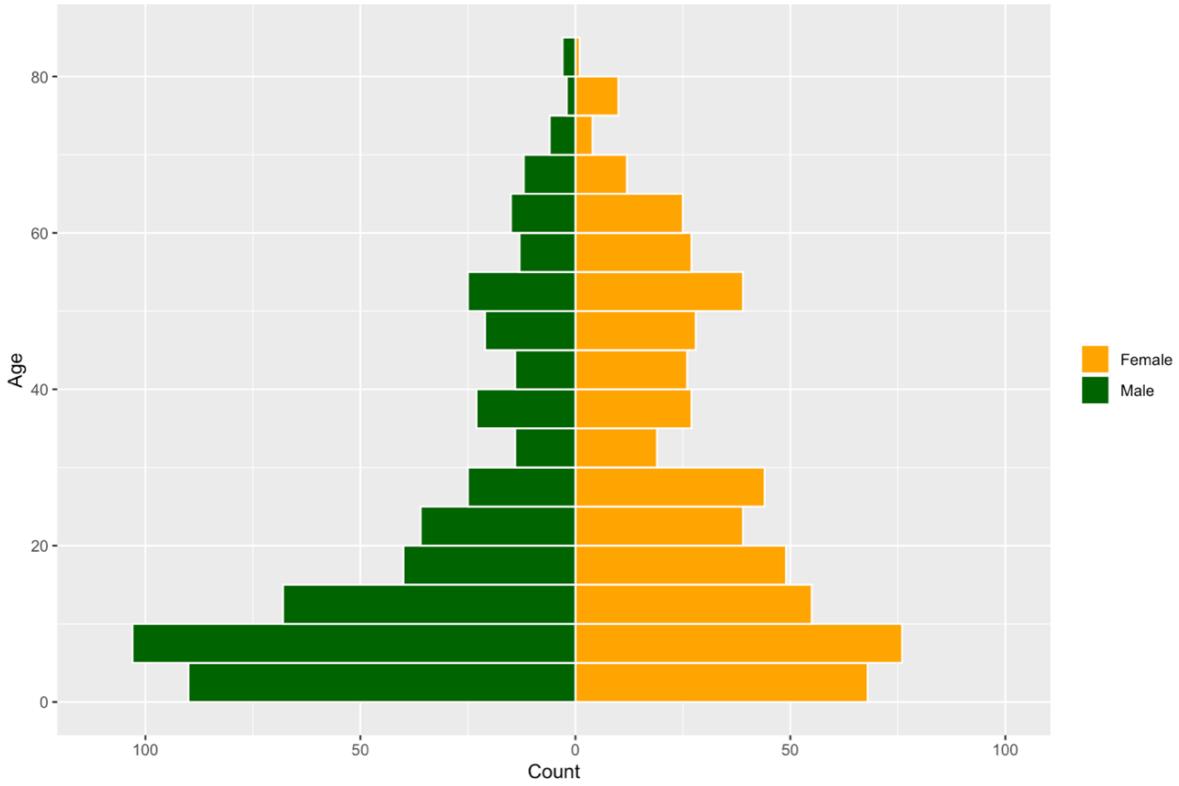

Figure 9 – Demographic pyramid showing a distribution of age and gender of the study population in Gabon which is indicative of high birth rates

The median age of the participants sampled in Gabon was 20 (IQR 8 – 44), with a mean of 27 (SD = 22).

Temperature of 98.5% of participants was normal. This can be explained by the study requirement of only sampling asymptomatic individuals. It is therefore difficult to determine what the cause of higher temperature was in the 1.5% of participants who had a temperature higher than 38.0°C.

3.3.2 Demographics of participants in Cameroon

For samples from Cameroon, the same data was analyzed, except for seasonal data. Indeed, only the first 504 samples of a much larger set of samples were delivered to and analyzed in Tübingen. As all these 504 samples were taken during one season, it is impossible to compare seasonal data in this current study. The remainder of the samples are currently stored in Cameroon and will be analyzed there. Future results from these samples will give an interesting look into a larger dataset, allowing for seasonal comparison.

Table 6 - Sociodemographic characteristics of the participants of the cross-sectional study in Cameroon

Baseline characteristic	Mbotto		Mbougam-Chefferie		Mibellon		Moinkoing		Ngatti		Ngatti-Bo		Overall	
	n	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
Gender														
Female	13	52%	33	67%	33	48%	83	59%	61	50%	44	52%	267	55%
Male	12	48%	16	33%	35	52%	58	41%	60	50%	41	48%	222	45%
Age														
1-4	3	12%	6	12%	6	9%	22	16%	12	10%	13	15%	62	13%
5-11	11	44%	16	33%	21	31%	37	26%	19	16%	28	33%	132	27%
12-19	2	8%	5	10%	12	18%	29	21%	54	45%	24	28%	126	26%
20-60	7	28%	21	43%	23	34%	51	36%	30	25%	20	24%	152	31%
> 60	2	8%	1	2%	6	9%	2	1%	6	5%	0		17	3 %
Fever														
Yes (>38°C)	0		0		5	7%	2	1%	1	1%	1	1%	9	2%
No (<38°C)	25	100%	49	100%	63	93%	139	99%	120	99%	84	99%	480	98%

BMI[a] (adults)														
N (adults)	9	100%	22	100%	29	100%	53	100%	36	100%	20	100%	169	100%
Low	3	33%	2	9%	5	17%	6	11%	0		1	5%	17	10%
Normal	3	33%	15	68%	16	55%	26	49%	19	53%	15	75%	94	56%
Overweight	1	11%	3	14%	4	14%	12	23%	14	39%	3	15%	37	22%
Obese	2	22%	2	9%	4	14%	9	17%	3	8%	1	5%	21	12%
Anemia[b]														
no	10	40%	15	31%	24	35%	43	30%	35	29%	50	59%	177	36%
mild	6	24%	8	16%	15	22%	34	24%	35	29%	13	15%	111	23%
moderate	8	32%	23	47%	23	34%	51	36%	44	36%	22	26%	171	35%
severe	1	4%	3	6%	6	9%	12	9%	7	6%	0		29	6%
not assessed	0		0		0		1	1%	0		0		1	
Total	25		49		68		141		121		85		489	

Note. N = 489

[a] BMI (starting at age 20): low= <18.5, normal= 18.5-24.9, overweight= 25 - 29.5, obese= >30

[b] Hemoglobin cut-offs as defined by the WHO

Of the 489 participants from Cameroon, 55% were female, and 45% male. We see here a slightly larger female population than in Gabon.

Ages from age 1 to 85 were recorded. The age pyramid (Figure 10), shows a larger bottom than Gabon, indicating an even younger population, with a high number of children and adolescents. This is, again, indicative of an expansive population with high birth rates. The median age of the participants sampled in Cameroon was 14 (IQR 7 – 28), with a mean of 20 (SD = 17), which is substantially younger than the population that was sampled in Gabon.

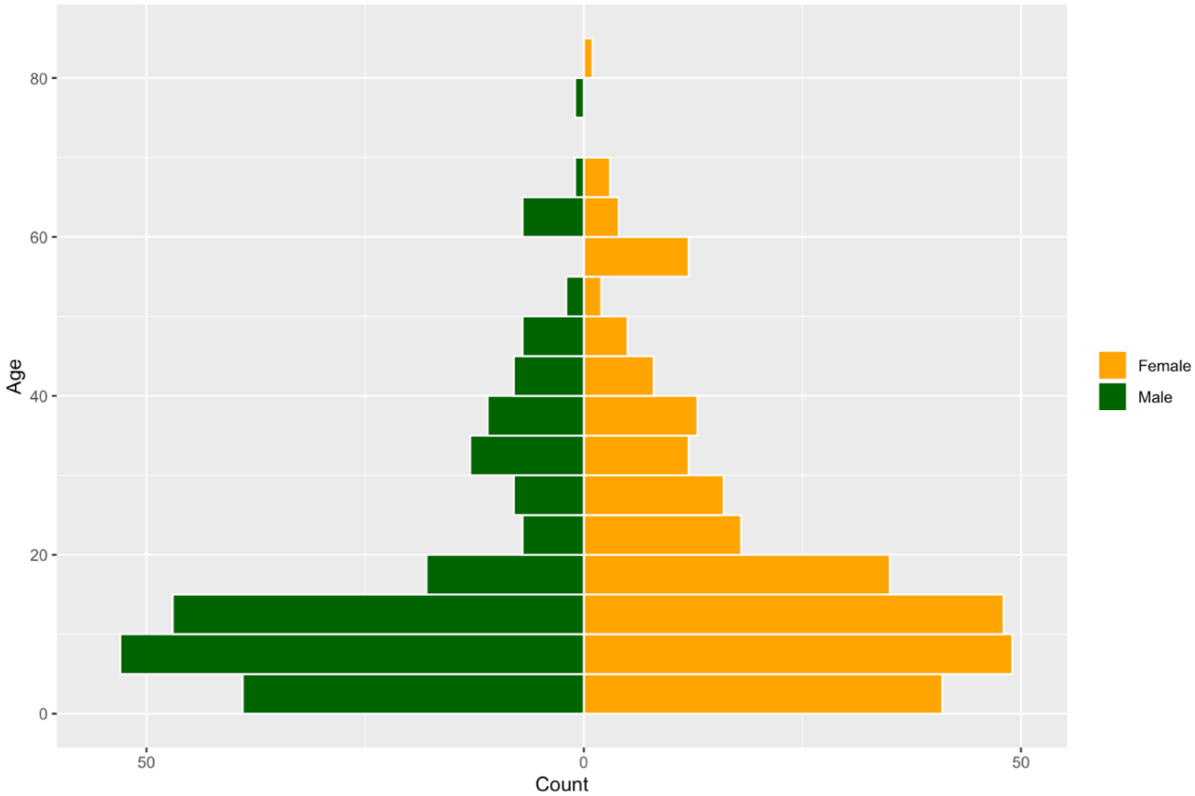

Figure 10 - Demographic pyramid showing a distribution of age and gender of the study population in Cameroon which is indicative of high birth rates

In Cameroon the body temperature of 98% of participants was normal. This can be explained by the study requirement of only sampling asymptomatic individuals. It is therefore again difficult to determine what the cause of higher temperature was in the 2% of participants who had a temperature higher than 38.0°C.

3.3.3 Body-Mass-Index

For the study participants > 19 years of age, a BMI (as defined by the WHO) was calculated. This was not done for children and adolescents, as the BMI in these age groups is not uniform to interpret and thus would be difficult to put into context in this thesis.

Of the adults aged 20 or older in Gabon, who represented about 50% of the study population, the majority, 59%, presented with a normal weight (BMI 18.5 - 24.9). 30% were overweight (BMI 25 – 29.9) or obese (BMI > 30), 11% were underweight with a BMI of < 18.5.

Among adults in Cameroon, who represented 35% of the study participants in this country, the majority, 56%, presented with a normal weight (BMI between 18.5 and 24.9). 34% were overweight (BMI 25 – 29.9) or obese (BMI > 30), 10% were underweight with a BMI of < 18.5.

It is to note that among the study populations, there are proportionally much less adults in Cameroon than in Gabon, speaking for a younger population in Cameroon as it can also be observed in the age pyramid above.

3.3.4 Hemoglobin levels

The hemoglobin level measured by point-of-care technology was transformed into the status of anemia, using hemoglobin levels to diagnose anemia at sea level as defined by the WHO (see Table 7). In this way, age and gender were taken into account, making the status of anemia comparable for all study participants.

Table 7 – Hemoglobin levels to diagnose anemia at sea level, as defined by the WHO (WHO/NMH/NHD/MNM/11.1)

Population	Non -Anaemia*	Anaemia*		
		Mild[a]	Moderate	Severe
Children 6 - 59 months of age	110 or higher	100-109	70-99	lower than 70
Children 5 - 11 years of age	115 or higher	110-114	80-109	lower than 80
Children 12 - 14 years of age	120 or higher	110-119	80-109	lower than 80
Non-pregnant women (15 years of age and above)	120 or higher	110-119	80-109	lower than 80
Pregnant women	110 or higher	100-109	70-99	lower than 70
Men (15 years of age and above)	130 or higher	110-129	80-109	lower than 80

± Adapted from references 5 and 6
* Haemoglobin in grams per litre
a "Mild" is a misnomer: iron deficiency is already advanced by the time anaemia is detected. The deficiency has consequences even when no anaemia is clinically apparent.

3.3.4.1 Anemia in Gabon

For 58 participants, 5% of all Gabonese study participants, no hemoglobin measure was performed. The calculations in this chapter are based on the 1010 hemoglobin measurements that have been recorded.

In Gabon, a little over half of the study participants whose hemoglobin levels were measured, 55%, were non anemic. Among the 45% of anemic individuals, 23% presented with mild anemia, 20% with moderate anemia and 2% with severe anemia (Figure 11).

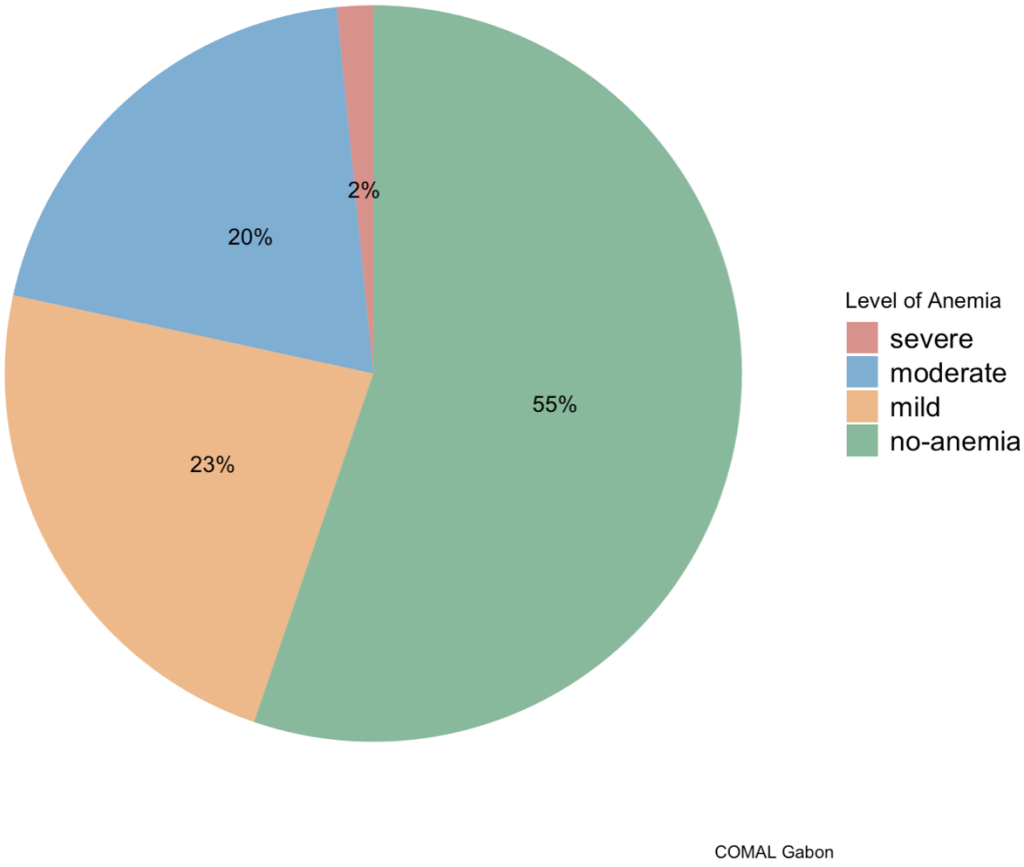

Figure 11 – Rates of anemia in the study participants in Gabon, showing that almost half of the population suffers from anemia

Most cases of severe anemia occurred in the age groups 1 to 4 and 20 to 60. Moderate anemia was mostly present in younger children, with a quarter of children aged 1 to 4 and a third of all children aged 5 to 11 suffering from moderate anemia. Mild anemia makes up at least one fifth of each age group and is present in over one third of adults older than 60 years (Table 8).

Table 8 - Anemia in all age groups in Gabon

	Age 1-4		Age 5-11		Age 12-19		Age 20-60		Age > 60	
	n	%	n	%	n	%	n	%	n	%
No anemia	49	*42.2*	110	*46.2*	80	*55.2*	269	*64.5*	50	*53.2*
Mild anemia	32	*27.6*	45	*18.9*	36	*24.8*	89	*21.3*	32	*34.0*
Moderate anemia	30	*25.9*	80	*33.6*	28	*19.3*	53	*12.7*	11	*11.7*
Severe anemia	5	*4.3*	3	*1.3*	1	*0.7*	6	*1.4*	1	*1.1*
Total	116	*100*	238	*100*	145	*100*	417	*100*	94	*100*

Taking a closer look at only children and adolescents (Figure 12 and Table 9), we see that especially in the younger children there are much higher rates of anemia than in adults.

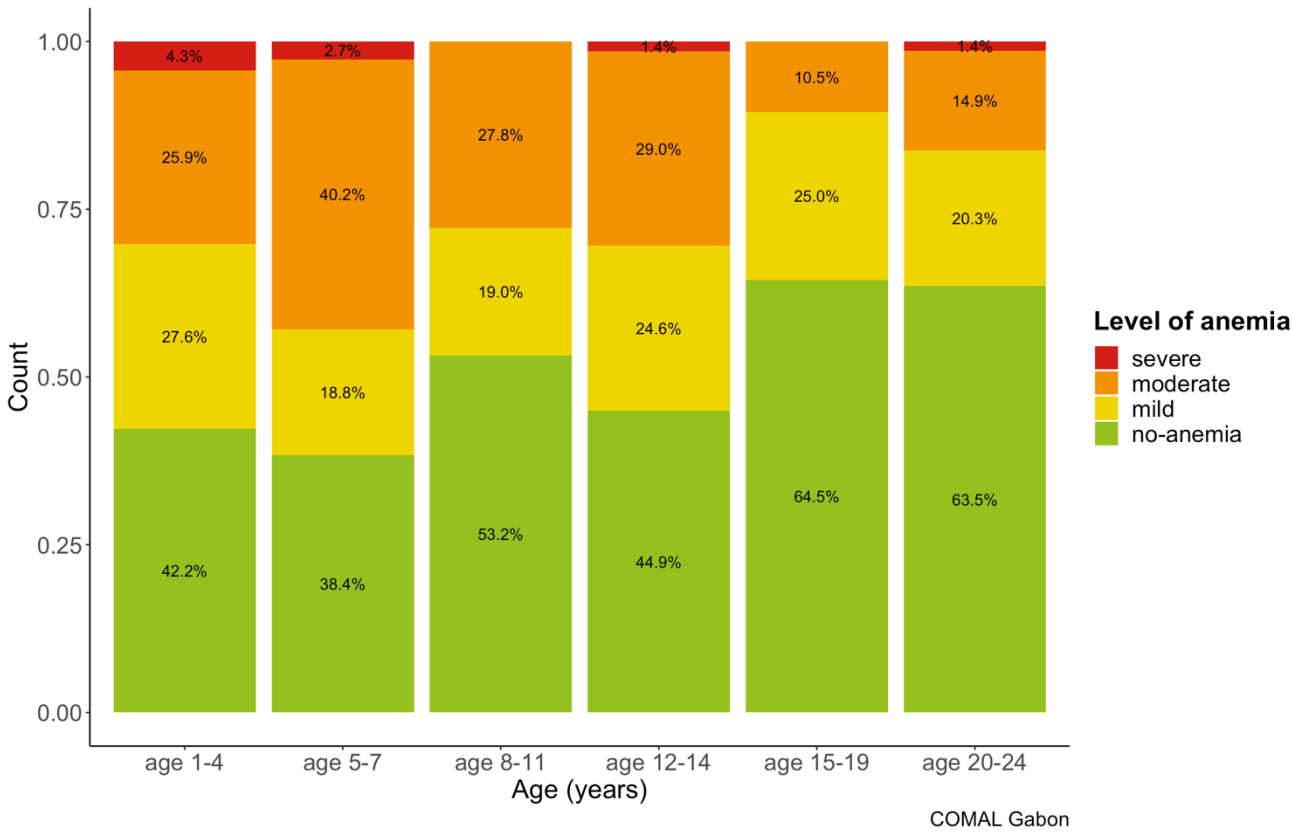

Figure 12 – Status of anemia in children, adolescents and young adults in Gabon, showing the higher overall rate of anemia in younger children

Severe anemia is mostly present in the youngest age group, children aged 1 to 4 years, with about 4%. In this age group there is also more mild anemia than in other age groups, with roughly 30% of all children aged 1 to 4 being mildly anemic.

In children aged 5 to 7 years, the burden of anemia is highest among all children, with 40% of moderate anemia and 3% of severe anemia. In the other age groups moderate anemia represents roughly 26-29% per group and even only 10.5% in 15–19-year-olds.

In children aged 8 to 11 the cases of anemia decrease, rising again in the group of teenagers aged 12 to 14, where about 25% are mildly anemic and 29% suffer from moderate anemia. In adolescents and young adults, the rate of anemia decreases substantially.

These differences are statistically significant, X^2 (15, N = 611) = 42.3, $p < 0.001$. Especially the high rate of severe anemia in children aged 1-4, the increase in moderate anemia in children aged 5-7 and the decrease in moderate anemia in adolescents aged 15-19 contribute to the significance of the chi-squared-test.

Table 9 – Levels of anemia in children in Gabon

	Age 1 - 4		Age 5 - 7		Age 8 - 11		Age 12 - 14	
	n	*%*	n	*%*	n	*%*	n	*%*
No anemia	49	*42.2*	43	*38.4*	67	*53.2*	31	*44.9*
Mild anemia	32	*27.6*	21	*18.8*	24	*19.0*	17	*24.6*
Moderate anemia	30	*25.9*	45	*40.2*	35	*27.8*	20	*29.0*
Severe anemia	5	*4.3*	3	*2.7*	0	*0.0*	1	*1.4*
Total	116	*100*	112	*100*	126	*100*	69	*100*

3.3.4.2 Anemia in Cameroon

In Cameroon, there were overall higher rates of anemia than in Gabon.

Only about 36% of all study participants in Cameroon were non anemic, as compared to 55% in Gabon. Among the anemic participants, 23% presented with mild anemia, similarly to the participants in Gabon. 35% of participants in Cameroon suffered from moderate anemia, as compared to 20% in Gabon, and 6% with severe anemia, as compared to 2% in Gabon (Figure 13).

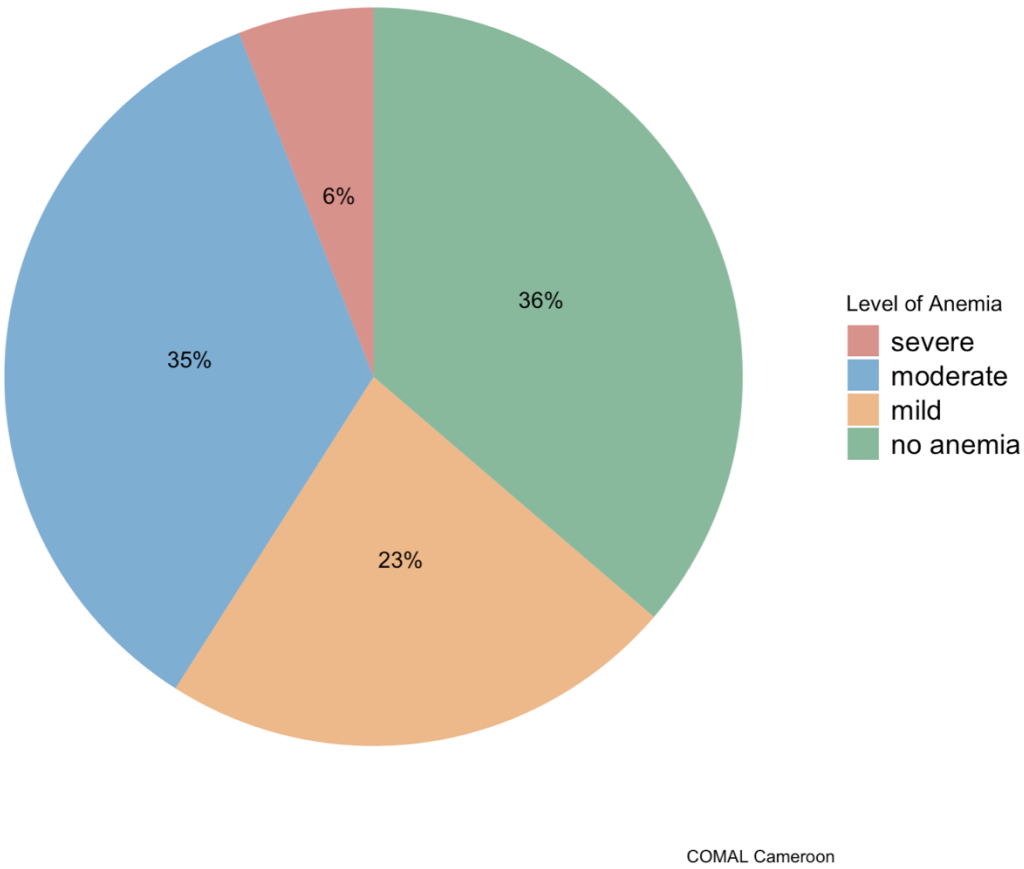

Figure 13 - Rates of anemia in the study participants in Cameroon, showing that almost two thirds of the population suffer from anemia

The large majority of severe anemia occurred in young children, with 22.6% of severe anemia among children aged 1 to 4. Moderate anemia was, as in Gabon, mostly prevalent in children aged 1 to 4 and 5 to 11 years. However, moderate anemia was much more prevalent in these age groups in Cameroon than in Gabon, with almost half of the children being moderately anemic.

Mild anemia on the other hand was most common in adolescents and adults aged 20 to 60, with about a third of those age groups suffering from mild anemia.

Table 10 - Anemia in all age groups in Cameroon

	Age 1-4		Age 5-11		Age 12-19		Age 20-60		Age > 60	
	n	*%*	n	*%*	n	*%*	n	*%*	n	*%*
No anemia	9	*14.5*	56	*42.4*	43	*34.4*	62	*40.8*	7	*41.2*
Mild anemia	9	*14.5*	8	*6.1*	41	*32.8*	50	*32.9*	3	*17.6*
Moderate anemia	30	*48.4*	61	*46.2*	36	*28.8*	39	*25.7*	5	*29.4*
Severe anemia	14	*22.6*	7	*5.3*	5	*4.0*	1	*0.7*	2	*11.8*
Total	62	*100*	132	*100*	125	*100*	152	*100*	17	*100*

When looking closer at only children and adolescents, we see a similar, albeit accentuated pattern, as the one in Gabon (see Table 11 and Figure 14).

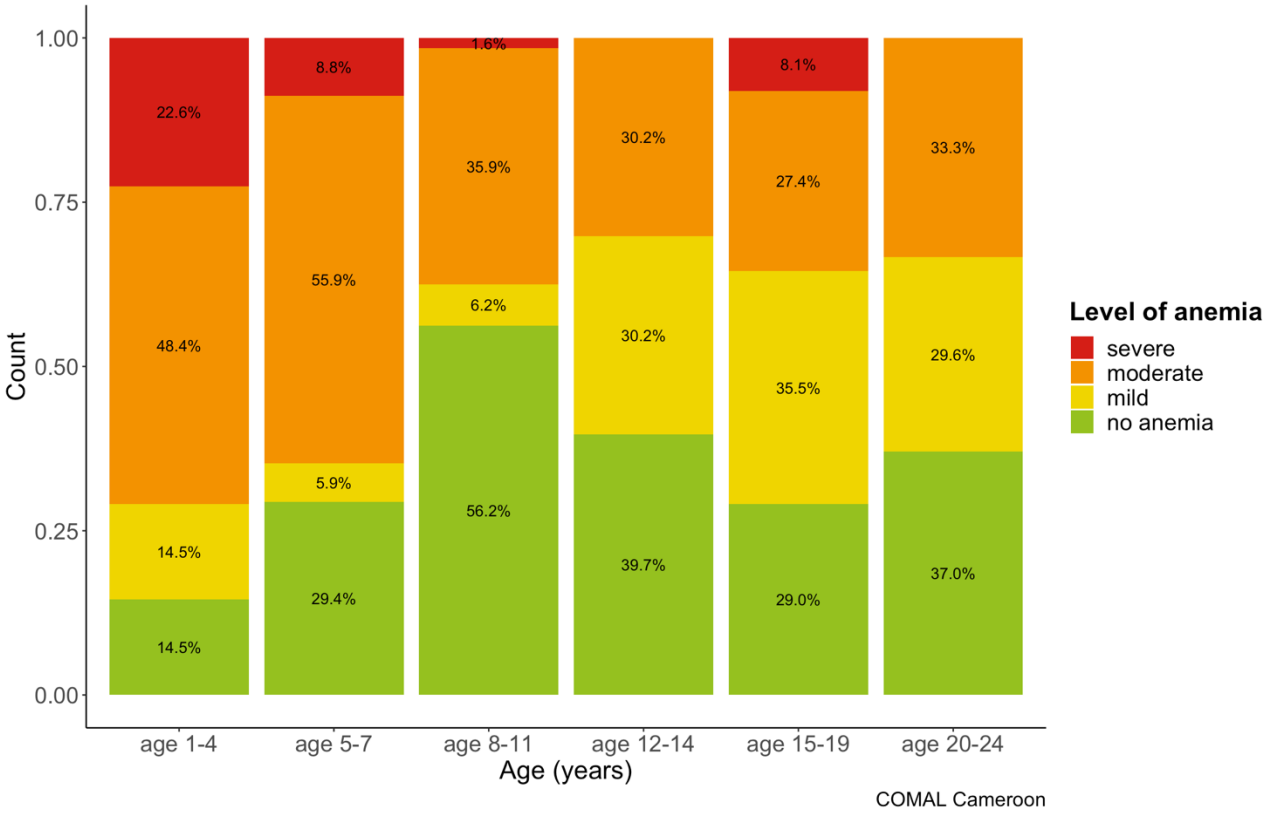

Figure 14 - Status of anemia in children, adolescents and young adults in Cameroon, showing very high rates of anemia in younger children

It is mainly the youngest kids from age 1 to 7 that suffer from the highest amount of anemia. In babies and toddlers aged 1 to 4 years, only 14.5% of all samples did not show any level of anemia. In this age group a striking 48.4% of the children suffer from moderate anemia, which, as will be further discussed below, can have detrimental effects on the development of these children. Additionally, in these youngest kids, 22.6% suffer from severe anemia, and 14.5% from mild anemia, making the proportion of children not affected by anemia very small.

Children from age 5 to 7, an even higher percentage of moderate anemia, 55.9%, while 8.8% of the children in this age group still suffer from severe anemia.

Looking onwards, in schoolchildren aged 8 to 11 years, there is an accentuated decrease in anemia cases, similar to the trends in Gabonese children, with over

half of the children not suffering from any kind of anemia. The highest number of cases in this age group, 35.9%, can be attributed to moderate anemia while there are only low levels of mild and severe anemia, with 6.2% and 1.6% respectively. In pubescent teenagers of the age group 12 to 14 cases of anemia rise again, with both 30% of mild anemia and 30% of moderate anemia cases .

Interestingly, adolescents and young adults from Cameroon do not see a decrease in anemia rates as can be observed in the samples from Gabon.

The differences in cases of anemia within the children and adolescents in Cameroon are statistically significant, X^2 (15, N = 347) = 83.3, $p < 0.001$. Especially the very high number of cases of severe anemia in the youngest age group and the increase of samples that are non-anemic in children aged 8-11 contribute to the significance of the Chi2-test.

Possible reasons for these differences in age groups, and the overall higher amount of anemia in children in Cameroon as compared to Gabon will be further discussed in the Discussion section.

Table 11 – Levels of anemia in children in Cameroon

	Age 1 - 4		Age 5 - 7		Age 8 - 11		Age 12 - 14	
	n	*%*	n	*%*	n	*%*	n	*%*
No anemia	9	*14.5*	20	*29.4*	36	*56.2*	25	*39.7*
Mild anemia	9	*14.5*	4	*5.9*	4	*6.2*	19	*30.2*
Moderate anemia	30	*48.4*	38	*55.9*	23	*35.9*	19	*30.2*
Severe anemia	14	*22.6*	6	*8.8*	1	*1.6*	0	

3.4 Prevalence of *Plasmodium malariae* and *falciparum*

For both countries, the prevalence of *Plasmodium malariae* and *Plasmodium falciparum* was assessed using light microscopy and qPCR. The results will be highlighted in the following section.

3.4.1 Ct Values reflect the amount of amplified genetic material

In order to understand the results obtained by qPCR, it is important to understand that the measured Ct value reflects the number of cycles of amplification of genetic material (in this case RNA), giving an indication about the parasitemia in the sample.

A higher Ct value indicates a need for more amplification cycles in order to detect a signal, a lower Ct value means that only a few cycles were needed to amplify enough material for it to become detectable. Therefore, a higher Ct value means there was less material to amplify (in our case less parasites per µL) and a lower Ct value indicates a higher amount of source material (in our case a higher parasitemia).

We can therefore infer that a higher Ct value reflects a lower parasitemia in the sample, and a lower Ct value reflects a higher parasitemia in the sample.

3.4.2 High prevalence of *P. malariae* and *P. falciparum* in Gabon

In this section, the prevalence of *Plasmodium malariae* and *falciparum* in Gabon, as measured by light microscopy and qPCR, will be presented.

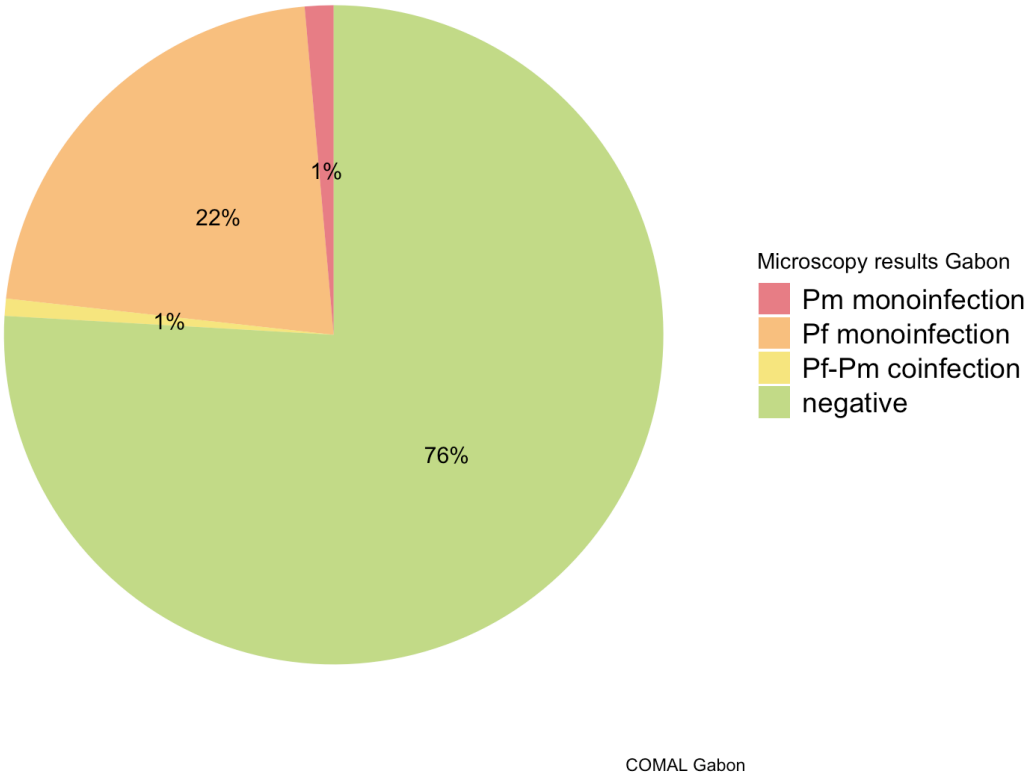

Figure 15 – Infections detected by light microscopy in Gabon

Through light microscopy, performed by expert microscopists in Gabon, 76% of all examined samples were found to be negative. 22% of the samples were identified as *P. falciparum*-mono-infections. *Plasmodium malariae* mono-infections were detected in 1.4% of the samples (15 out of 1068 samples), co-infections of *P. falciparum* and *P. malariae* in only 0.8% of samples (9 samples). Additionally, one *P. falciparum – P. ovale*-co-infection was identified.

This is in stark contrast with regards to the results obtained by qPCR.

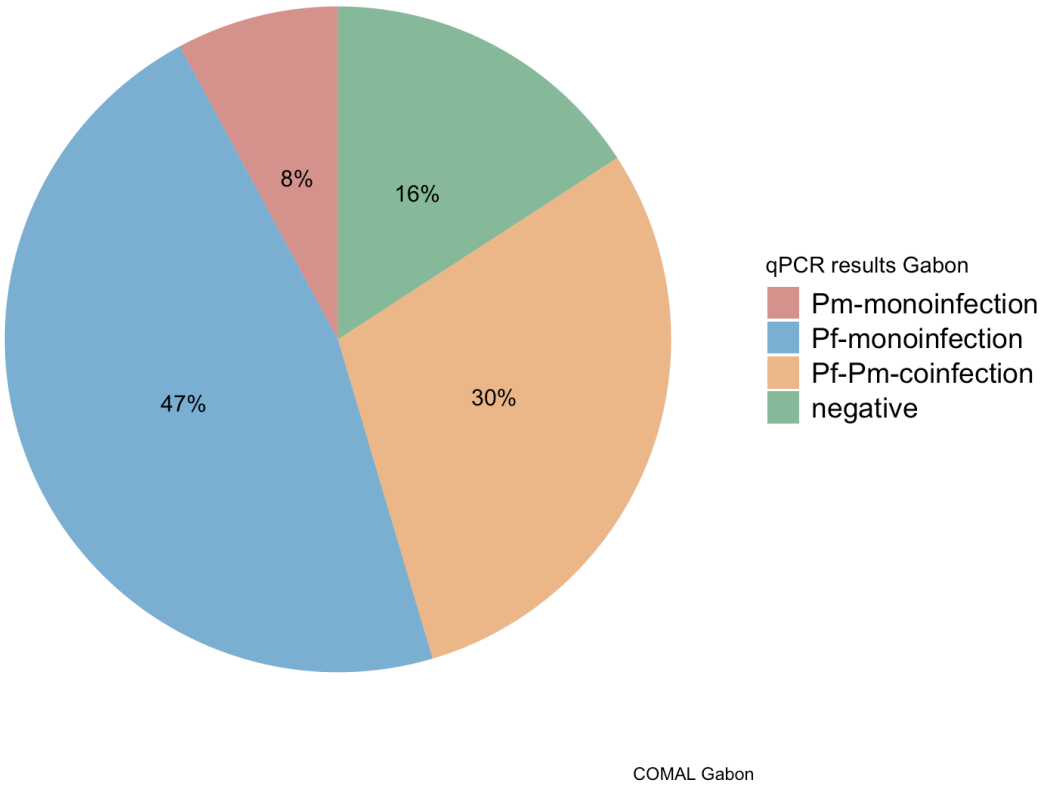

Figure 16 – Infections detected by qPCR in Gabon

By highly sensitive qPCR, only 16% of all 1068 samples were negative. Of the positively tested samples there were 47% of *P. falciparum*-monoinfections, 8% of *P. malariae*-mono-infections and 30% *P. falciparum-P. malariae*-co-infections.

These numbers show a much larger amount of *Plasmodium malariae* mono- and co-infections with *Plasmodium falciparum* than observed by light microscopy.

It is additionally interesting to note, that the overall amount of *Plasmodium falciparum* infections (counting both mono- and co-infections), which covers about 77% of all samples, is almost double the amount of *Plasmodium malariae* infections which is present in about 38% of samples.

This could be due to a number of factors in vector and parasite behavior as well as the *Plasmodium* life cycle and will be further discussed in the Discussion section.

When looking at malaria infections in Gabon in different age groups, multiple differences can be observed between age groups, but also between results obtained by light microscopy and qPCR (Figure 17 & Table 12).

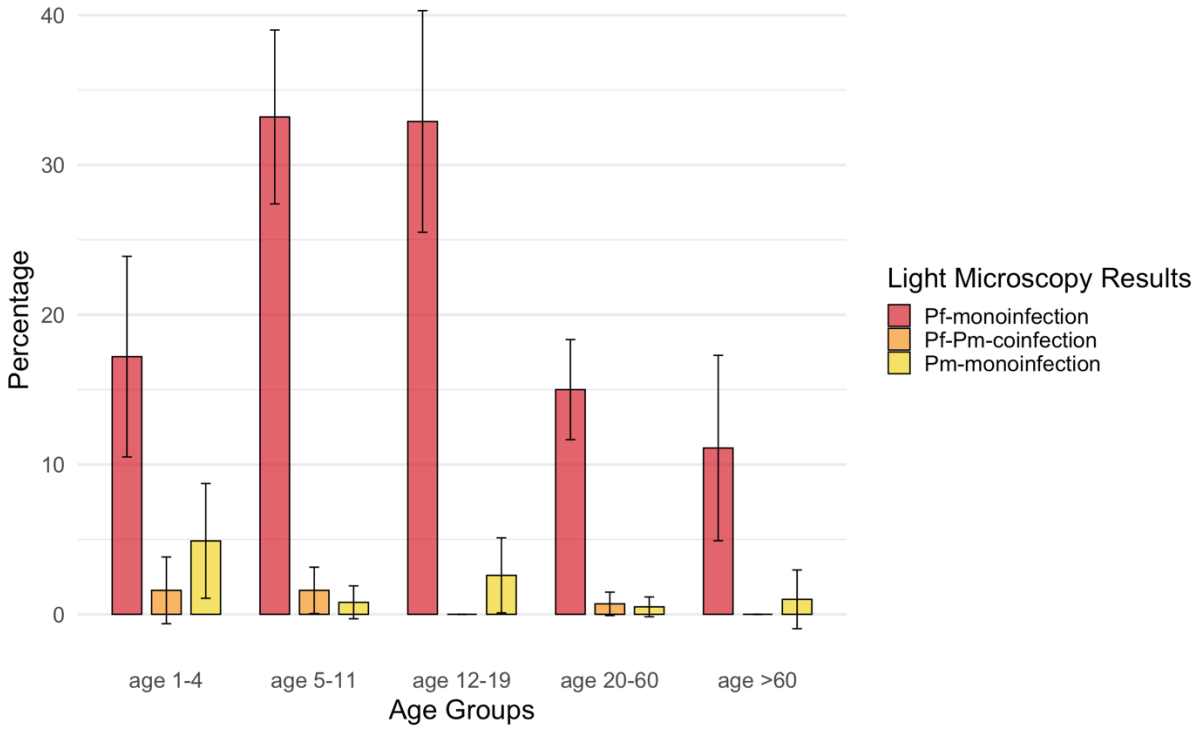

Figure 17 – Prevalence (in %) of P. falciparum and P. malariae found by light microscopy in different age groups in Gabon, including error bars indicating the 95% confidence interval

For instance, for infections diagnosed by light microscopy, in ages 5-11 and 12-19, with 33.2% and 32.9% respectively there are almost double as many *Plasmodium falciparum* mono-infections as in the other age groups.

Plasmodium malariae monoinfections on the other hand are, again as obtained by light microscopy, most prevalent in the youngest age group, making up 4.9% of infections in children of age 1-4. A second peak can be observed in young adults aged 12-19, with 2.6% of infections in this age group being identified as *P. malariae*.

The results obtained by qPCR (Figure 18) paint a different picture. When considering *P. falciparum* mono-infections, the highest number of infected samples can be found in the youngest age groups, namely 56.6% in children

aged 1-4 years and 53.4% in children aged 5-11 years. Over the age groups, a gradual decline of infected samples can be found with rising age, with, in comparison, only 38.4% of all samples being *P. falciparum* mono-infected in study participants over 60 years old.

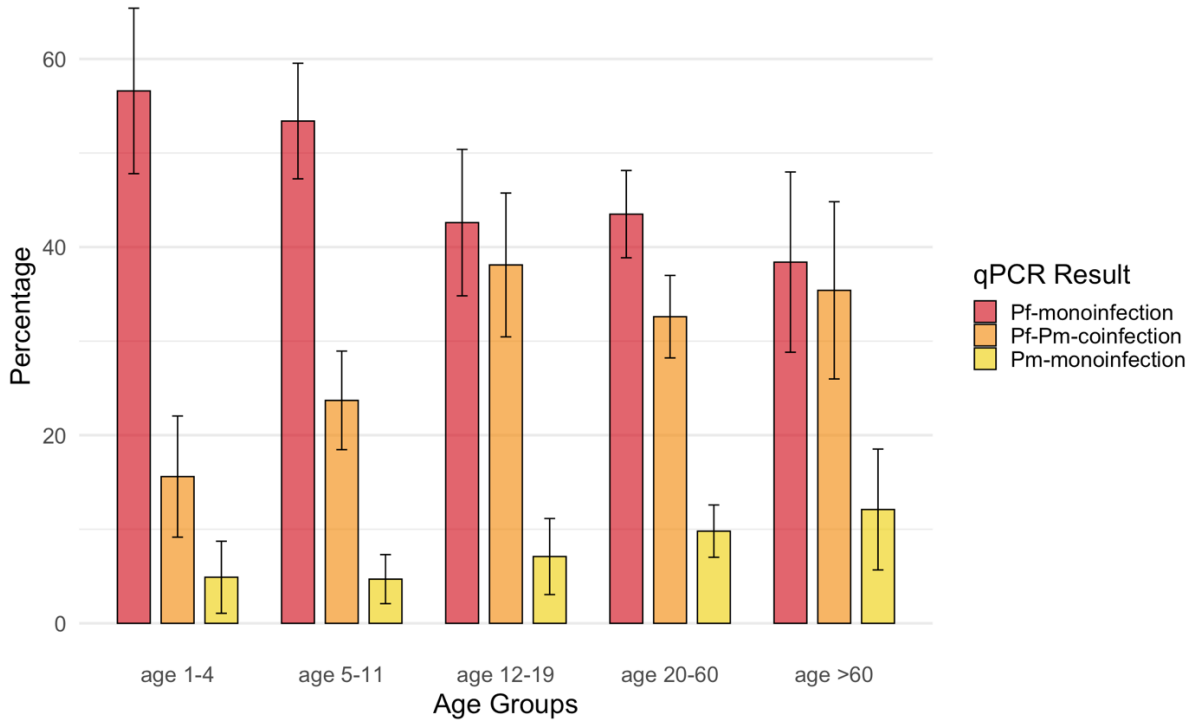

Figure 18 – Prevalence (in %) of P. falciparum and P. malariae found by qPCR in different age groups in Gabon, including error bars indicating the 95% confidence interval

For *Plasmodium malariae* monoinfections on the other hand, a gradual incline of the number of infected samples from younger to older participants can be observed. In the age group of 1-4, 4.9% of samples are mono-infected with *P. malariae*. In adults over 60 years old, the *P. malariae* mono-infection rate has risen to 12.1% of samples in this age group.

As identified by qPCR, the rate of negatively tested samples decreases with age, suggesting a higher rate of chronic sub-clinical *Plasmodium* infection in adults.

Table 12 – Prevalence in different age groups of Plasmodium malariae and falciparum identified by microscopy and qPCR in Gabon

Plasmodium spp. infection	Age 1- 4		Age 5 - 11		Age 12 - 19		Age 20 - 60		Age > 60		Total	
	n	%	*n*	%	*n*	%	*n*	%	n	%	n	%
Microscopy												
P. falciparum-monoinfection	21	*17.2%*	84	*33.2%*	51	*32.9%*	66	*15.0%*	11	*11.1%*	233	*21,8%*
P. malariae-monoinfection	6	*4.9%*	2	*0.8%*	4	*2.6%*	2	*0.5%*	1	*1.0%*	15	*1,4%*
P. falciparum - P. malariae-co-infection	2	*1.6%*	4	*1.6%*	0		3	*0.7%*	0		9	*0,8%*
P. falciparum-P. ovale-co-infection	0		1	*0.4%*	0		0		0		1	*0,09%*
Negative	93	*76.2%*	162	*64%*	100	*64.5%*	368	*83.8%*	87	*87.9%*	810	*75,8%*
PCR												
P. falciparum-monoinfection	69	*56.6%*	135	*53.4%*	66	*42.6%*	190	*43.3%*	38	*38.4%*	498	*46.6%*
P. malariae-monoinfection	6	*4.9%*	12	*4.7%*	11	*7.1%*	44	*10.0%*	12	*12.1%*	85	*8%*
P. falciparum - P. malariae-co-infection	19	*15.6%*	60	*23.7%*	59	*38.1%*	143	*32.6%*	35	*35.4%*	316	*29.6%*
Negative	28	*23%*	46	*18.2%*	19	*12.3%*	62	*14.1%*	14	*14.1%*	169	*15.8%*
Total	122		253		155		439		99		1068	*100%*

3.4.3 Comparison of qPCR and light microscopy findings in Gabon

The results obtained by light microscopy do not reflect the results that the highly sensitive qPCR yielded.

80% of *P. malariae* mono-infections, 78% of *P. falciparum* mono-infections and 61% of mixed infections were microscopically classified as negative samples, indicating a high level of sub-microscopic infections.

A few samples show that not all infections were classified correctly, as for example 11 % of *P. malariae* mono-infections were microscopically identified as *P. falciparum* mono-infections.

Table 13 gives an overview of the exact numbers of infections in Gabonese samples as identified by qPCR and light microscopy.

Table 13 – Comparison of the results obtained by highly sensitive qPCR and by light microscopy in samples from Gabon

Results by qPCR →	*P. malariae* mono-infection		*P. falciparum* mono-infection		*P. f – P. m* co-infection		No infection by qPCR		Row Total
Results by light microscopy ↓	n	*%*	n	*%*	n	*%*	n	*%*	n
P. malariae mono-infection	6	*7.1*	4	*0.8*	4	*1.3*	1	*0.6*	15
P. falciparum mono-infection	9	*10.7*	100	*20.0*	113	*35.8*	11	*6.5*	233
P. f – P. m co-infection	2	*2.4*	2	*0.4*	4	*1.3*	1	*0.6*	9
P. f – P. o co-infection	0		0		1	*0.3*	0		1
No infection by microscopy	67	*79.8*	393	*78.0*	194	*61.4*	156	92.3	810
Column Total	84	*100%*	499	*100%*	316	*100%*	169	100%	1068

When comparing median Ct values of microscopically negative yet qPCR positive samples the values are higher (*P. malariae* median Ct value of 25.9 (IQR 22.6 – 31.6), *P. falciparum* median Ct value of 23.7 (IQR 19.2 – 31.8)) than the median Ct values in microscopically and qPCR positive samples (P. malariae median Ct value of 23.8 (IQR 19.9 – 29.0), *P. falciparum* median Ct value of 16.3 (IQR 13.8 – 20.0).

3.4.4 High prevalence of P. malariae and P. falciparum in Cameroon

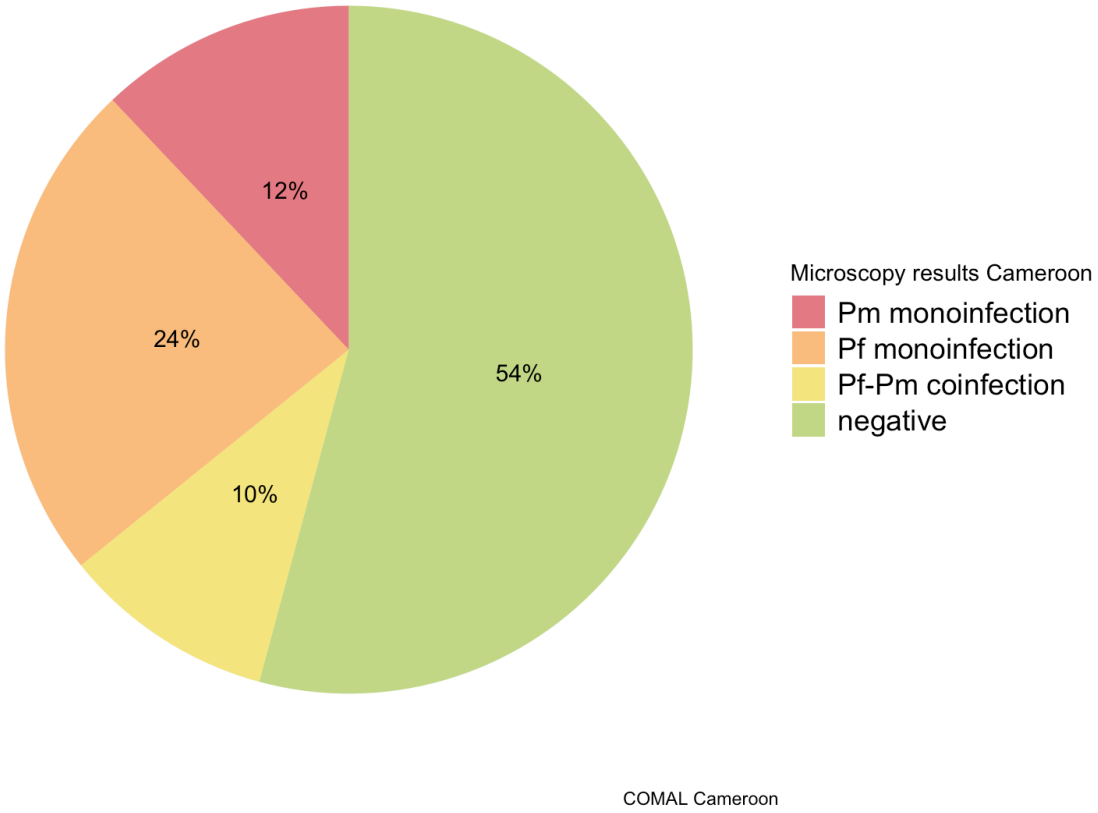

Figure 19 – Infections detected by light microscopy in Cameroon

Through light microscopy, performed by expert microscopists in Cameroon, a little over half of all examined samples, 54%, was negative.

Almost 24% of all samples were classified as *Plasmodium falciparum* mono-infections (116 out of 489 samples). In contrast to the results in Gabon, in Cameroon 12% of all samples were found to be a *P. malariae* mono-infection by microscopy (59 out of 489 samples). In 10% of all samples, a *P. falciparum* – *P. malariae*-co-infection was identified (49 out of 489 samples).

Similarly to Gabon, in Cameroon too the results by light microscopy vary strongly with regards to the results obtained by qPCR.

By highly sensitive qPCR, only 4% of all 489 samples were negative. Of the positively tested samples the qPCR identified 45% as *P. falciparum* mono-

infections, 4% as *P. malariae* mono-infections and 48% as *P. falciparum – P. malariae*-co-infections.

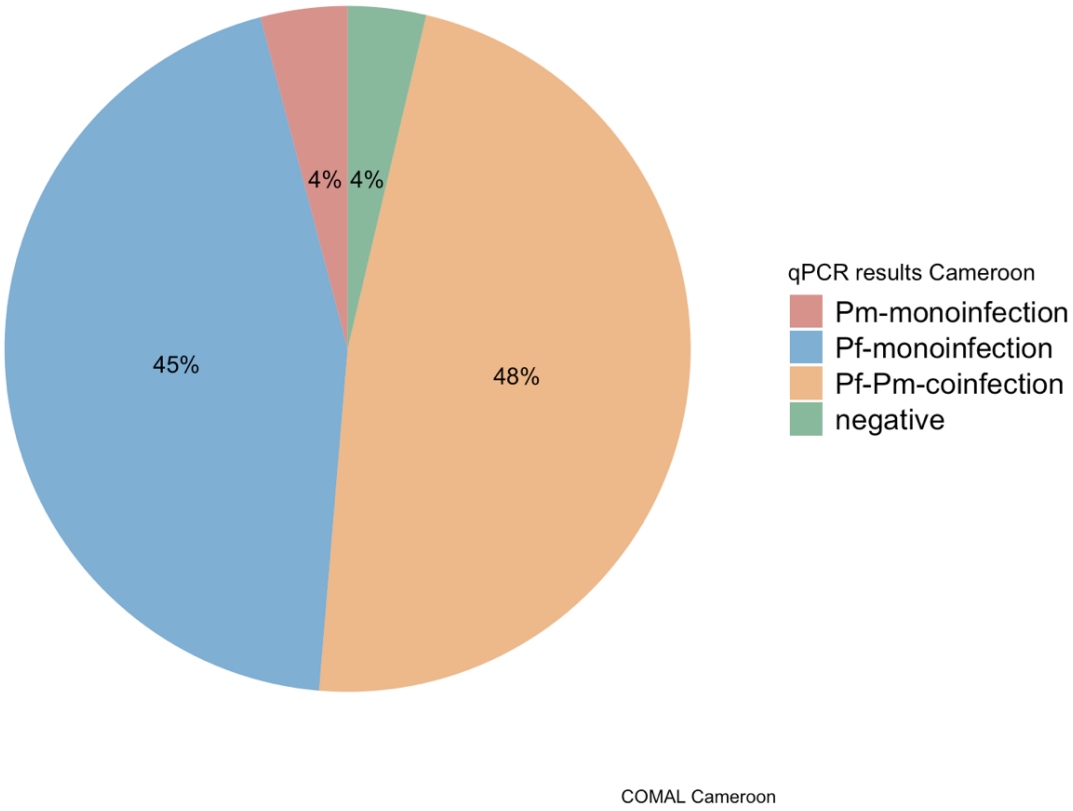

Figure 20 – Infections detected by qPCR in Cameroon

As in Gabon, in Cameroon the overall amount of *Plasmodium falciparum* infections (counting both mono- and co-infections), which covers a staggering 93% of all samples, is almost double the amount of *Plasmodium malariae* infections which is present in about 52% of all samples.

When taking a closer look at the prevalence of infections in different age groups we see a slightly different picture than in Gabon.

By light microscopy, high numbers of *Plasmodium falciparum* mono-infections were detected in children and young adults. These range from 25.8% in children aged 1 to 4 years to 26.2% in adolescents and, with the highest amount of *P. falciparum* mono-infections, 37.1%, in children aged 5 to 11 years. In adults, the infection rate as detected by light microscopy does not reach more than 12%.

Similar to the *P. malariae* mono-infection rates, the number of samples having been classified positive by light microscopy for a co-infection of *P. falciparum* and *P. malariae* is quite high, at an average 10% of all samples. Interestingly, the detected amount of co-infections is highest among children, with 21% in children aged 1 to 4 years, and slowly decreasing with each age group, with only 2.6% of co-infections detected in adults aged 20 to 60 years. Due to the low number of study participants over the age of 60 years in Cameroon, the confidence intervals in this age group cross zero, making it difficult to draw valid conclusions for individuals of this age group.

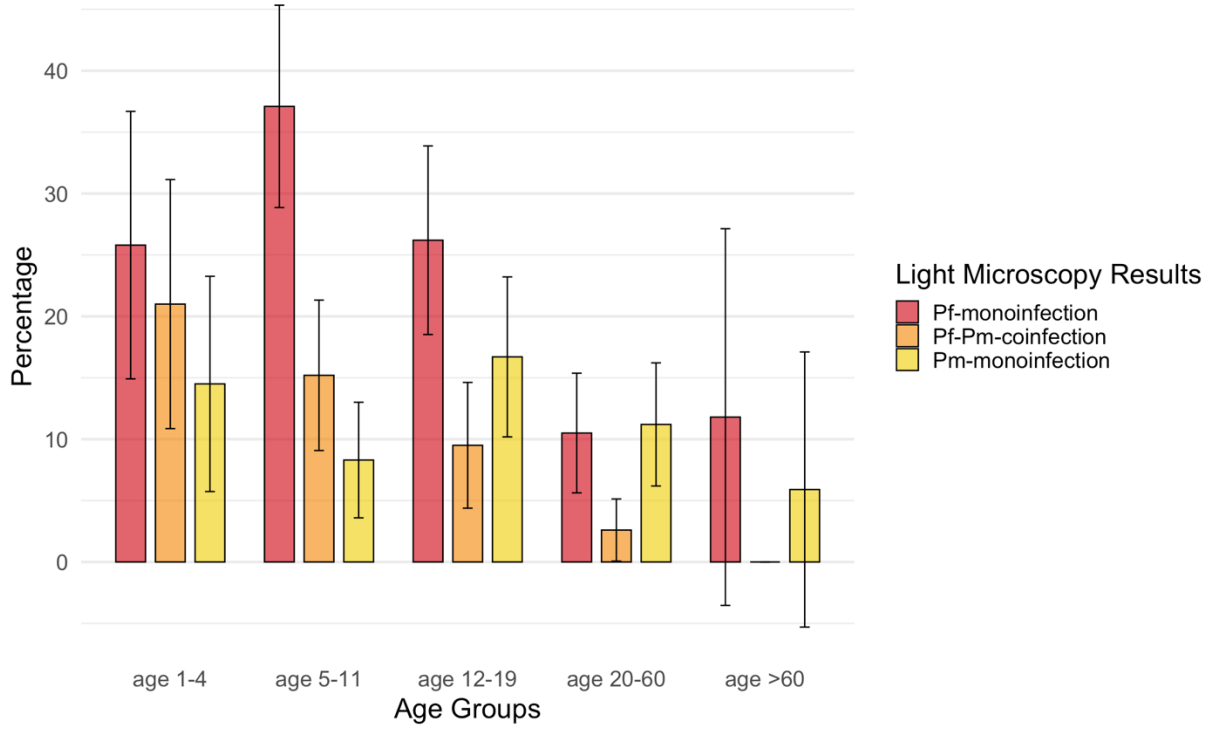

Figure 21 - Prevalence (in %) of P. falciparum and P. malariae found by light microscopy in different age groups in Cameroon, including error bars indicating the 95% confidence interval

The results obtained by highly sensitive qPCR show different numbers.

With 44.6% there is a high overall amount of *P. falciparum* mono-infections in the Cameroonian samples, reaching its peaks in the youngest children (ages 1 to 4) with 55.8% and in adults aged 20-60 with 55.3%.

P. malariae mono-infections were detected in 4.1% of the samples, with a similar prevalence in the different age groups.

Almost 50% of all samples were identified as a co-infection of the two pathogens, with the highest number of mixed infections, 61.1%, being present in adolescents aged 12 to 19, and the lowest, 35.5%, in children aged 1 to 4 years.

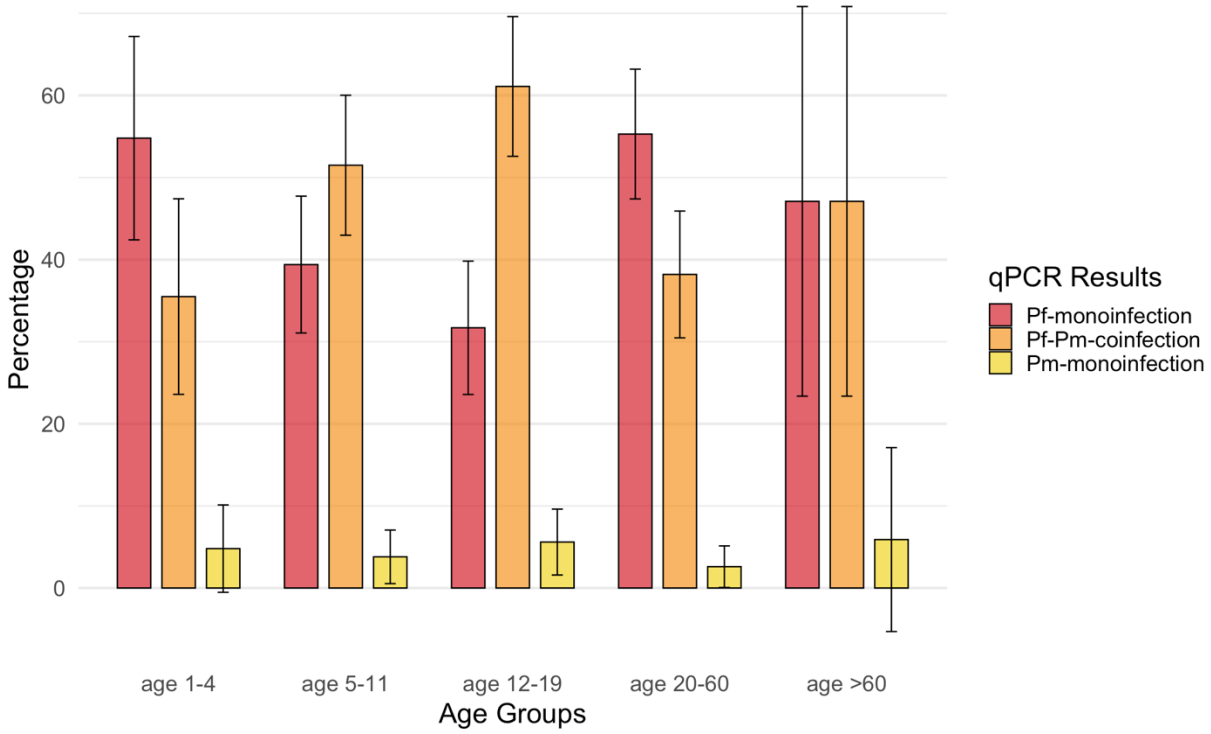

Figure 22 – Prevalence (in %) of P. falciparum and P. malariae found by qPCR in different age groups in Cameroon, including error bars indicating the 95% confidence interval

Table 14 - Prevalence in different age groups of Plasmodium malariae and falciparum as identified by microscopy and qPCR in Cameroon

Plasmodium spp. infection	Age 1- 4		Age 5 - 11		Age 12 - 19		Age 20 - 60		Age > 60		Total	
	n	%	*n*	%	*n*	%	*n*	%	n	%	n	%
Microscopy												
P. falciparum-monoinfection	16	*25.8%*	49	*37.1%*	33	*26.2%*	16	*10.5%*	2	*11.8%*	116	*23.7%*
P. malariae-monoinfection	9	*14.5%*	11	*8.3%*	21	*16.7%*	17	*11.2%*	1	*5.9%*	59	*12.1%*
P. falciparum - P. malariae-co-infection	13	*21.0%*	20	*15.2%*	12	*9.5%*	4	*2.6%*	0		49	*10%*
Negative	24	*38.7%*	52	*39.4%*	60	*47.6%*	115	*75.7%*	14	*82.4%*	265	*54.2%*
PCR												
P. falciparum-monoinfection	34	*54.8%*	52	*39.4%*	40	*31.7%*	84	*55.3%*	8	*47.1%*	218	*44.6%*
P. malariae-monoinfection	3	*4.8%*	5	*3.8%*	7	*5.6%*	4	*2.6%*	1	*5.9%*	20	*4.1%*
P. falciparum - P. malariae-co-infection	22	*35.5%*	68	*51.5%*	77	*61.1%*	58	*38.2%*	8	*47.1%*	233	*47.6%*
Negative	3	*4.8%*	7	*5.3%*	2	*1.6%*	6	*3.9%*	0		18	*3.7%*
Total	62		132		126		152		17		489	*100%*

3.4.5 Comparison of qPCR and light microscopy findings in Cameroon

In the Cameroonian samples 40% of *P. malariae* mono-infections, 63% of *P. falciparum* mono-infections and 46% of mixed infections were microscopically classified as negative samples. This indicates a substantial level of sub-microscopic infections, albeit less than in Gabon.

Table 15 shows similar results to Gabon when it comes to erroneous microscopical diagnoses. 7 out of 20 *Plasmodium malariae* mono-infections were identified as *P. falciparum* mono-infections by light microscopy and 2 as mixed infections. An interesting finding is that of all the *P. falciparum* mono-infections, 13% were identified as *P. malariae* mono-infections and 7.3% as mixed infections.

Table 15 – Comparison of the results obtained by highly sensitive qPCR and by light microscopy in samples from Cameroon

Results by qPCR →	*P. malariae* mono-infection		*P. falciparum* mono-infection		*P. f – P. m* co-infection		No infection by qPCR		Row Total
Results by light microscopy ↓	n	*%*	n	*%*	n	*%*	n	*%*	n
P. malariae mono-infection	3	*15.0*	28	*12.8*	25	*10.7*	3	*16.7*	59
P. falciparum mono-infection	7	*35.0*	36	*16.5*	71	*30.5*	2	*11.1*	116
P. f – P. m co-infection	2	*10.0*	16	*7.3*	31	*13.3*	0		49
No infection by microscopy	8	*40.0*	138	*63.3*	106	*45.5*	13	*72.2*	265
Column Total	20	*100%*	218	*100%*	233	*100%*	18	*100%*	489

As in Gabon, when comparing median Ct values of microscopically negative yet PCR positive samples the values are higher (*P. malariae* median Ct value of 28.2 (IQR 24.0 – 34.3), *P. falciparum* median Ct value of 22.2 (IQR 18.3 – 28.9) than the median Ct values in microscopically and PCR positive samples (*P. malariae* median Ct value of 25.3 (IQR 22.0 – 29.5), *P. falciparum* median Ct value of 15.7 (IQR 13.6 – 19.6)).

3.5 Differentiation of results into mono- and co-infections

A goal of this study, next to describing the epidemiology of *Plasmodium malariae* in Gabon and Cameroon, was to understand how *P. malariae* might interact with *P. falciparum*, and what factors could be playing a role in this interaction.

While these factors will be discussed later, it is important to look at the differences that can be found when we separate our results into mono- and co-infections and compare the Ct values obtained by qPCR.

3.5.1 Comparison of mono- versus co-infections in Gabon

As was stated above, in Gabon there were 8% of *P. malariae* mono-infections, 46.6% *P. falciparum* mono-infections and 29.6% co-infections of *P. malariae* with *P. falciparum*, as identified by qPCR.

3.5.1.1 Plasmodium malariae Ct values are lower in mono-infections than in co-infections in Gabon

The Ct values obtained for *Plasmodium malariae* mono-infections are spread out from 13.0 to 45.0, with a median-value of 24.00 (IQR 20.7 – 28.4), and a mean-value of 24.8 (SD = 6.7).

In co-infections of *P. malariae* and *P. falciparum*, the Ct values obtained for *P. malariae* range from 9.3 to 40.8, with a median-value of 25.9 (IQR 22.0 – 31.4), and a mean-value of 26.8 (SD = 6.0).

When comparing these Ct values which were obtained by highly sensitive qPCR on *P. malariae* RNA, it is interesting to see that the median Ct value of *P. malariae* monoinfections is lower than the median *P. malariae* Ct value of samples that are co-infected with *P. falciparum*. This has been visually presented in Figure 23.

A Welsh two-samples t-test shows that this difference is statistically significant, with $t(125) = -2.5$, $p = 0.01$ and a 95% confidence interval of -3.5 to – 0.4.

This suggests that in the average *Plasmodium malariae* mono-infection in Gabon there is a higher relative *P. malariae* parasitemia than when *P. malariae* comes in co-infections with *P. falciparum*, where there seems to be lower *P. malariae* parasitemia in comparison.

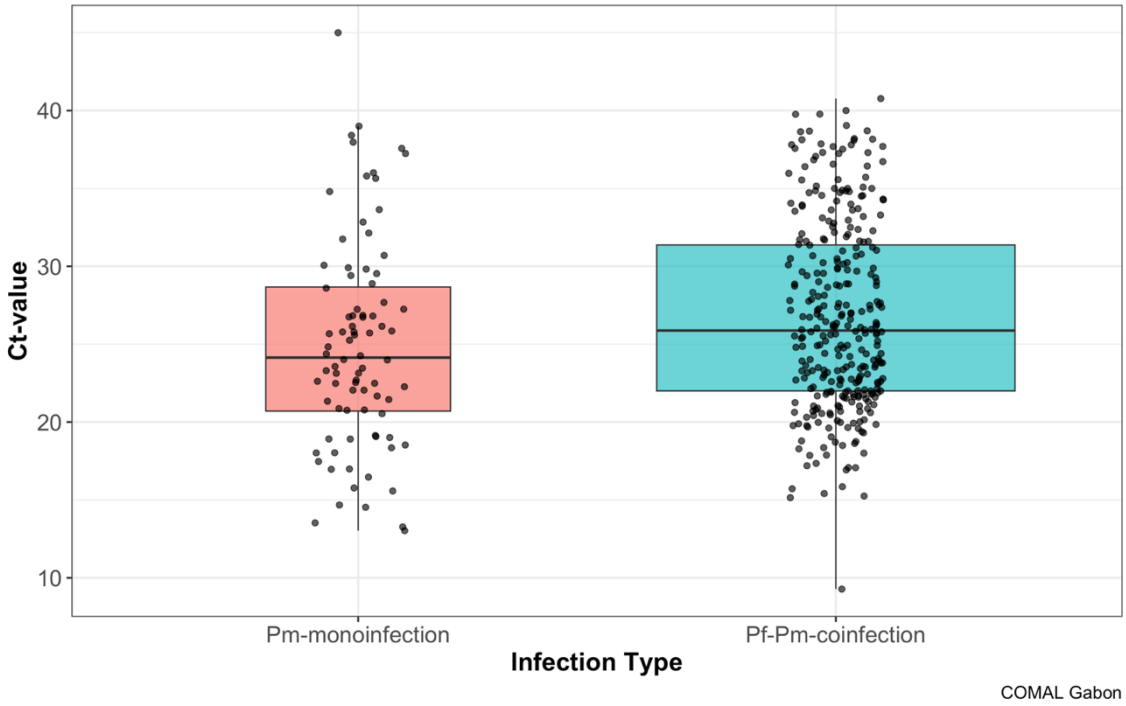

Figure 23 – Ct values of P. malariae in mono-infections are significantly lower than in co-infections with P. falciparum in samples from Gabon (p = 0.01)

Splitting up the results by age, this phenomenon is true for almost all age groups (Figure 24). Only in samples obtained from individuals > 60 years there is a trend to higher Ct values in *P. malariae* mono-infections, suggesting an impact of acquired immunity. This will be further highlighted in the section on "Age and *Plasmodium* infections" below.

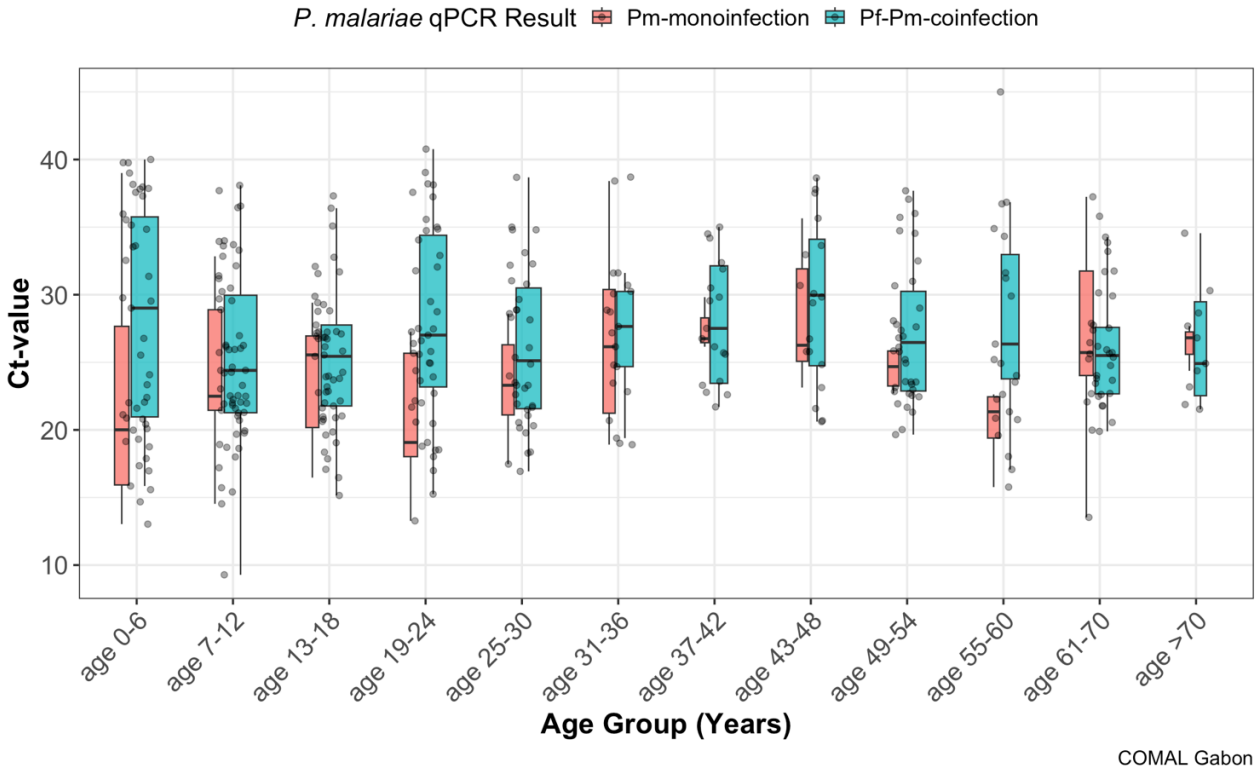

Figure 24 – P. malariae Ct value variations in P. malariae infected samples in different age groups in Gabon, split up into mono- and co-infections

The significance of the difference between Ct values in mono-versus co-infections is independent of any effect that age might have on this relationship, as linear regression shows. It reveals that there is indeed no significant effect of age on the differences between Ct values, with a P-value of 0.3 (Figure 25).

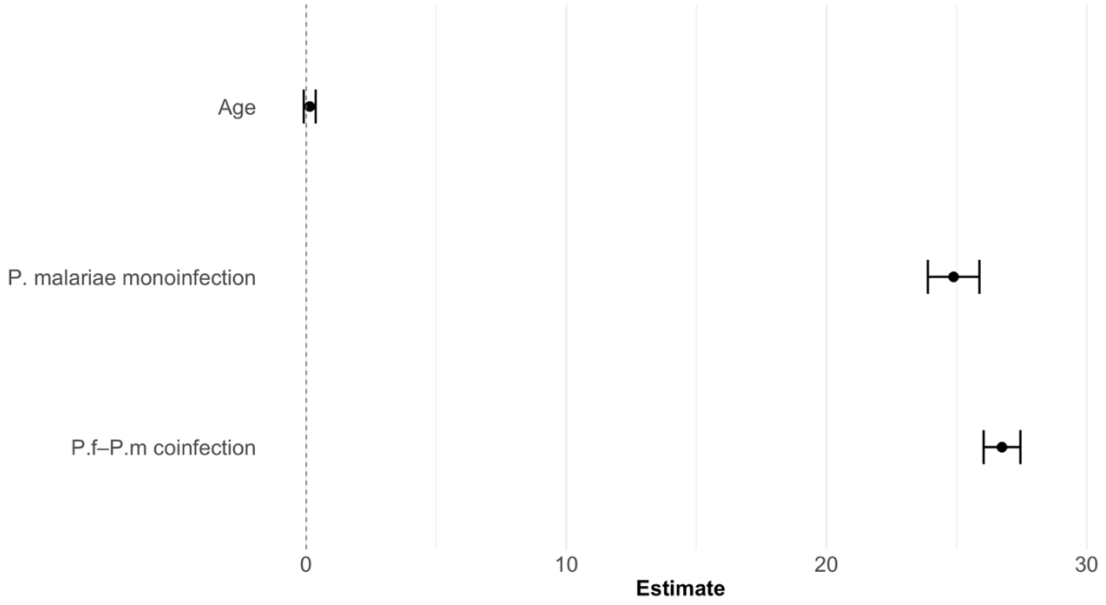

Figure 25 – Linear model showing no significant effect of age on P. malariae Ct values in samples from Gabon (pm_ct ~ pcr_res + scale(age))

3.5.1.2 Plasmodium falciparum Ct values are higher in mono-infections than in co-infections in Gabon

Taking a closer look at the results obtained by qPCR for *P. falciparum* in Gabon, a different picture arises.

P. falciparum Ct values range from 8.0 to 43.3 in monoinfections and 6.7 to 36.7 in coinfected samples.

For P. falciparum, the median Ct value in *P. falciparum* mono-infections is at 25.0 (IQR 19.1 – 33.4), while for P. falciparum co-infections with *P. malariae*, it is much lower, at 18.4 (IQR 15.8 – 22.0).

This suggests, that the parasitemia in *P. falciparum* mono-infections is lower than when it comes together with a *P. malariae* infection. Indeed, in mixed infections it seems that *P. falciparum* parasitemia is relevantly higher, when judging by the much lower Ct value in co-infections (Figure 26).

Again, this difference is significant, with $t(812) = 13.2$, $p < 0{,}001$ and a 95% confidence interval of 5.3 to 7.1 obtained by Welsh's t-test.

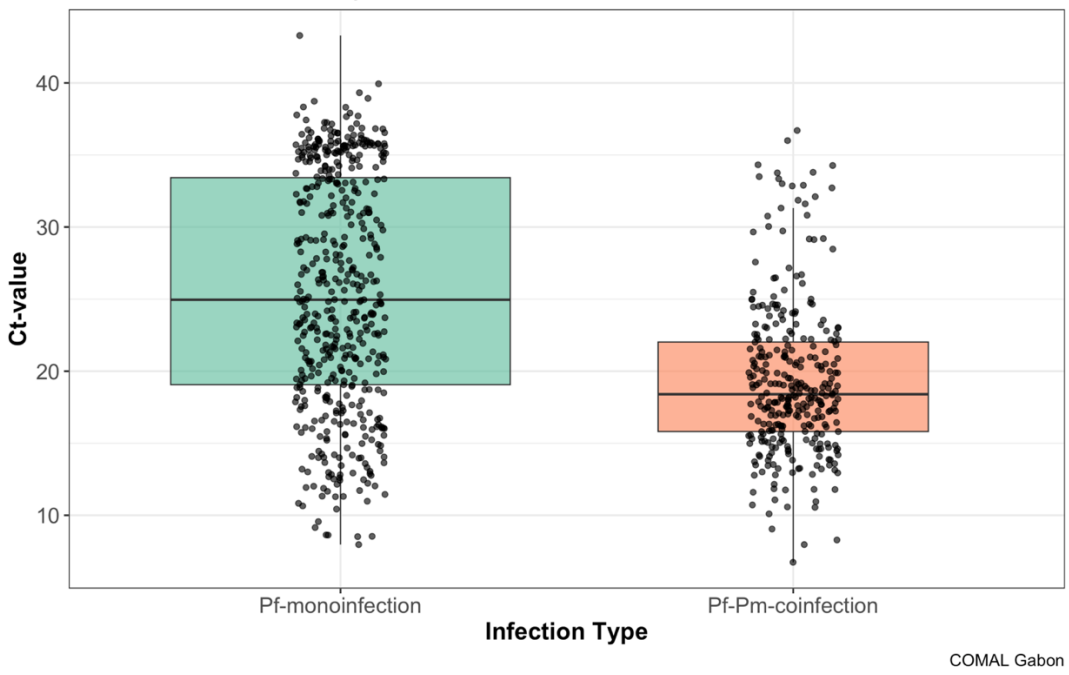

Figure 26 - Ct values of P. falciparum in mono-infections are significantly higher than in co-infections with P. malariae in samples from Gabon ($p < 0.001$)

When splitting the analysis by age groups, it can be observed that this phenomenon persists along all ages (Figure 27).

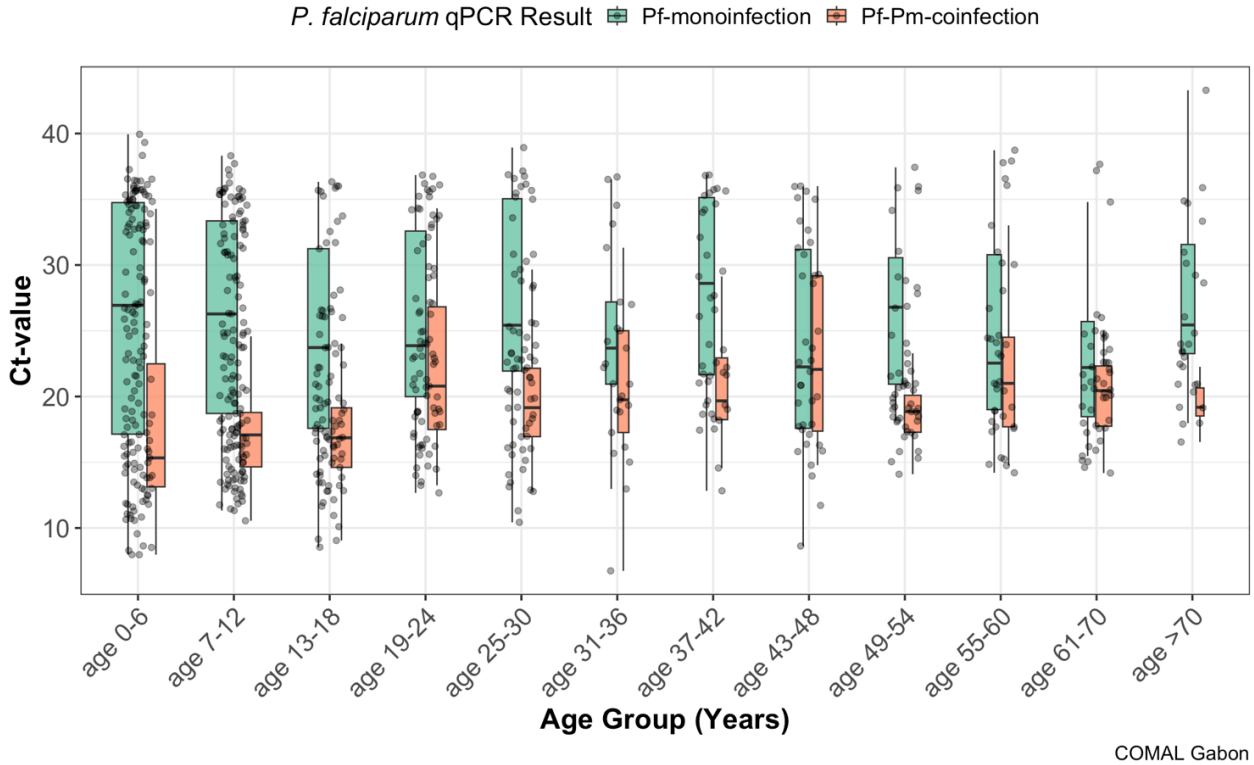

Figure 27 - P. falciparum Ct value variations in P. falciparum infected samples in different age groups in Gabon, split up into mono- and co-infection

A linear regression model (Figure 28), with a p-value of 0.1, shows no significant effect of age on the Ct values in *P. falciparum* infected samples in Gabon.

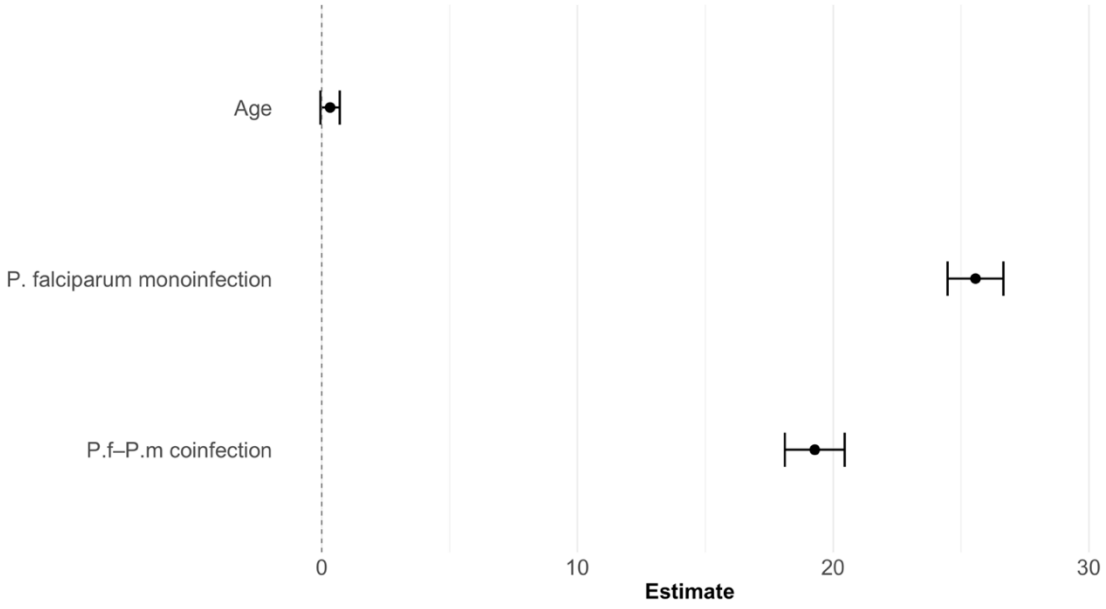

Figure 28 - Linear model showing no significant effect of age on P. falciparum Ct values in samples from Gabon (pf_ct ~ pcr_res + scale(age))

3.5.2 Comparison of mono- versus co-infections in Cameroon

For the samples from Cameroon, 4% of *P. malariae* mono-infections were found, 45% of *P. falciparum* mono-infections and a staggering 48% of co-infections of *P. malariae* with *P. falciparum*, as identified by qPCR.

3.5.2.1 Plasmodium malariae Ct values are lower in mono-infections than in co-infections in Cameroon

The Ct values obtained for *Plasmodium malariae* mono-infections are spread out from 14.8 to 36.2, with a median-value of 22.4 (IQR 19.0 – 26.1) and a mean-value of 23.3 (SD = 6.4).

In co-infections of *P. malariae* and *P. falciparum*, the Ct values obtained for *P. malariae* range from 12.7 to 50.5, with a median-value of 26.9 (IQR 23.3 – 31.7) and a mean-value of 27.5 (SD = 5.6).

Again, as in the data from Gabon, there is a visible difference of the median Ct value of *P. malariae* monoinfections when compared to the median *P. malariae* Ct value of samples that also contain an infection with *P. falciparum*. This has been visualized in Figure 29.

A Welsh two-samples t-test shows that this difference is statistically significant, t(21.5) = -2.9, p < 0,01 and a 95% confidence interval of -7.3 to -1.2.

This suggests that we see the same trend in Cameroon as we saw in Gabon: in the average *Plasmodium malariae* mono-infection there is a higher *P. malariae* parasitemia than when *P. malariae* comes in co-infections with *P. falciparum*, where there seems to be lower *P. malariae* parasitemia in comparison.

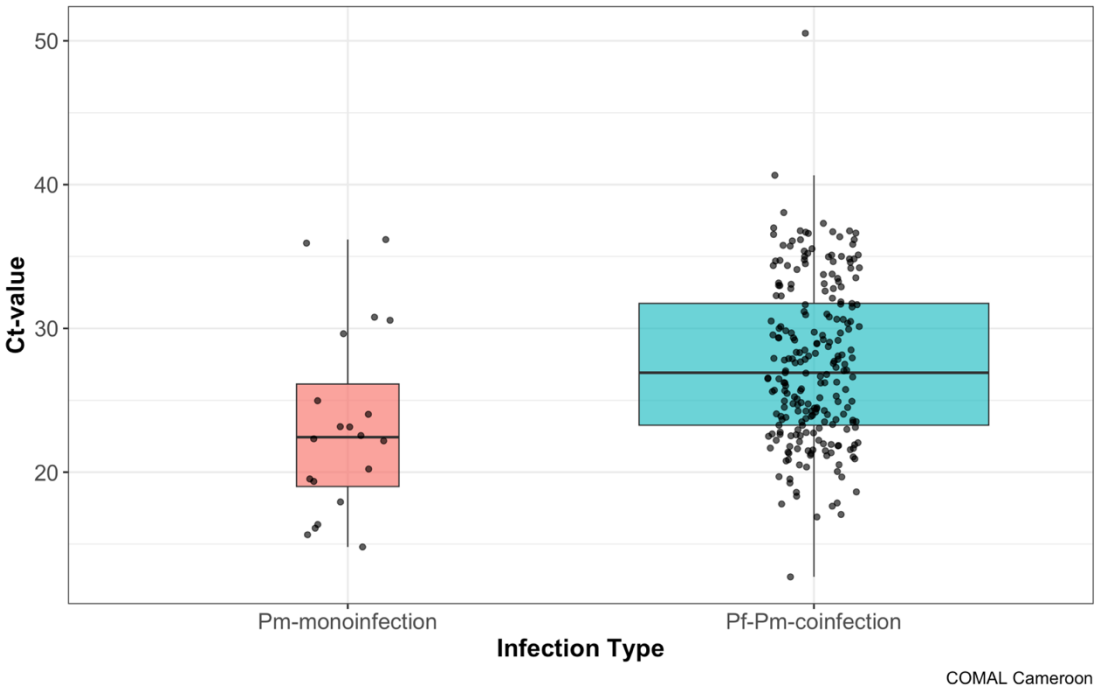

Figure 29 - Ct values of P. malariae in mono-infections are significantly lower than in co-infections with P. falciparum in samples from Cameroon (p < 0.01)

However, when the box plot is split up by age groups, it becomes obvious that the low absolute number of *P. malariae* mono-infections makes the interpretation of these results very difficult, as there are simply not enough *P. malariae* mono-infections in most age groups to present them statistically (Figure 30).

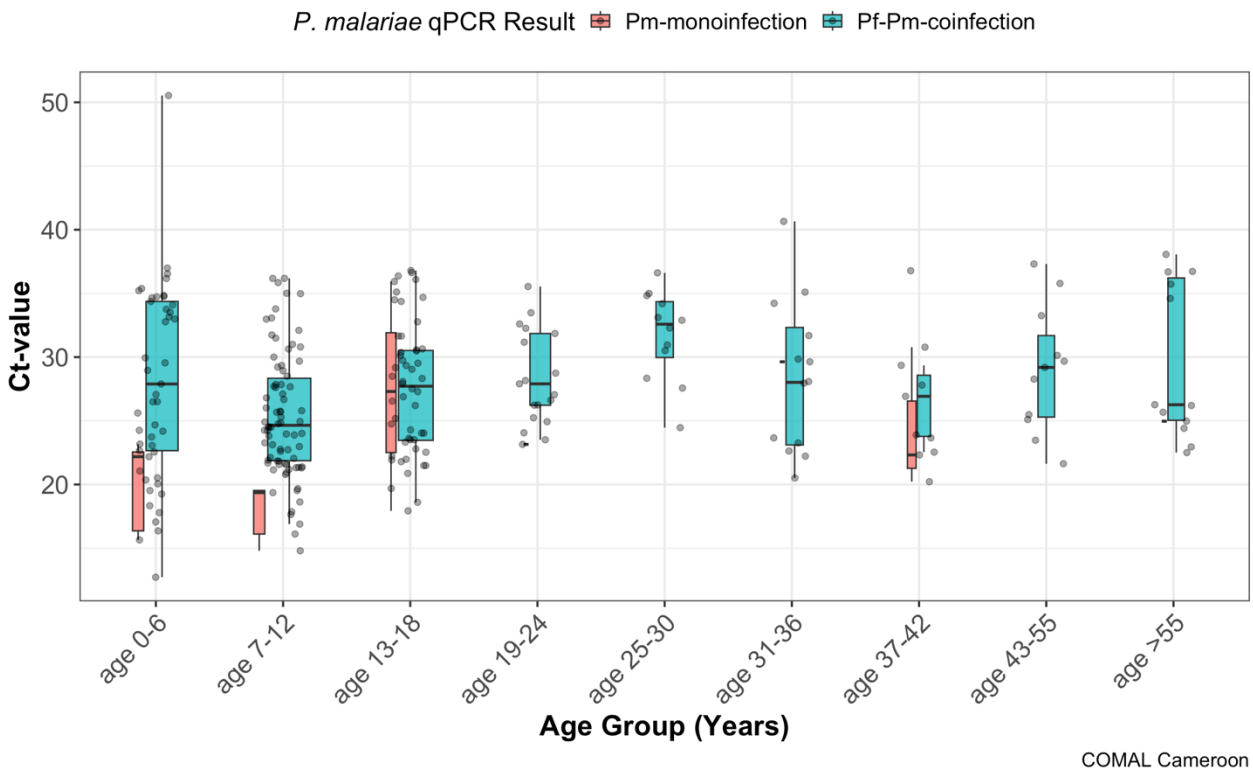

Figure 30 - P. malariae Ct value variations in P. malariae infected samples in different age groups in Cameroon, split up into mono- and co-infections

In *P. malariae* infected samples in Cameroon, a linear regression model shows that age significantly impacts Ct values ($p < 0.01$), even though much less than the kind of infection (Figure 31). This suggests some influence of acquired immunity on Ct values.

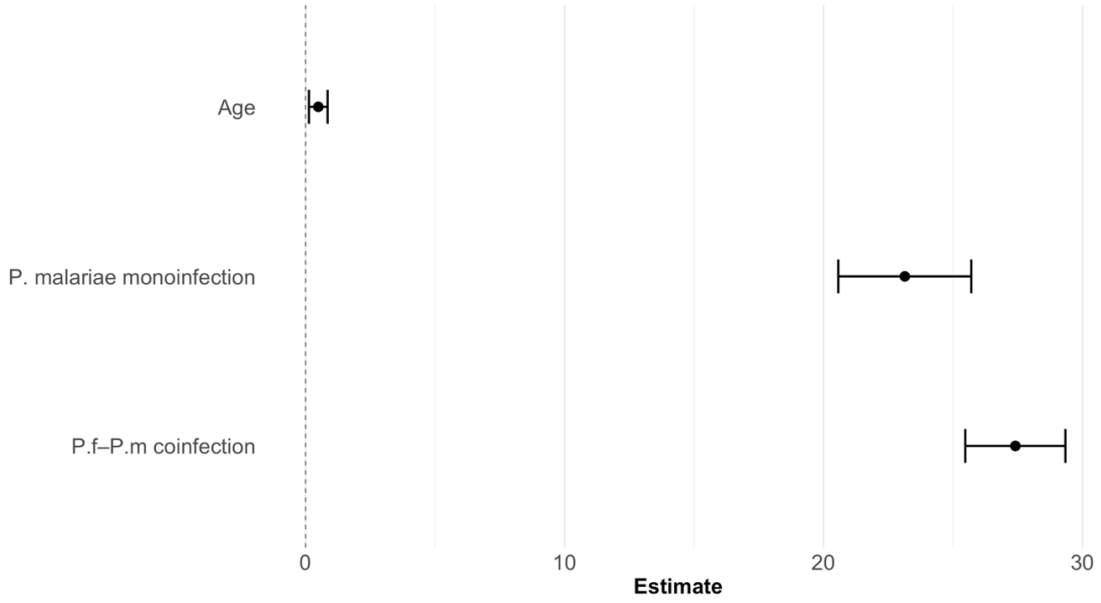

Figure 31 – Linear model showing a significant effect of age on P. malariae Ct values in samples from Cameroon ($p < 0.01$) (pm_ct ~ pcr_res + scale(age))

To sum up, for the *P. malariae* values from Cameroon the low number of *P. malariae* mono-infections presents a limitation to the validity of these statistical calculations that we need to keep in mind when looking at the results. However, based on what has been shown for the data from Gabon, it seems that the trend for the results from Cameroon goes into the same direction. Additionally, there seems to be some effect of acquired immunity.

The future analysis of the remaining samples from Cameroon will certainly give further power to these assumptions.

3.5.2.2 Plasmodium falciparum Ct values are higher in mono-infections than in co-infections in Cameroon

Regarding *P. falciparum* in the samples from Cameroon a similar trend as in Gabon can be observed (Figure 32).

P. falciparum Ct values range from 6.0 to 37.2 in monoinfections and 9.2 to 35.0 in coinfected samples.

For *P. falciparum*, the median Ct value in *P. falciparum* monoinfections is at 22.5 (IQR 17.1 – 31.3), while for *P. falciparum* co-infections with *P. malariae*, it is again much lower, at 17.4 (IQR 14.7 – 20.9).

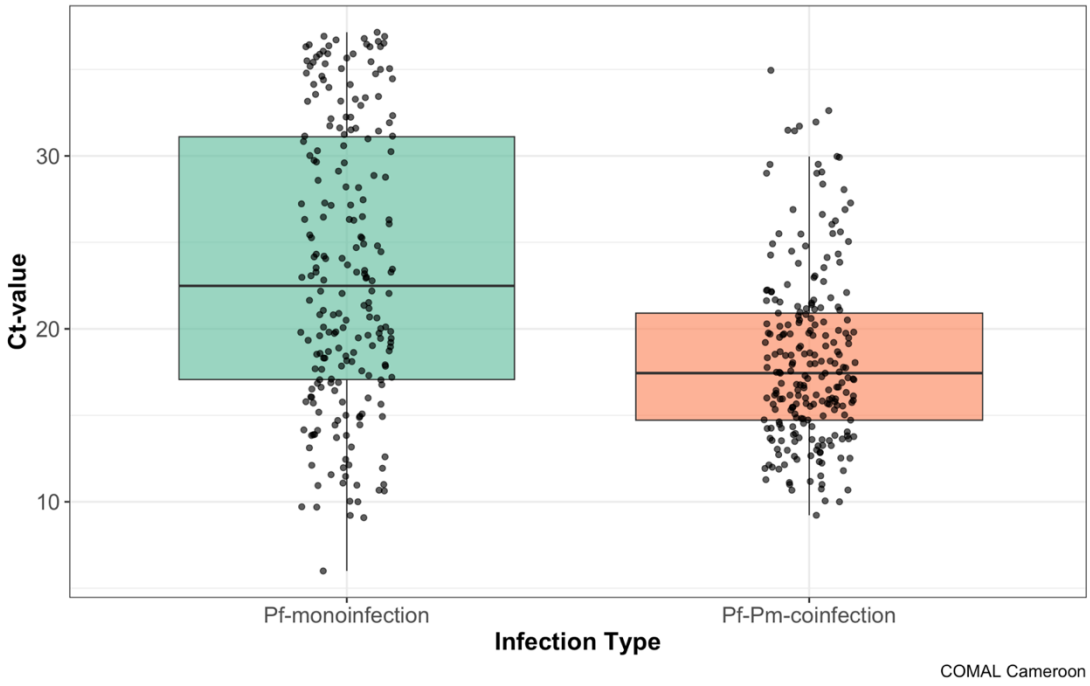

Figure 32 - Ct values of P. falciparum in mono-infections are significantly higher than in co-infections with P. malariae in samples from Cameroon (p < 0.001)

This suggests, that the parasitemia in *P. falciparum* monoinfections is lower than when it comes together with a *P. malariae* infection where it seems that *P. falciparum* parasitemia is relevantly higher, when judging by the much lower Ct value in co-infections.

This difference is statistically significant, with t(358) = 8.1, p < 0,001 and a 95% confidence interval of 3.9 to 6.4 obtained by Welsh's t-test.

However, as for *P. malariae* infected samples, there is a non-negligible effect of age on Ct values. This can be observed in Figure 33 which shows that while Ct values of *P. falciparum* mono-infections are always higher than Ct values in

mixed infections with *P. malariae*, they also have an overall tendency to rise with higher age.

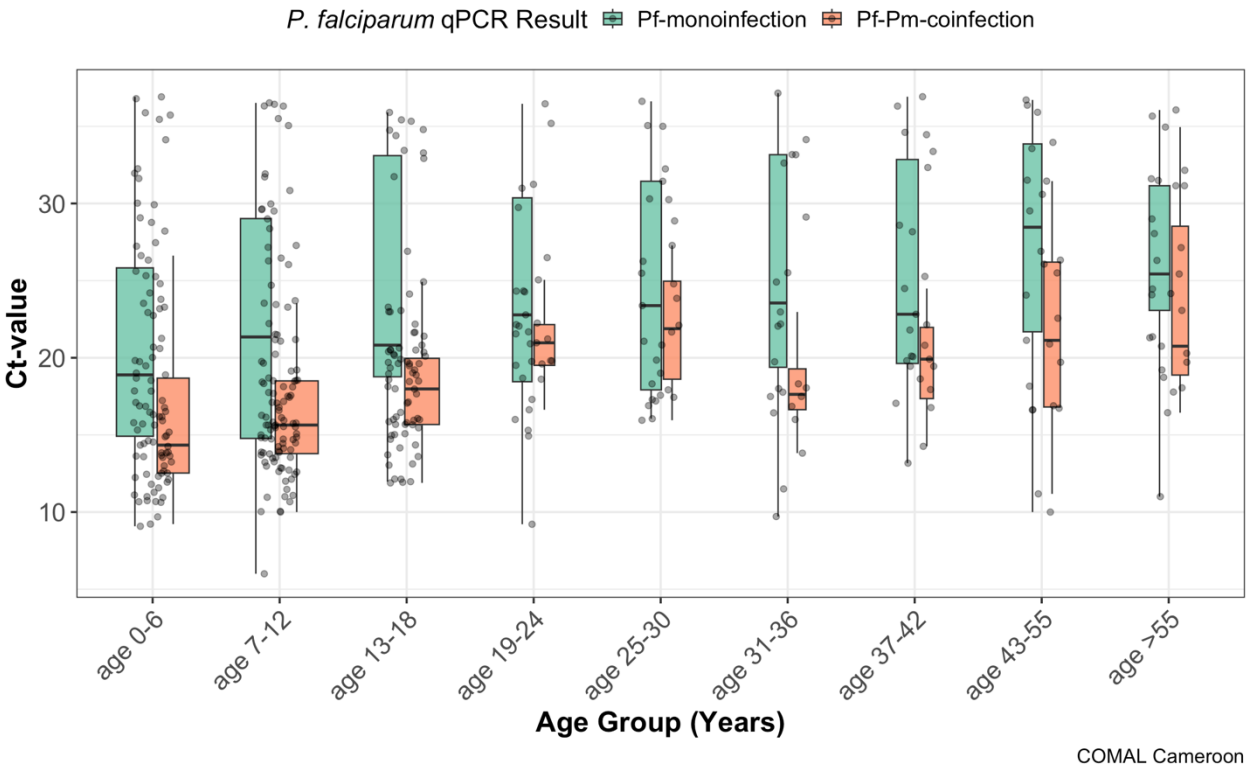

Figure 33 - P. falciparum Ct value variations in P. falciparum infected samples in different age groups in Cameroon, split up into mono- and co-infections

This effect of age on Ct values for *P. falciparum* infected samples is highly significant with a p-value of < 0.001 as can be observed in the linear regression model below (Figure 34).

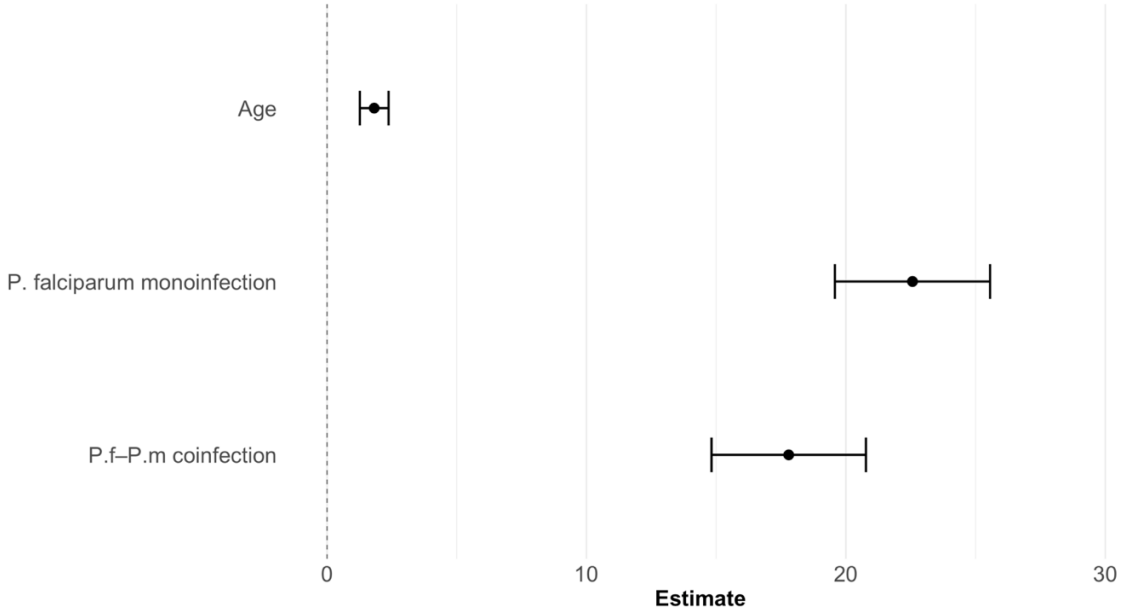

Figure 34 – Linear model showing a significant effect of age on P. falciparum Ct values in samples from Cameroon (p < 0.001) (pf_ct ~ pcr_res + scale(age))

3.5.2.3 Summary of the observed changes in median Ct values in mono- versus co-infections

The Figures above show that for both the samples from Gabon and Cameroon there seems to be a similar trend.

In mono-infections with *Plasmodium malariae* the median *P. malariae* Ct values are lower than in co-infections of *P. malariae* and *P. falciparum*. This suggests a higher relative parasitemia of *P. malariae* in mono-infections and could mean that there is indeed an interaction happening between the two parasites that influences the parasitemia of both.

The data derived from *Plasmodium falciparum* infections shows an inverse trend. In *P. falciparum* mono-infections the median Ct values are higher than in co-infections with *P. malariae*, suggesting that *P. falciparum* profits off the concurrent presence of *P. malariae* and has a higher parasitemia in co-infections than in mono-infections. Whether this is an indicator for an interaction between *P. falciparum* and *P. malariae* is, at this point, difficult to hypothesize. The Ct values that were found for *P. falciparum* in this study are to be regarded with caution, as the non-specific primers used in the qPCR reaction might have

altered the qPCR results. This issue is further explained in the section 'Limitations' (see "4.11.1 Non-specific *Plasmodium falciparum* primers and their effects")

In Gabon, age does not impact the Ct values, while there is a statistically significant impact of age on Ct values in Cameroon, suggesting a certain effect of acquired immunity.

Tables 16 and 17 show an overview of the Ct values and the t-test results.

Table 16 – Overview of Ct Values Obtained by qPCR of Samples from Gabon and Cameroon

	P. malariae Mono-infection		*P. malariae* Co-infection		*P. falciparum* Mono-infection		*P. falciparum* Co-infection	
	Gabon	Cam.	Gabon	Cam.	Gabon	Cam.	Gabon	Cam.
Lowest Ct value	13.03	14.80	9.28	12.72	7.97	6.00	6.74	9.22
Highest Ct value	45.00	36.18	40.77	50.53	43.29	37.15	36.70	34.95
Median Ct	24.00	22.43	25.93	26.92	24.96	22.48	18.40	17.44
(IQR)	(20.7 – 28.4)	(19.0 – 26.1)	(22.0 – 31.4)	(23.3 – 31.7)	(19.1 – 33.4)	(17.1 – 31.1)	(15.8 – 22.0)	(14.7 – 20.9)
Mean Ct	24.83	23.27	26.80	27.53	25.54	23.41	19.33	18.25

Table 17 – Comparison of mean Ct values in mono- and co-infections of P. malariae and P. falciparum by Welsh's t-test

	Gabon		Cameroon	
	P. malariae Mono- vs Co-infection	*P. falciparum* Mono- vs Co-infection	*P. malariae* Mono- vs Co-infection	*P. falciparum* Mono- vs Co-infection
Welsh's t-test	t(125) = -2.5	t(812) = 13.2	t(21.5) = -2.9	t(358) = 8.1
95% CI	-3.5 to -0.4	5.3 to 7.1	-7.3 to – 1.2	3.9 to 6.4
P-value	p = 0,01	p < 0,001	p < 0,01	p < 0,001

3.6 Correlations of qPCR results with relevant anthropometric data

When assessing the epidemiological impact of a certain pathogen, it is important to see it in the context of different anthropometric measures.

In this section the correlations of the results obtained by qPCR in Gabon and Cameroon with the data regarding age, seasonality and hemoglobin levels will be shown in detail.

3.6.1 Age and Plasmodium infection

Statistical analyses show that for both Gabon and Cameroon, there are differences in the median age of participants depending on the kind of infection they have. These will be highlighted in the following section.

3.6.1.1 Age and infection in Gabon

Figure 35 shows that the median age of infected study participants varies by kind of infection. Especially the higher median age of *P. malariae* mono-infected individuals stands out.

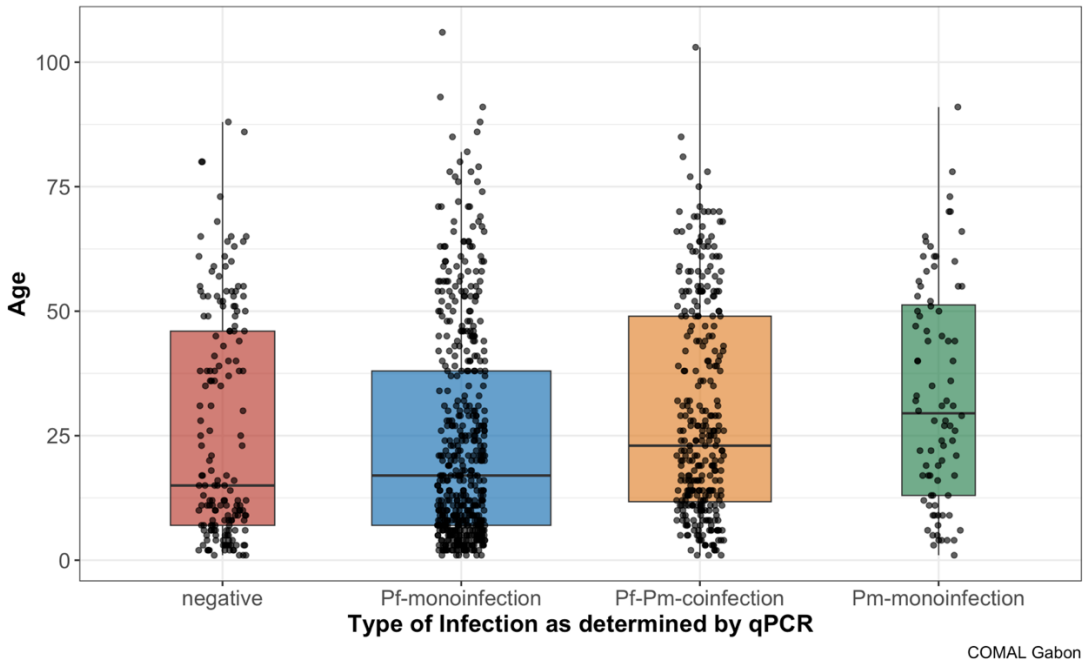

Figure 35 – Variations in age in different types of infections in samples from Gabon, showing a higher median age in P. malariae mono-infected individuals

A linear regression model (Figure 36) shows that the higher median age in *Plasmodium malariae* mono-infections is significantly different ($p < 0.05$) from the median age of the other *Plasmodium* infected study participants.

These results suggest a significant relationship between older age and *P. malariae* mono-infections.

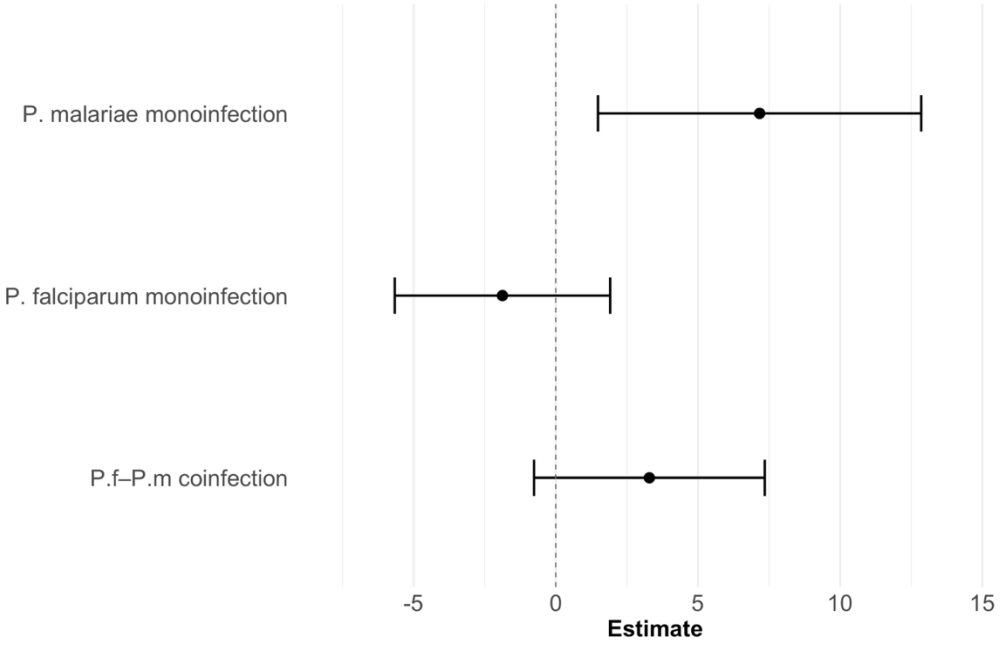

Figure 36 – Linear model of age and qPCR results for samples from Gabon (age ~ pcr_res), showing a significant relationship between age and P. malariae mono-infections ($p < 0.05$)

In Cameroon there is not one kind of infection standing out against the others when looking at the median age of the infected individuals (Figure 37).

However, the low number of *P. malariae* mono-infections might figure as a confounding factor in this analysis.

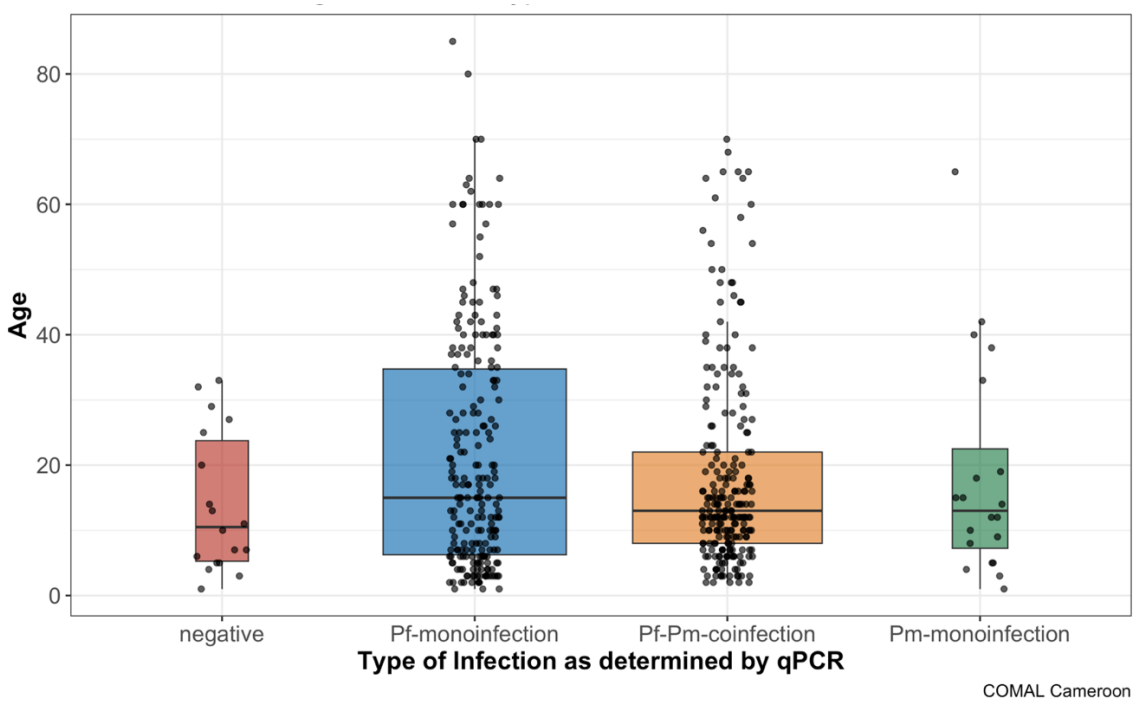

Figure 37 – Variations in age in different types of infections in samples from Cameroon

As expected, the linear model of the relationship between age and *Plasmodium* infection in Cameroon (Figure 38) shows no statistically significant relationship between the different kinds of infections and the median age of the sampled individuals.

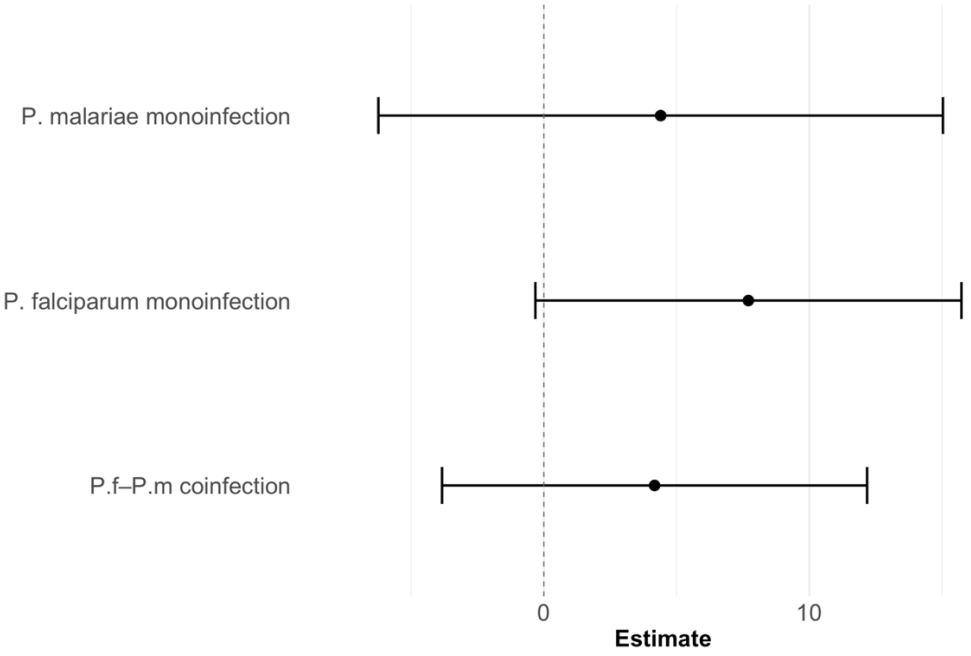

Figure 38 - Linear model of age and qPCR results for samples from Cameroon (age ~ pcr_res), showing no significant relationships between the different variables

However, as we have seen above, the very low number of *Plasmodium malariae* mono-infections in the samples from Cameroon makes this statistical comparison difficult and serves as a limitation of this study.

3.6.1.2 Correlation analyses of age and Ct values in Gabon

To further compare not only infection but also the estimated parasitemia, several analyses were conducted for both countries. The Ct value, as explained above, serves as a surrogate parameter for estimated parasitemia, with a high Ct value indicating lower parasitemia and vice versa.

First, age was correlated with all Ct values of *Plasmodium malariae* or *Plasmodium falciparum* respectively. In a next step, the Ct-results were split into mono- and co-infections and each one was again compared with the ages of all the study participants.

Figure 39 and Figure 40 show that for the overall *Plasmodium malariae* Ct values from the Gabon samples (R = 0.048; p = 0.34) as well as for the *Plasmodium falciparum* Ct values (R = 0.009; p = 0.8), there seems to be no significant correlation between parasitemia and age.

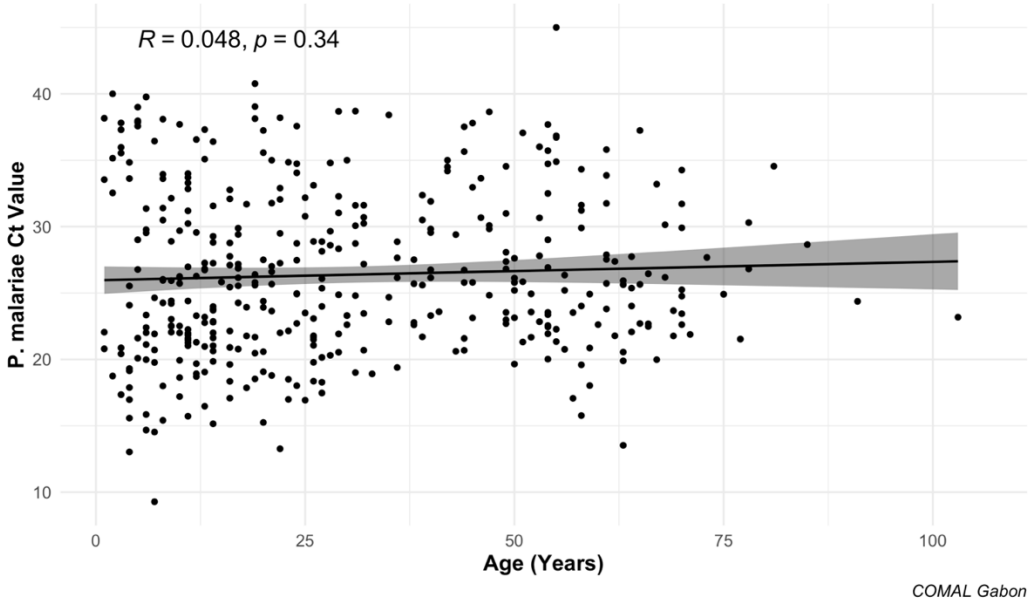

Figure 39 – Pearson's correlation analysis of all P. malariae Ct values with age, showing no correlation of P. malariae Ct values with age in Gabon

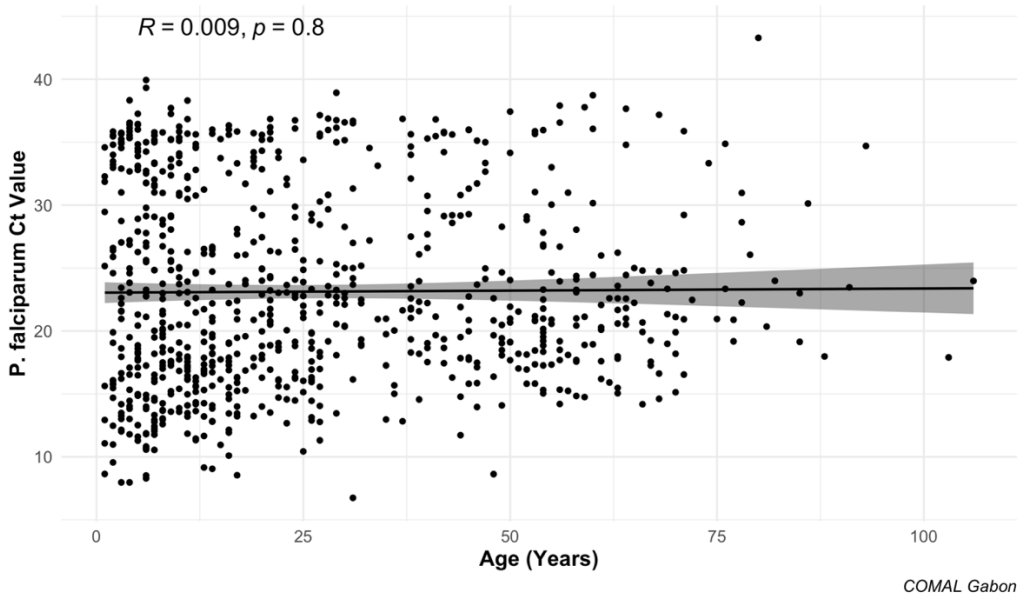

Figure 40 – Pearson's correlation analysis of all P. falciparum Ct values with age, showing no correlation of P. falciparum Ct values with age in Gabon

When only considering *P. malariae* mono-infections, there is a low degree of positive correlation of parasitemia with age, with R = 0.19 and p = 0.084. Figure 41 shows a trend of higher Ct values in older study participants. This, however, is not a statistically significant correlation, as the p-value of 0.084 shows, possibly due to the low number of *Plasmodium malariae* mono-infections to analyze.

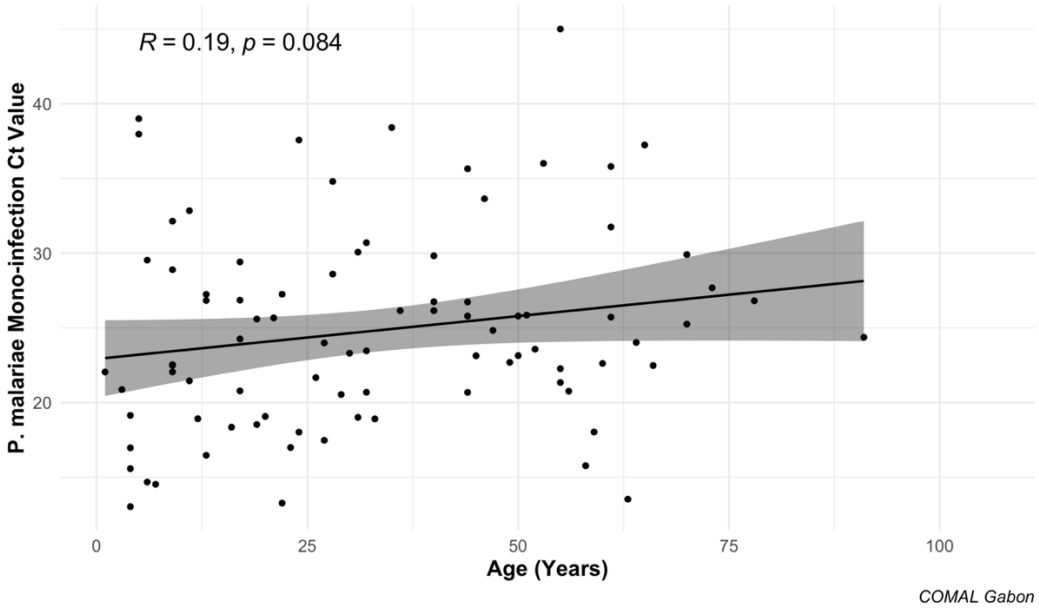

Figure 41 - Pearson's correlation analysis of only P. malariae mono-infection Ct values with age in Gabon, showing a low degree of positive correlation of Ct values and age

The Ct values of *P. falciparum* mono-infections from samples collected in Gabon do not correlate with age (Figure 42).

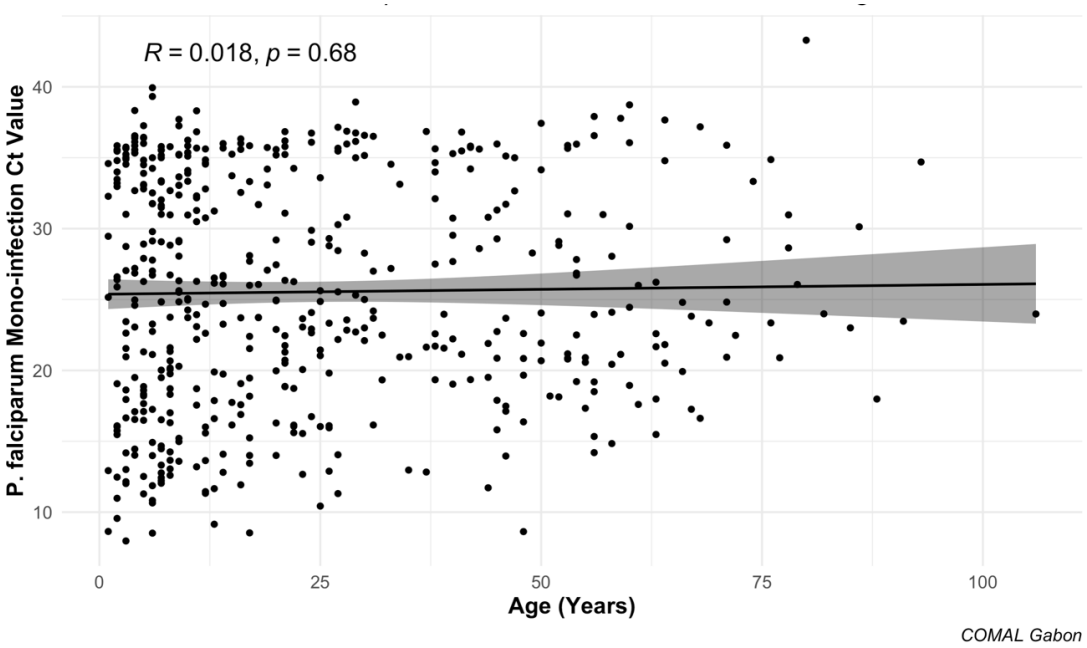

Figure 42 - Pearson's correlation analysis of Ct values in mono-infections of P. falciparum with age in Gabon, showing no correlation of Ct values and age

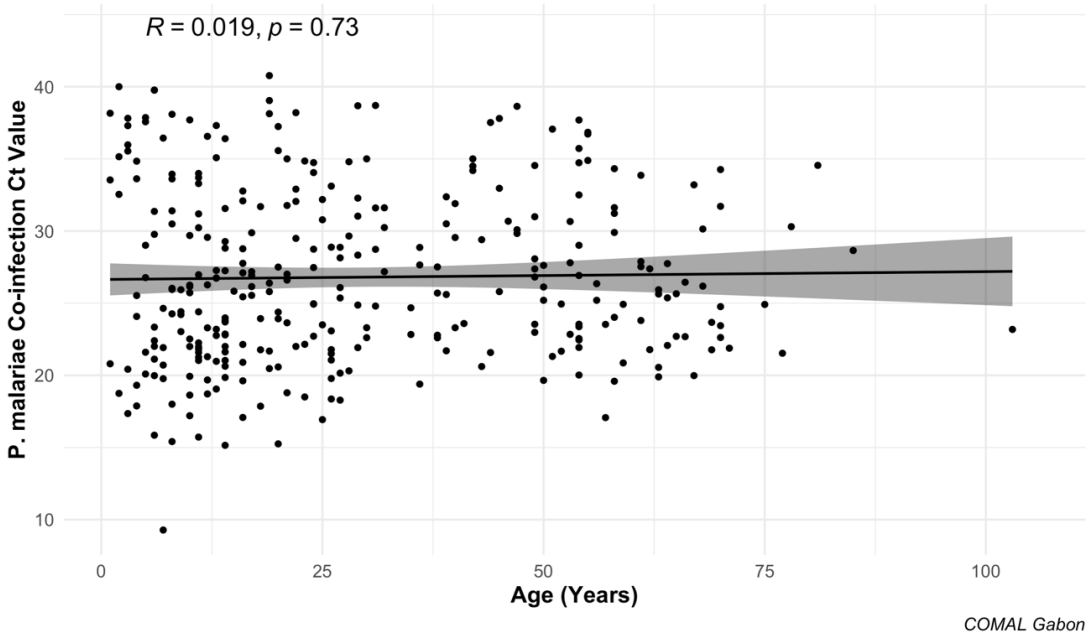

Figure 43 - Pearson's correlation analysis of Ct values of P. malariae in co-infections of P. malariae and P. falciparum with age in Gabon, showing no correlation of Ct values and age

In co-infections of both kinds of *Plasmodium* (Figures 43 & 44) it is the co-infection Ct values of *P. falciparum* which show a positive correlation with age (R = 0.16 and p < 0.005).

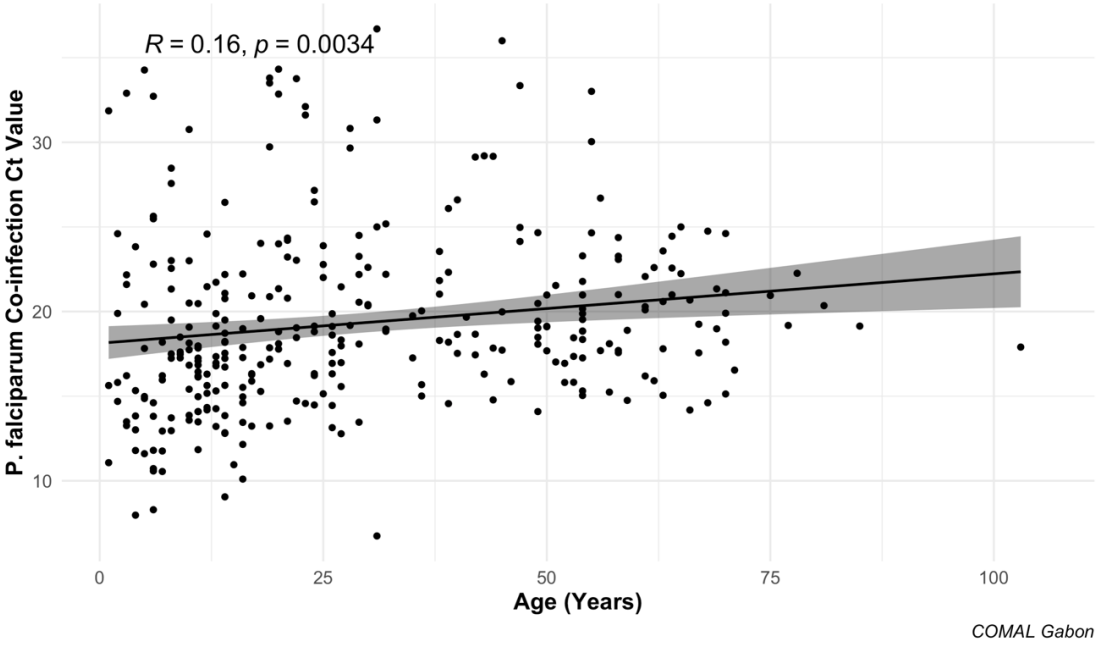

Figure 44 - Pearson's correlation analysis of Ct values of P. falciparum in co-infections of P. malariae and P. falciparum with age in Gabon, showing a positive correlation of Ct values and age

3.6.1.3 Cameroon and age

When correlating age and Ct values in Cameroon we see a different trend than in the sample results from Gabon.

In the analysis of all *P. malariae* sample results there is a correlation of the Ct values with age (R = 0.18; p < 0.005) (Figure 45).

The same applies to the *P. falciparum* Ct values. They correlate strongly with age (R = 0.31; p < 0.001) (Figure 46).

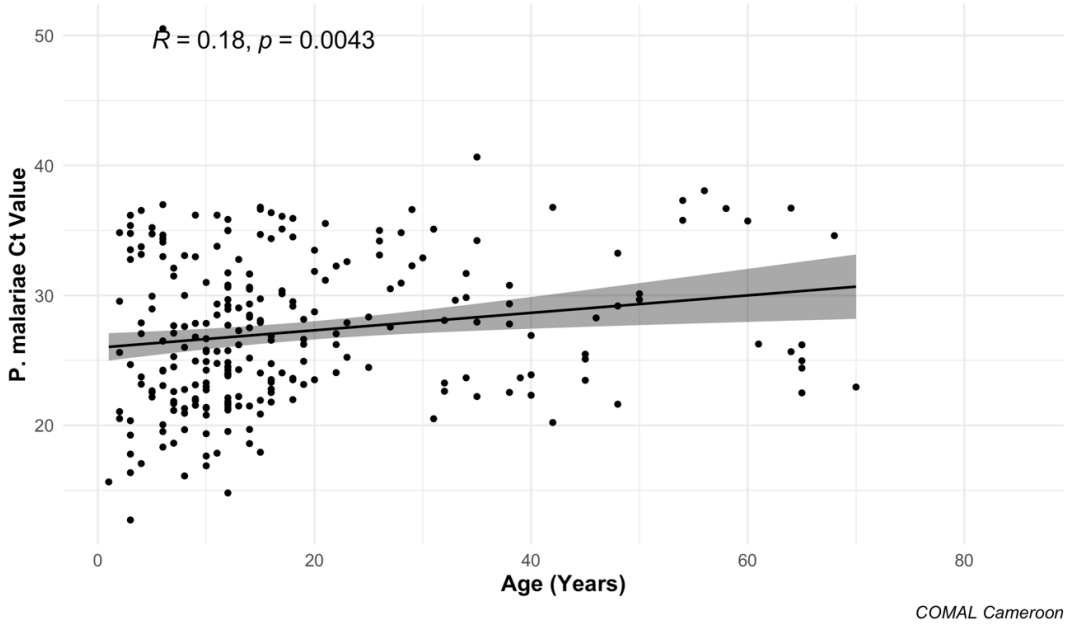

Figure 45 - Pearson's correlation analysis of all P. malariae Ct values with age in Cameroon, showing a low degree of positive correlation of age and Ct values

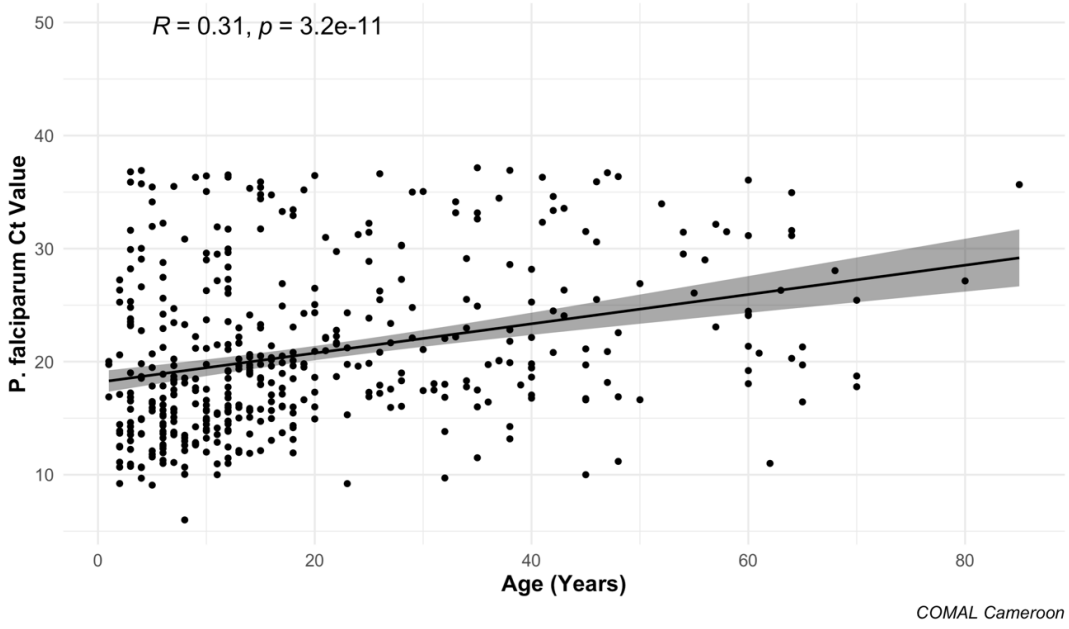

Figure 46 - Pearson's correlation analysis of all P. falciparum Ct values with age in Cameroon, showing a strong positive correlation of age and Ct values

When splitting up the analysis to only look at mono-infected and co-infected sample results separately, it can be observed that for mono-infected samples there also is a positive correlation.

For the *Plasmodium malariae* samples from Cameroon (Figure 47) this correlation is not significant as there aren't enough samples to analyze (R = 0.26; p = 0.26). The *Plasmodium falciparum* mono-infection Ct values (Figure 48) however correlate strongly with age (R = 0.24; p < 0.001).

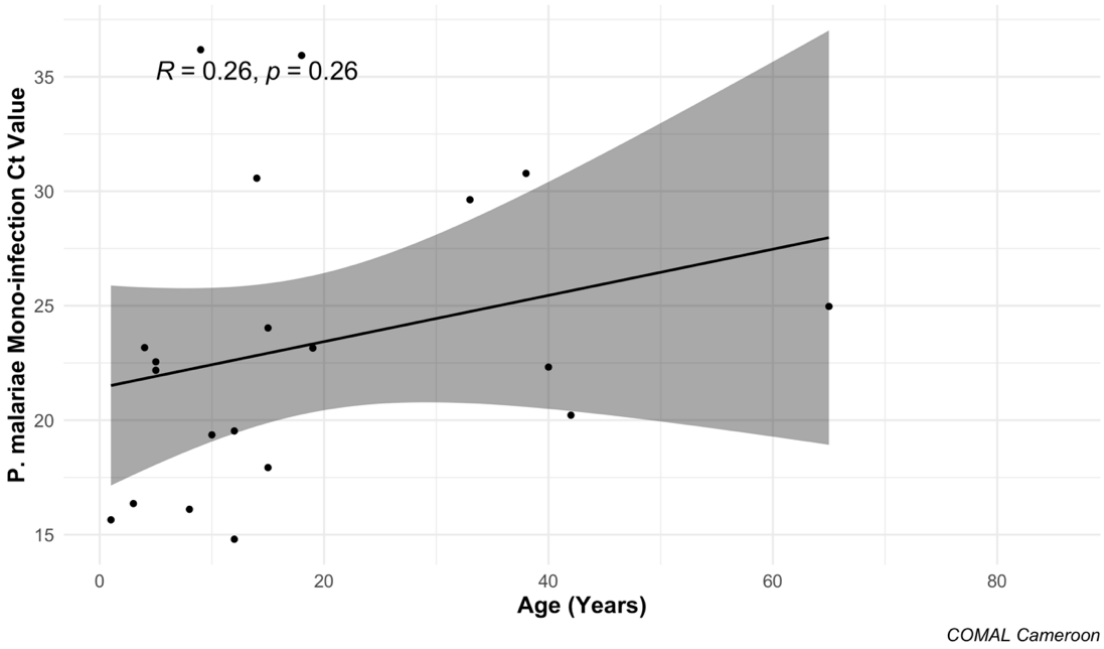

Figure 47 - Pearson's correlation analysis of Ct values in mono-infections of P. malariae with age in Cameroon, showing no significant correlation of age and Ct values

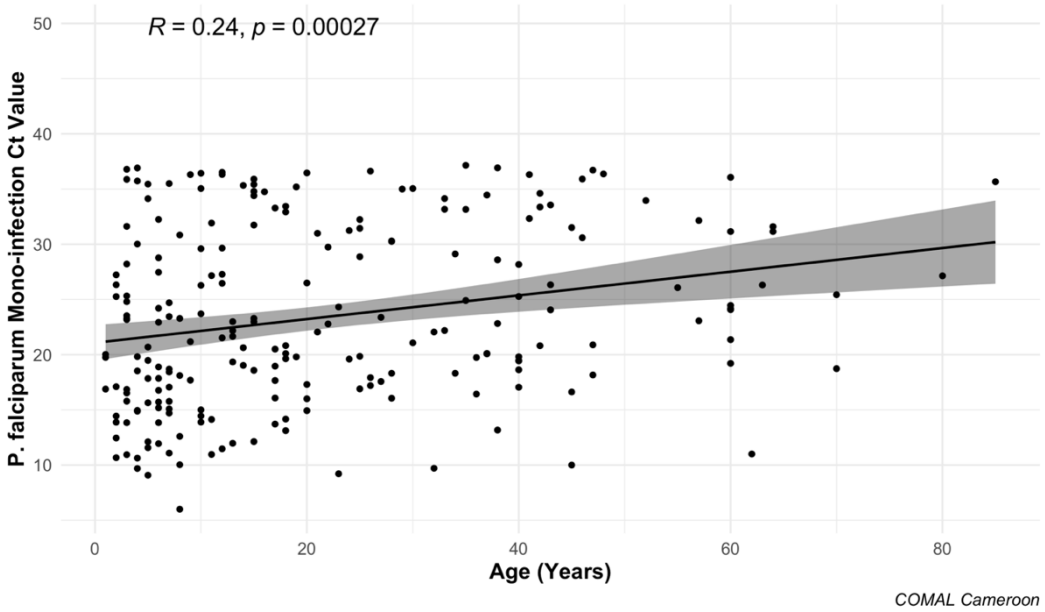

Figure 48 - Pearson's correlation analysis of Ct values in mono-infections of P. falciparum with age in Cameroon, indicating a high degree of positive correlation of age and Ct values

In co-infected samples in Cameroon, there is a significant correlation of Ct values with age for both *P. malariae* (R = 0.18; p < 0.01) (Figure 49) and *P. falciparum* (R = 0.38; p < 0.001) (Figure 50).

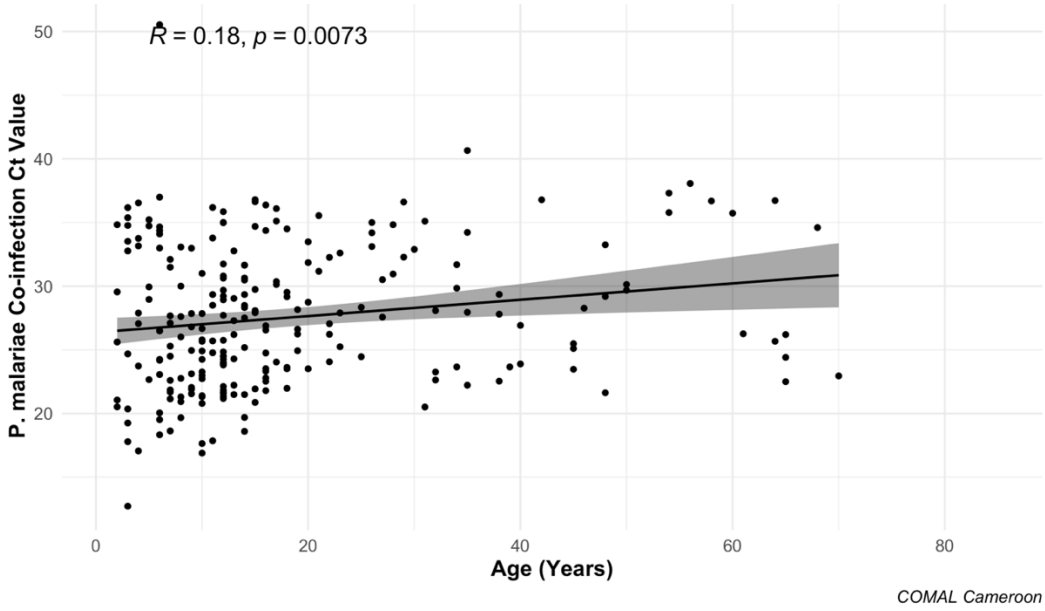

Figure 49 - Pearson's correlation analysis of P. malariae Ct values in co-infections of P. malariae and P. falciparum with age in Cameroon, indicating a low degree of positive correlation of age and Ct values

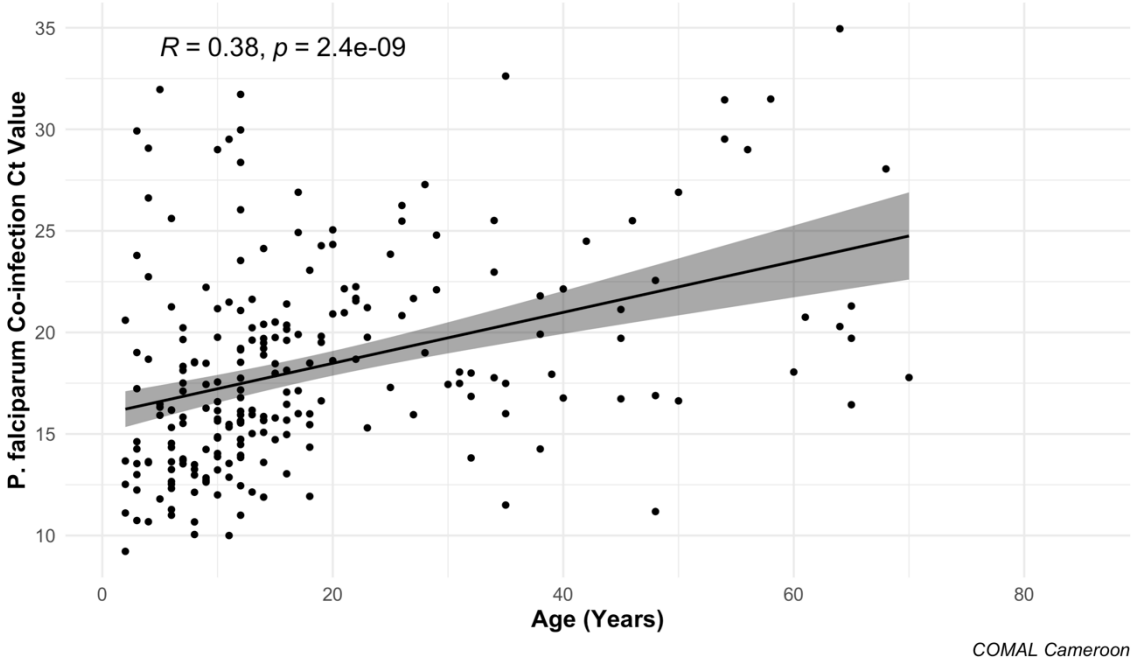

Figure 50 - Pearson's correlation analysis of P. falciparum Ct values in co-infections of P. malariae and P. falciparum with age in Cameroon, indicating a high degree of positive correlation of age and Ct values

To summarize, in Gabon only Ct values from *P. malariae* mono-infections and Ct values from P. falciparum co-infections show a certain degree of positive correlation with age.

In Cameroon on the other hand there is positive correlation for the Ct values of all the different kinds of infection except for *P. malariae* mono-infections, which is most likely due to the low number of samples for this kind of infection.

The implications of these correlation analyses of Ct values with age will be further discussed in the Discussion section.

3.6.2 Seasons and Plasmodium infection in Gabon

As already mentioned, the relationship between seasons and *Plasmodium* infections could only be assessed for the samples from Gabon.

Overall, 542 samples that were collected in the dry season (from December to January and May to September) and 511 samples that were collected in the rainy season (from October to November and February to April) in Gabon were included into this study.

Figure 51 shows an overview if the different infections detected by qPCR in the two different seasons.

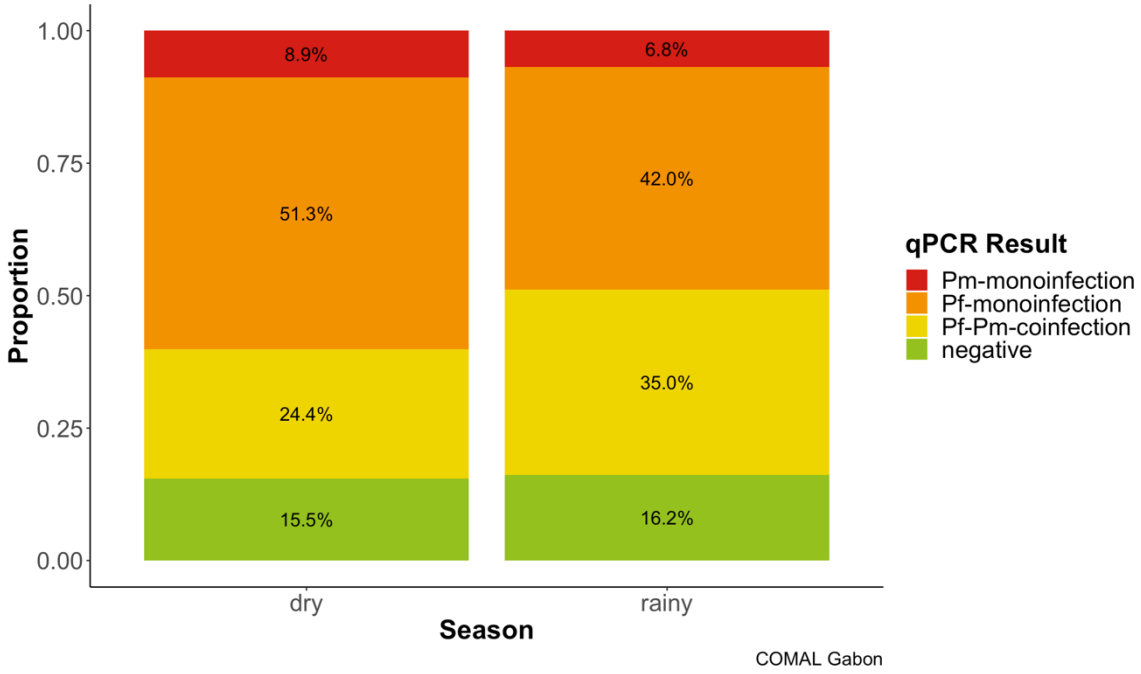

Figure 51 – Infections in dry and rainy seasons, as detected by qPCR

There was almost the same number of infected samples both in rainy and dry seasons. In the dry season there were 9% of *P. malariae* mono-infections while there were 7% in the rainy season. *P. falciparum* mono-infections were also more prevalent in the dry season with 51% of infected samples, and 42% in the rainy season.

Overall, with 35%, there were more mixed infections of *Plasmodium falciparum* and *malariae* in the rainy season, while there were 24% in the dry season.

The exact numbers can be seen in Table 18.

Table 18 – Overview of the number of infections in dry and rainy seasons, as detected by qPCR

	Dry Season		Rainy Season	
	n	*%*	n	*%*
P. malariae mono-infection	48	*8.9*	35	*6.8*
P.m-P.f-co-infection	132	*24.4*	180	*35.2*
P. falciparum mono-infection	278	*51.3*	213	*41.7*
No infection	84	*15.5*	83	*16.2*

The changes in prevalence of *P. falciparum* and *P. malariae* infections in Gabon over the months of the study period can be observed in Figure 52. Interestingly, both pathogens seem to follow a similar pattern, except for the sampling period of August 2021 (dry season), where the prevalence of *P. falciparum* increases while the *P. malariae* prevalence decreases.

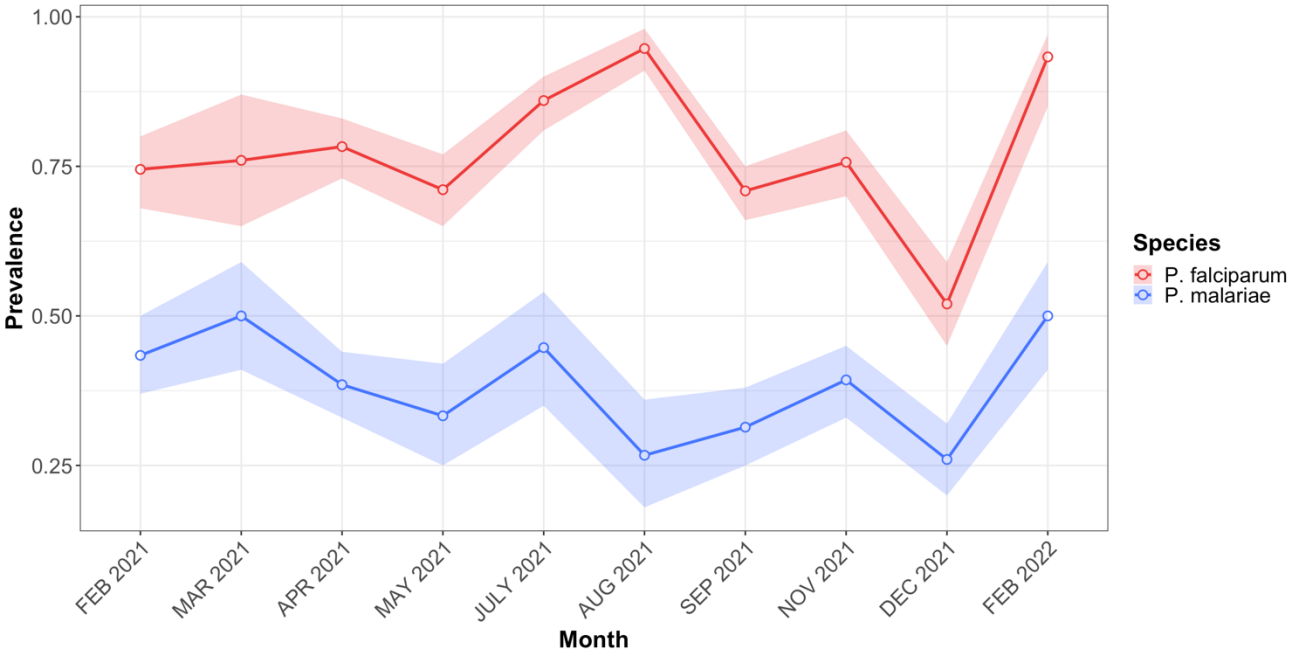

Figure 52 – Prevalence of Plasmodium malariae in samples from Gabon, with a 95% confidence interval, detected by highly sensitive qPCR; Seasons: DEC-JAN (small dry), MAY-SEP (long dry), OCT-NOV (small rainy), FEB-APR (long rainy)

When not only looking at infection but specifically at Ct values of positive infections, the median Ct values of both pathogens also seem to follow a similar pattern, except in May, the beginning of the long dry season. A rise in median *P. falciparum* Ct values with a concomitant fall of median *P. malariae* Ct values can be observed (Figure 53) indicating a decrease of *P. falciparum* parasitemia and an increase of *P. malariae* parasitemia. Afterwards however, in the samples taken in July, the inverse trend can be observed, with median *P. falciparum* Ct values falling and median *P. malariae* Ct values rising again.

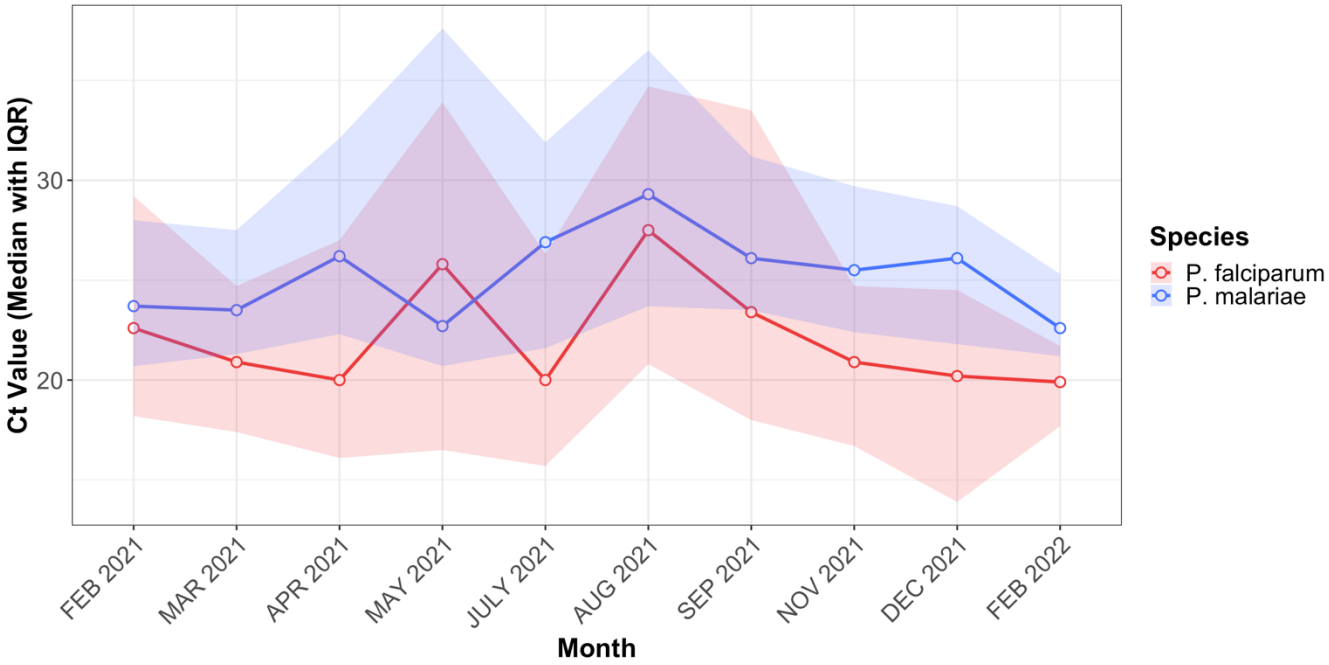

Figure 53 - Median Ct values in samples from Gabon (with IQR) over the course of the sampling period; Seasons: DEC-JAN (small dry), MAY-SEP (long dry), OCT-NOV (small rainy), FEB-APR (long rainy)

Overall, Figure 53 shows that *P. malariae* Ct values are almost consistently higher than *P. falciparum*, indicating a generally lower parasitemia.

In the rainy season, there is a lower median *P. malariae* Ct value as compared to the dry season (Figure 54). Compared by Welsh's t-test, assuming unequal variance, this difference is significant ($t(373) = 2.6$, $p = 0.01$, 95% confidence interval 0.4 - 2.8).

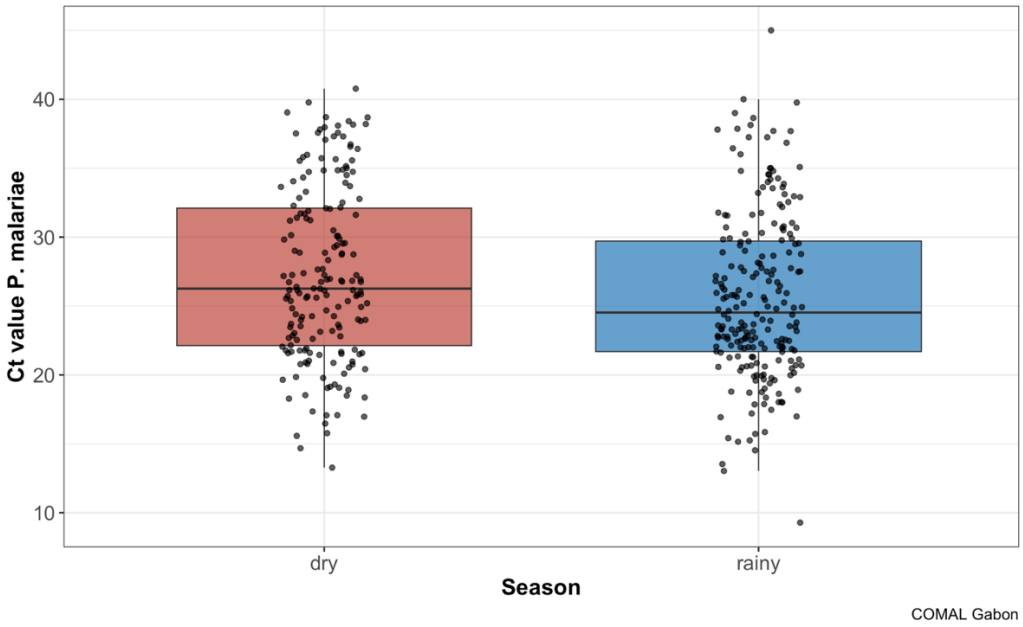

Figure 54 – Plasmodium malariae Ct values are significantly higher in the dry season than in the rainy seasons (p = 0.01)

As for *Plasmodium falciparum*, the same relationship can be observed. Figure 55 shows that Ct values in the dry season are on average higher than in the rainy season. Compared by Welsh's t-test, this difference is highly significant ($t(796) = 3.9$, $p < 0.001$, 95% confidence interval 1.1 to 3.2).

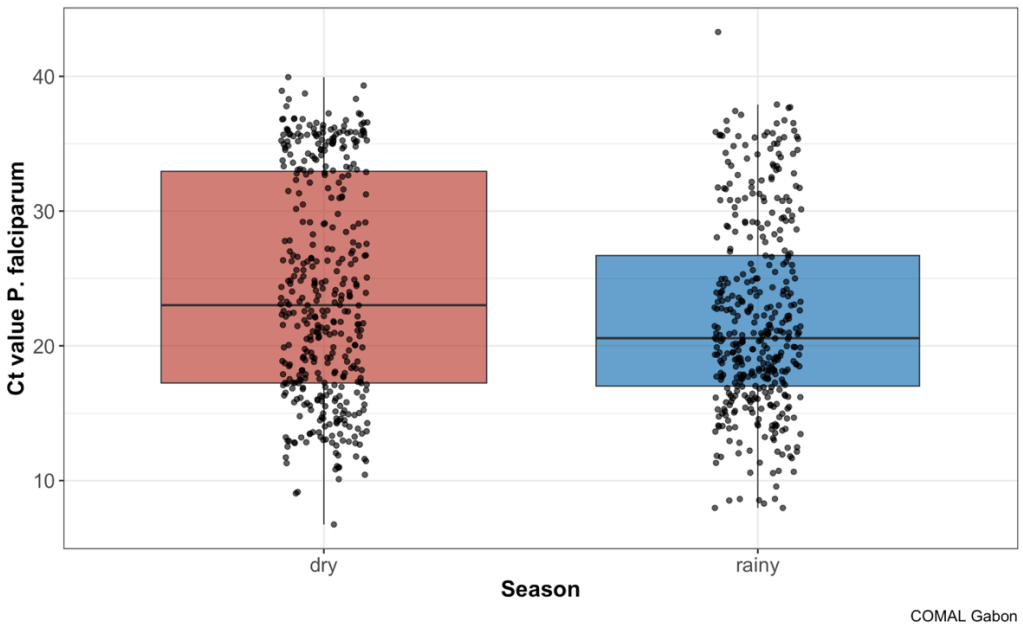

Figure 55 - Plasmodium falciparum Ct values are significantly higher in the dry than in the rainy season (p < 0.001)

3.6.3 Hemoglobin levels and Plasmodium infection

As we have seen above, there are high rates of anemia both in Gabon and in Cameroon. This section aims to compare status of infection, Ct levels and levels of anemia in different age groups to understand whether there might be an effect of subclinical infection on anemia.

Figures 56 and 57 show the median hemoglobin levels in *Plasmodium* infected versus negatively tested study participants for Gabon and Cameroon.

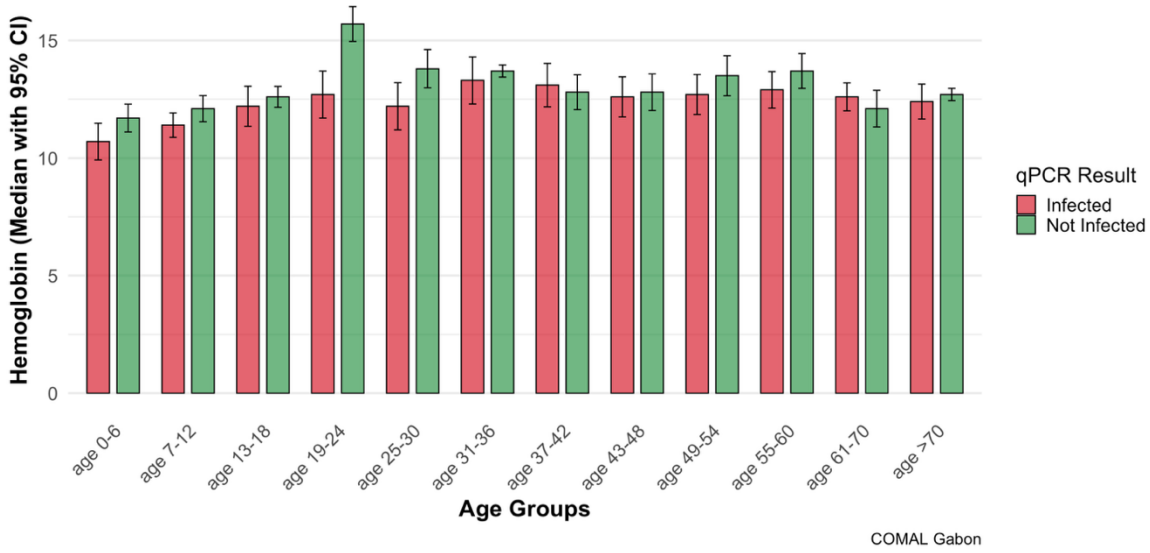

Figure 56 – Median hemoglobin levels in g/dL, including the 95% confidence interval, in infected versus negatively tested samples in Gabon

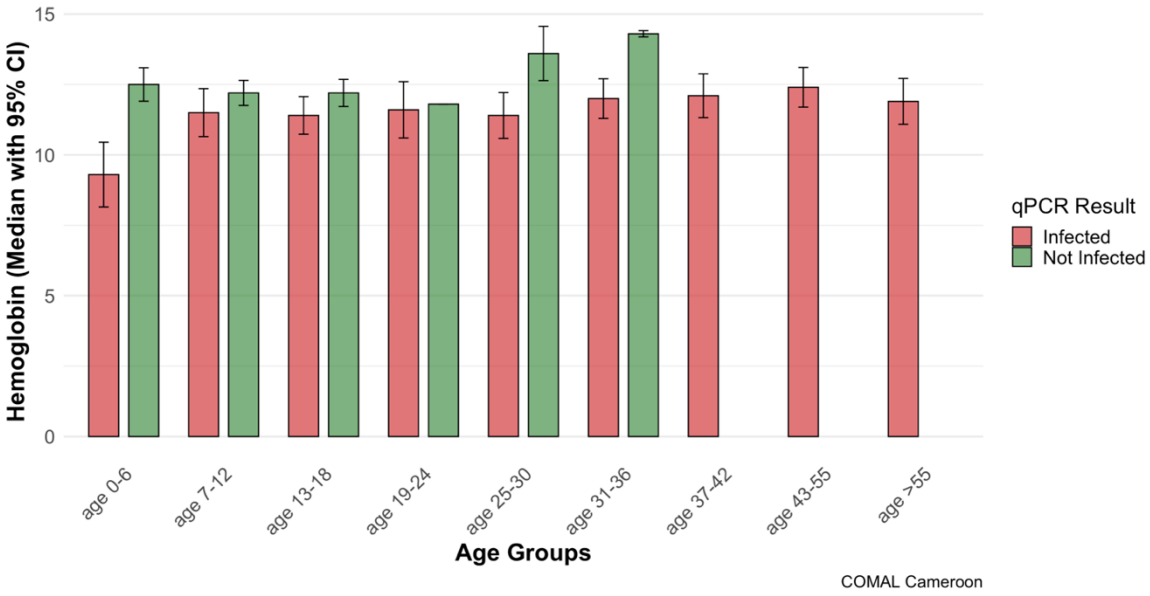

Figure 57 – Median hemoglobin levels in infected versus negatively tested samples in Cameroon (note: there were no negative samples of individuals above the age of 36 years in Cameroon)

In both countries, infected individuals almost always have a lower median hemoglobin level than non-infected individuals. A one-way ANOVA showed this difference to be significant for both Gabon ($F = 4.2$, $p < 0.05$) and Cameroon ($F = 12.6$, $p < 0.001$).

When comparing hemoglobin levels with Ct values, there is no significant correlation of *Plasmodium malariae* Ct values and hemoglobin in both Gabon and Cameroon (Figures 58 & 59).

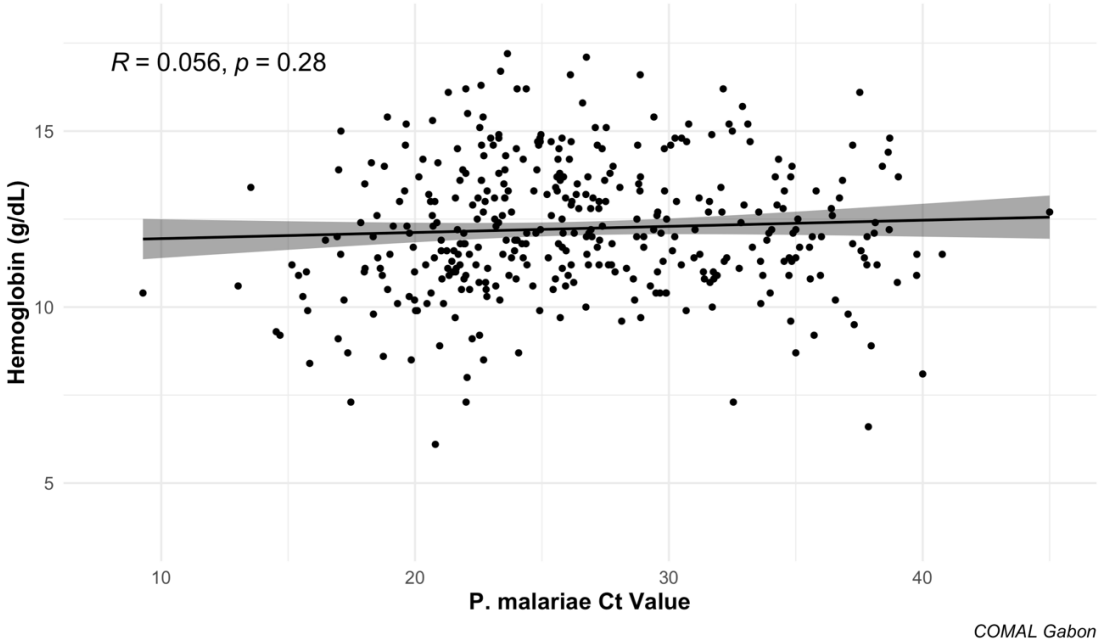

Figure 58 – Pearson's correlation analysis of P. malariae Ct values and hemoglobin levels (g/dL) in Gabon, showing no correlation

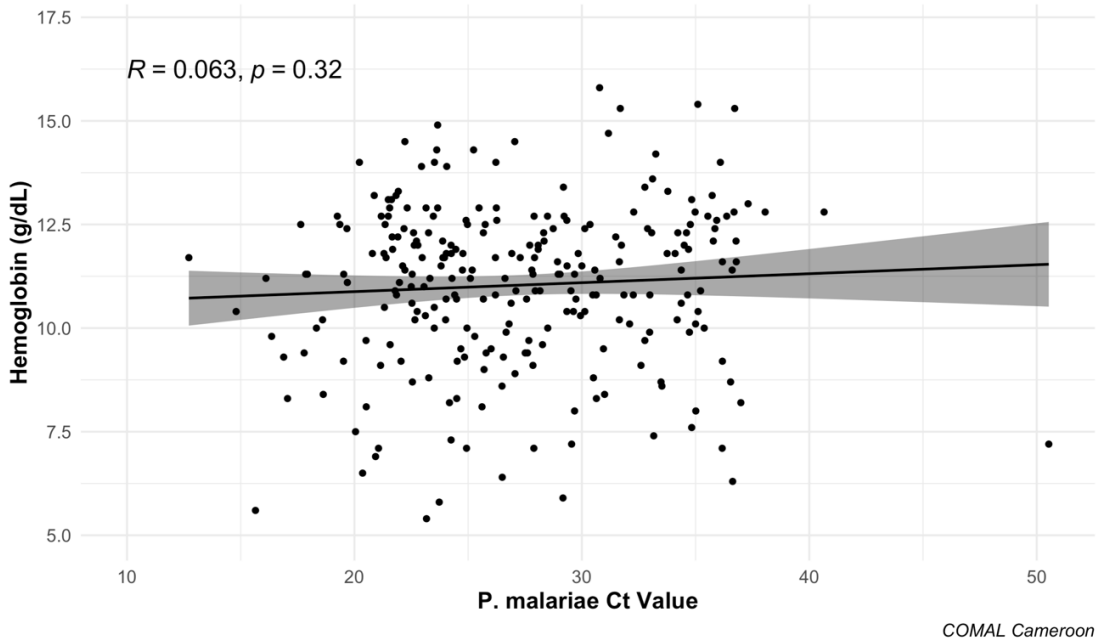

Figure 59 – Pearson's correlation analysis of P. malariae Ct values and hemoglobin levels (g/dL) in Cameroon, showing no correlation

For *Plasmodium falciparum* however, there is a significant correlation of Ct values with hemoglobin in both countries.

In Gabon it is a low positive correlation (R = 0.076; p < 0.05, Figure 60), while in Cameroon a more accentuated positive correlation exists (R = 0.22; p < 0.01, Figure 61), indicating a relevant effect of higher parasitemia infections with *P. falciparum* on hemoglobin levels.

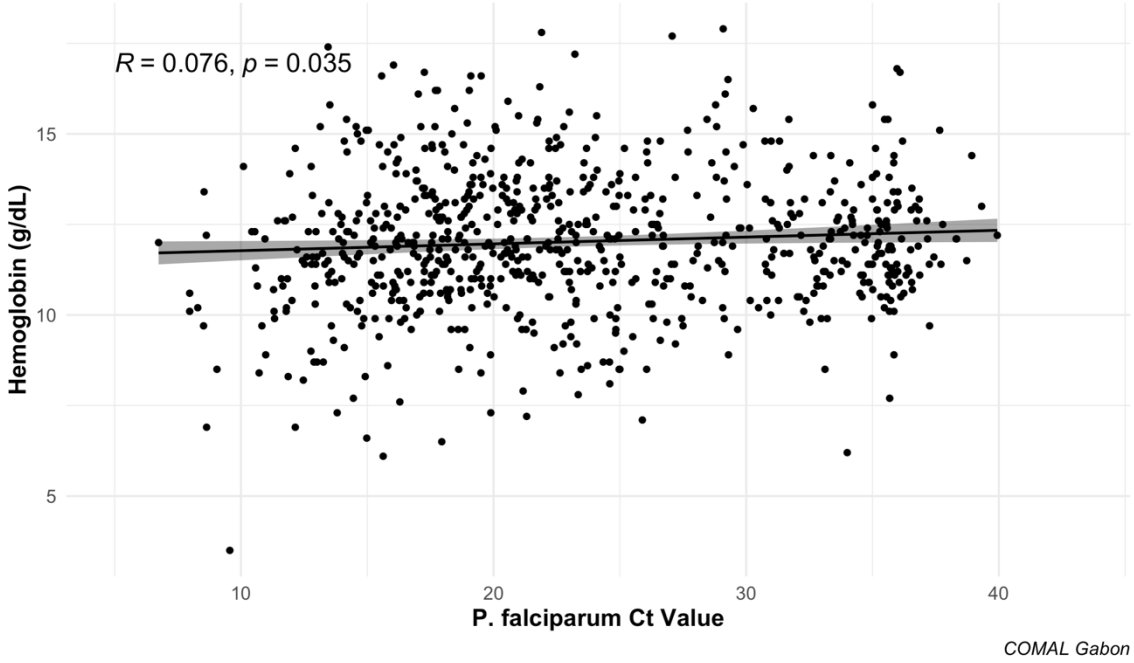

Figure 60 – Pearson's correlation analysis of P. falciparum Ct values and hemoglobin levels (g/dL) in Gabon, showing a low positive correlation

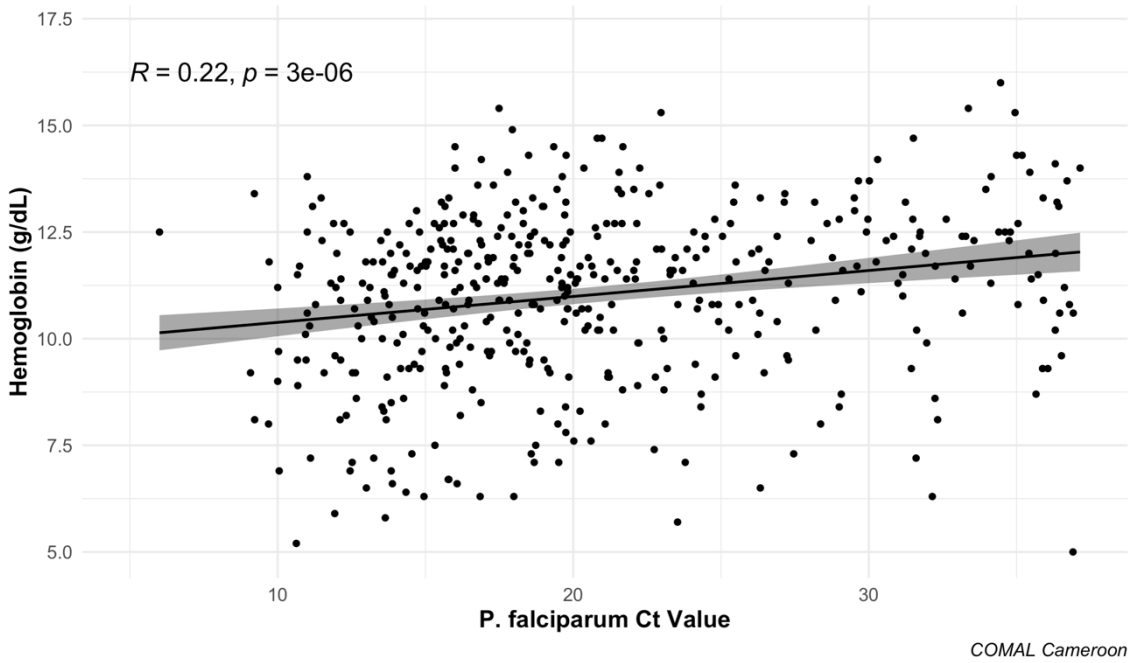

Figure 61 – Pearson's correlation analysis of P. falciparum Ct values and hemoglobin levels (g/dL) in Cameroon, showing a positive correlation

In a linear model, alongside age, regression reveals that there is indeed no effect of *Plasmodium malariae* infection on the level of hemoglobin, while there is a distinct negative effect of *Plasmodium falciparum* infection on hemoglobin values (Figures 62 & 63). This effect is present in both the samples from Gabon ($p < 0.05$) and Cameroon ($p < 0.01$). For both countries, there is a statistically significant positive relationship of age with hemoglobin, which is physiological.

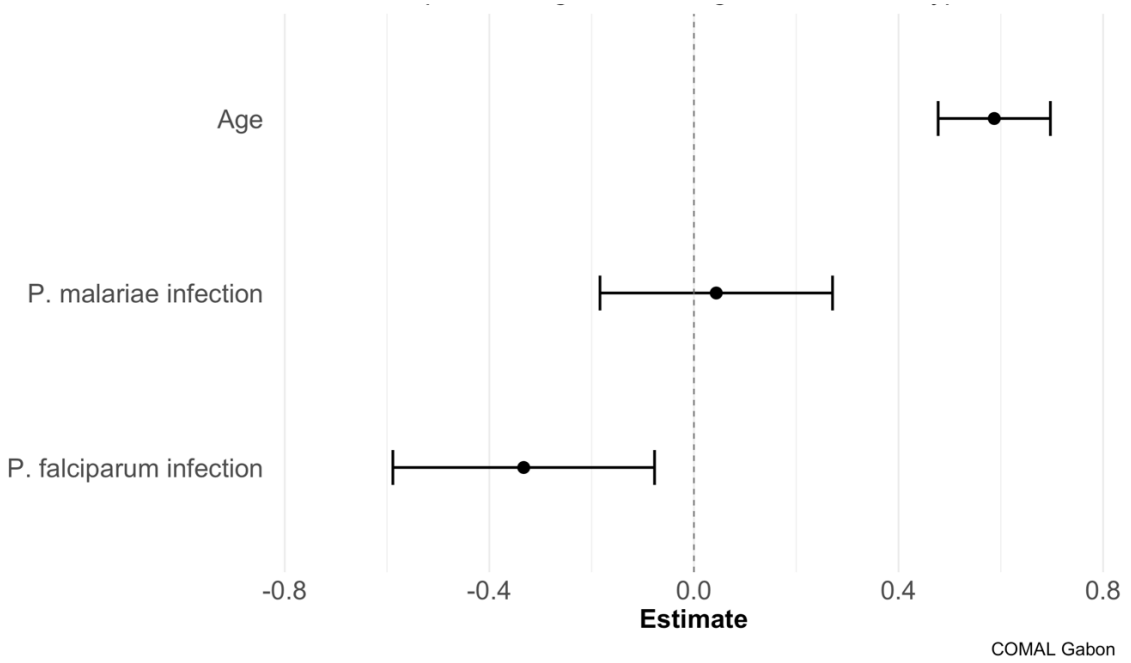

Figure 62 - Regression model highlighting the relationship of age and status of infection with hemoglobin in all samples in Gabon (hemoglobin ~ scale(age) + pcr_pm + pcr_pf)

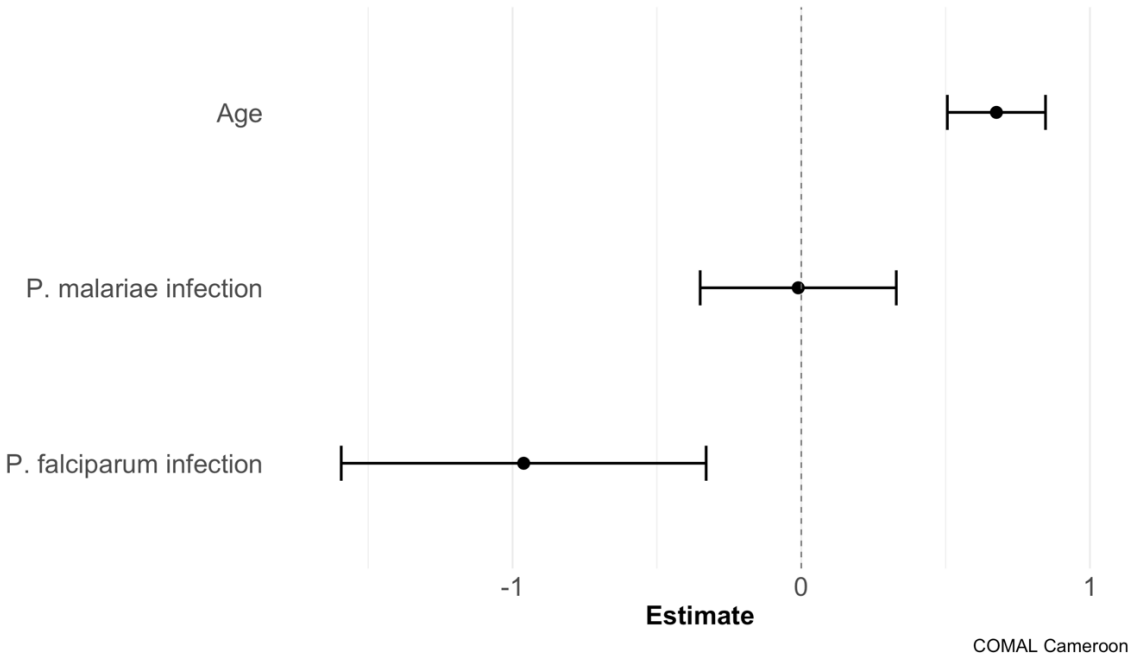

Figure 63 – Regression model highlighting the relationship of age and status of infection with hemoglobin in all samples in Cameroon (hemoglobin ~ scale(age) + pcr_pm + pcr_pf)

In children and adolescents, the results are different.

Figures 64 & 65 show that in children and adolescents up to the age of 19 years, the median hemoglobin level is consistently lower in children infected with *P. malariae* compared to the children who are tested negative for *P. malariae*.

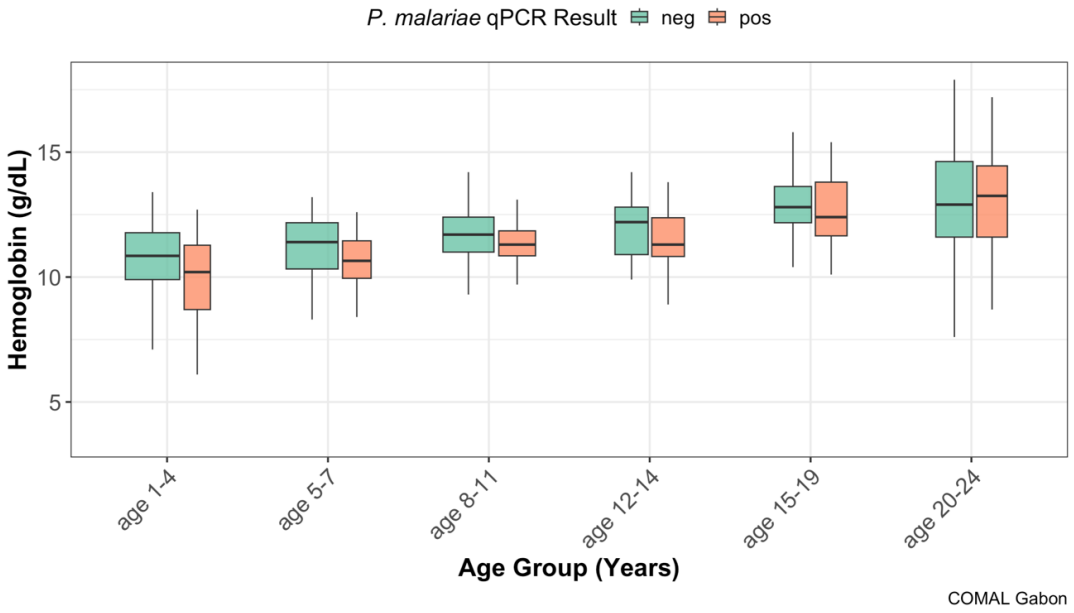

Figure 64 – Boxplot of median hemoglobin levels in children, adolescents and young adults in Gabon, in P. malariae positive versus negative samples

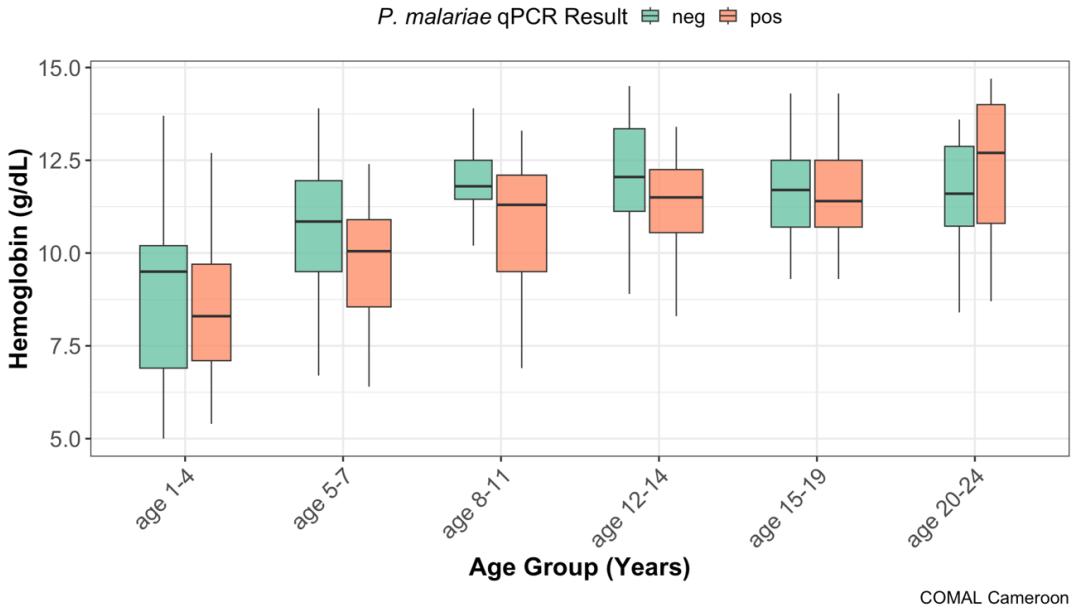

Figure 65 – Boxplot of median hemoglobin levels in children, adolescents and young adults in Cameroon, in P. malariae positive versus negative samples

The scatterplots below (Figures 66 & 67) confirm this trend towards lower levels of hemoglobin in children and adolescents infected with *Plasmodium malariae*, for both Gabon and Cameroon.

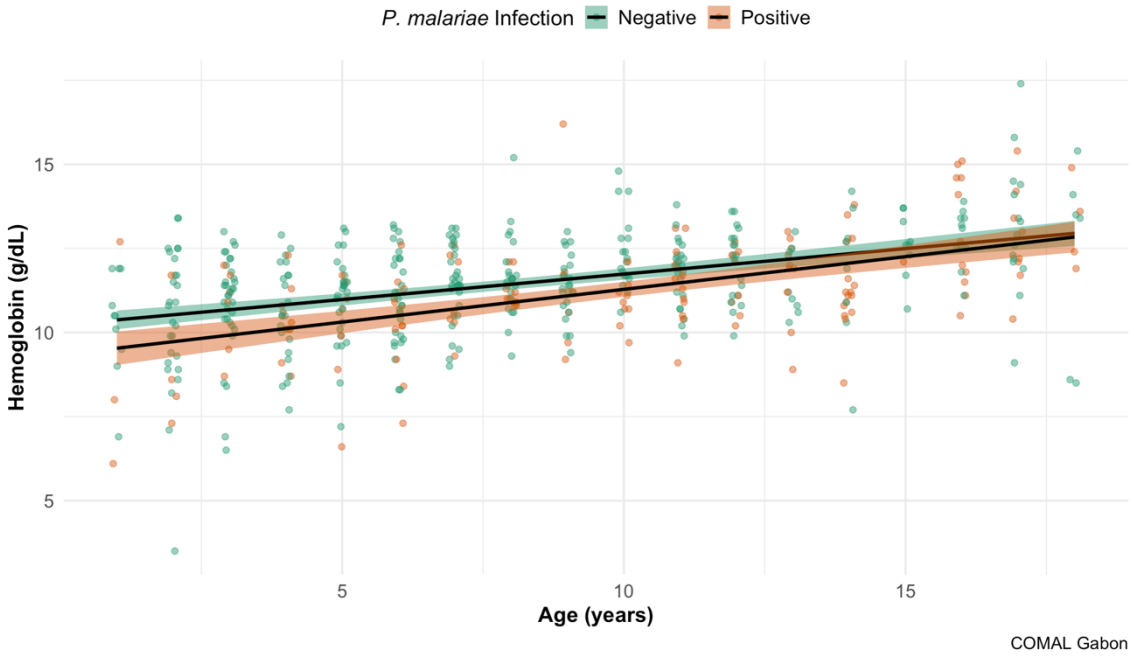

Figure 66 – Difference in hemoglobin levels in P. malariae infected versus non-infected children < 18 years in Gabon

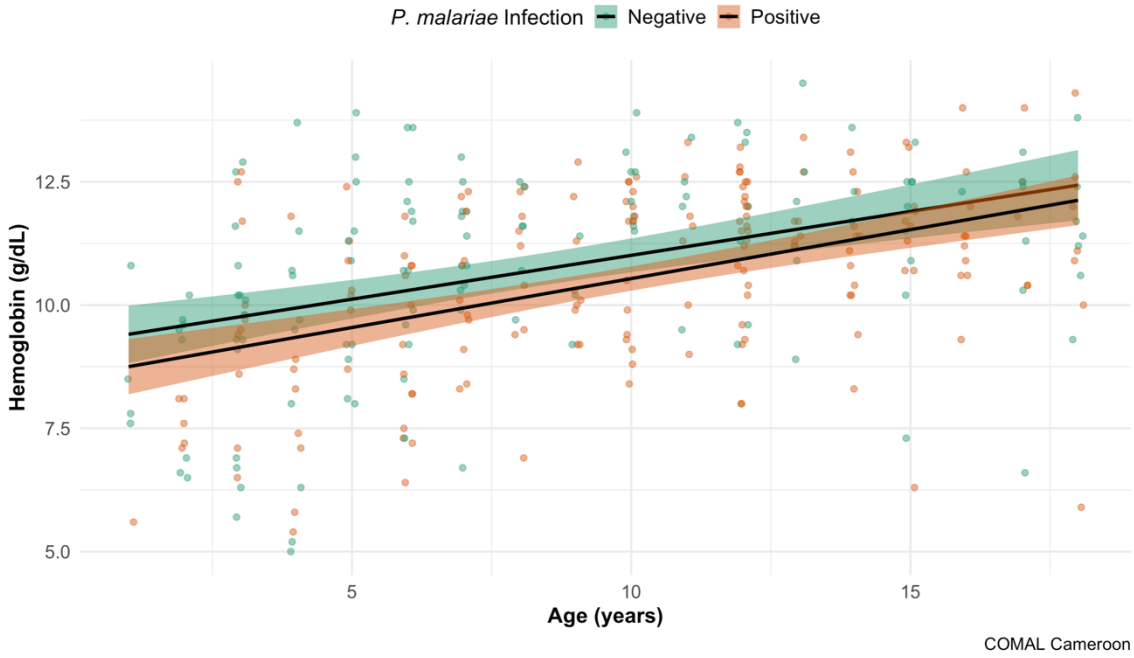

Figure 67 - Difference in hemoglobin levels in P. malariae infected versus non-infected children < 18 years in Cameroon

Linear regression reveals the statistical significance of these results. Figures 68 & 69 show that a significant relationship between a subclinical *Plasmodium malariae* infection and the level of hemoglobin can be inferred.

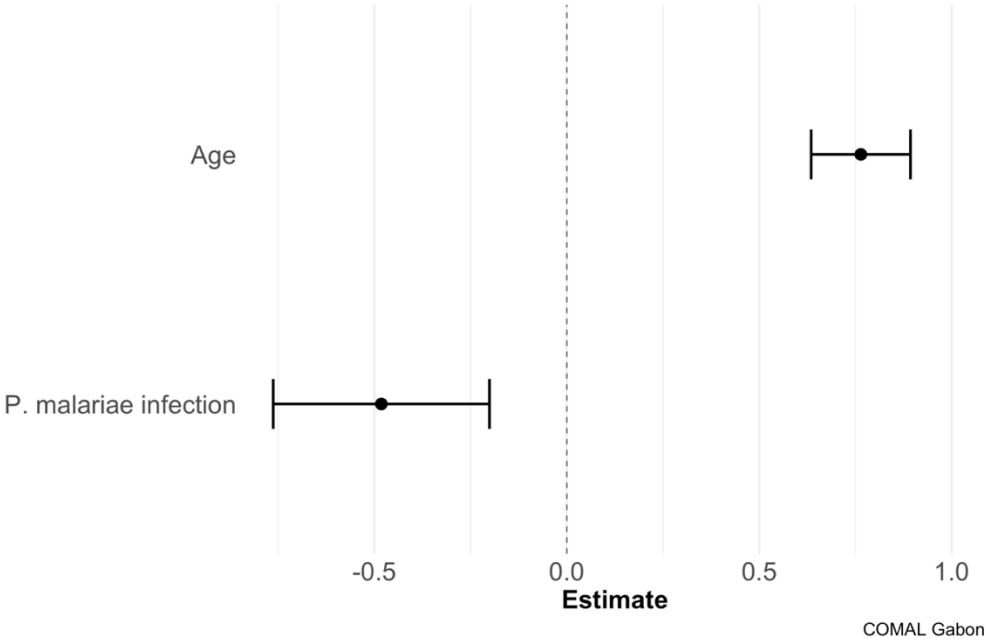

Figure 68 - Relationship of hemoglobin with age and status of Plasmodium malariae infection in children <18 years in Gabon (p < 0.001) (hemoglobin ~ scale(age) + pcr_pm)

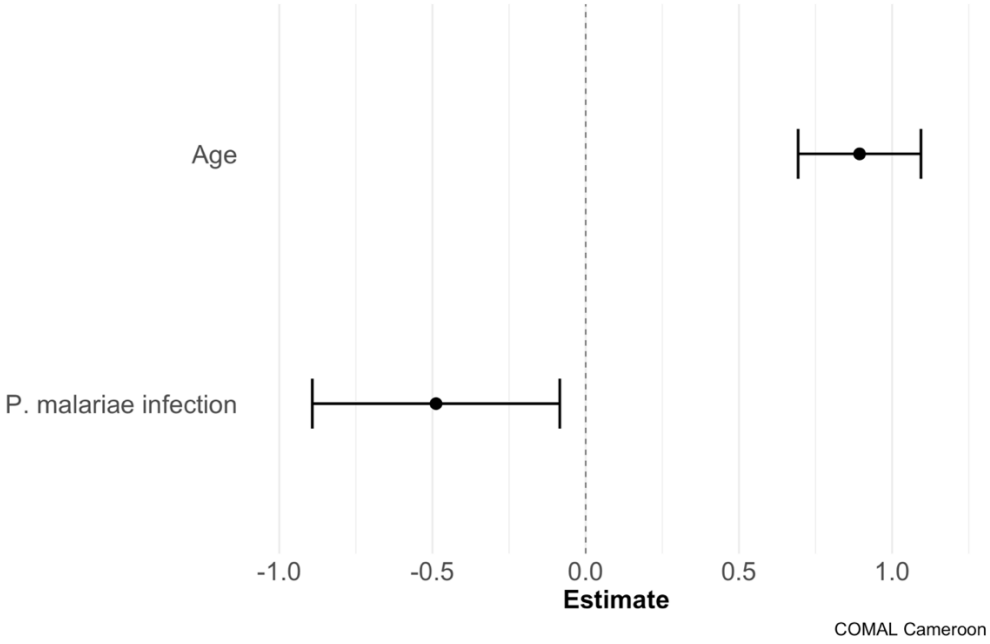

Figure 69 - Relationship of hemoglobin with age and status of Plasmodium malariae infection in children <18 years in Cameroon (p < 0.05) (hemoglobin ~ scale(age) + pcr_pm)

These regression analyses show that both in Gabon ($p < 0.001$) and in Cameroon ($p < 0.05$) there is an impact of subclinical *Plasmodium malariae* infection on hemoglobin levels in children. As above, the models show the significant physiological effect of age on hemoglobin levels.

3.6.4 Temperature

In Gabon, of the 16 participants with a temperature higher than 38.0°C 14 had a positive qPCR. 6 of these were co-infections of *P. malariae* and *P. falciparum*, 2 *P. malariae* monoinfections and 6 *P. falciparum* mono-infections.

In Cameroon, of the 9 participants with a temperature higher than 38.0°C all tested positive for a malaria infection in qPCR. 3 of these were co-infections of *P. malariae* and *P. falciparum*, 1 *P. malariae* monoinfections and 5 *P. falciparum* mono-infections.

As this study focuses on asymptomatic individuals, there will be no statistical analyses on the relationship between temperature and *P. malariae* infection on the examined data as this would likely be skewed.

3.7 Summary of results

For both countries, Gabon and Cameroon, age pyramids show a large bottom and a narrowing top, indicating the typical distribution of a predominantly young population, with a high number of children and adolescents. A little more than half of both sampled populations were women (52% in Gabon and 55% in Cameroon).

45% of participants (452 of 1010) in Gabon whose hemoglobin level was measured were anemic. Of those 45%, 42% (192 of 452) were adults over the age of 20 and 58% (260 of 452) were children and adolescents up to the age of 19. High numbers of severe and moderate anemia occurred in the age groups 1-4 and 5-11 years.

In Cameroon 64% (311 of 489) of the included study participants were anemic. Of these 64%, 32% (100 of 311) were adults over the age of 20 and 68% (211 of 311) were children and adolescents up to the age of 19. In Cameroon there were even higher numbers of severe and moderate anemia in children up to the age of 11 than in Gabon.

Prevalence by light microscopy showed 76% negative samples in Gabon. 22% of the samples were identified as *P. falciparum*-mono-infections by light-microscopy, and 1.4% *Plasmodium malariae* mono-infections. By microscopy, co-infections of *P. falciparum* and *P. malariae* were found in 0.8% of samples. Additionally, one *P. falciparum – P. ovale*-co-infection was identified by light microscopy.

In Cameroon 54% of samples were identified as negative by light microscopy. 24% of all samples were classified as *Plasmodium falciparum* mono-infections and 12% of all samples were found to be a *P. malariae* mono-infection. In 10% of all samples, a *P. falciparum – P. malariae*-co-infection was identified by light microscopy.

Using the method of highly sensitive qPCR, higher overall numbers of infected samples were found. In Gabon, only 16% of all samples were found to be negative. There were 47% of *P. falciparum*-monoinfections, 8% of *P. malariae*-mono-infections and 30% *P. falciparum – P. malariae*-co-infections.

In Cameroon, qPCR results revealed only 4% of all samples to be negative. 45% were identified as *P. falciparum* mono-infections, 4% as *P. malariae* mono-infections and 48% as *P. falciparum – P. malariae*-co-infections.

Comparison of results by light microscopy and qPCR shows lower median Ct values of the samples correctly identified as positive by microscopy and higher median Ct values of the samples that were incorrectly found to be negative by microscopy. These results may indicate that the samples falsely identified as negative by microscopy are indeed submicroscopic infections. Overall, there were more submicroscopic infections in Gabon than in Cameroon.

Comparing median Ct values in mono- versus co-infections in both Gabonese and Cameroonian samples suggests that in the average *Plasmodium malariae* mono-infection there is a higher parasitemia (as indicated by the lower median Ct value) than when *P. malariae* is detected as part of a co-infection with *P. falciparum*, where there seems to be lower *P. malariae* parasitemia (as indicated by the higher median Ct value) in comparison.

The inverse is shown for *Plasmodium falciparum*. In samples from both countries, the parasitemia in *P. falciparum* mono-infections is lower (with a higher median Ct value) than when it comes together with a *P. malariae* infection where it seems that *P. falciparum* parasitemia is relevantly higher (with a lower median Ct value).

When correlating qPCR results with age, regression analysis shows that in Gabon individuals with *P. malariae* mono-infections are significantly older than the those with other kinds of *Plasmodium* infection. This however cannot be shown for qPCR results in Cameroon, possibly due to the very low number of *P. malariae* mono-infections that were detected.

When performing a Pearson's correlation analysis for age and Ct values, a low positive correlation of *P. malariae* mono-infection Ct values with age can be observed in samples from Gabon. For *P. falciparum* positive samples, only Ct-values from co-infected samples correlate positively with age in Gabon.

In Cameroonian samples, both *P. malariae* and *P. falciparum* Ct values correlate positively with age. This indicates a higher Ct value the older the infected person is, and points towards a certain degree of acquired immunity with age.

The seasonal analysis was only possible for the samples from Gabon. There was almost the same number of infected samples both in rainy and dry seasons. In the dry season there were 9% of *P. malariae* mono-infections while there were 7% in the rainy season. *P. falciparum* mono-infections were more prevalent in the dry season with 51% of infected samples, versus 42% in the rainy season. Conversely, there were more mixed infections of *Plasmodium*

falciparum and *malariae* in the rainy season, 35%, compared to 24% in the dry season.

When looking at Ct values and seasonality, the Ct values are significantly lower for both *P. malariae* and *P. falciparum* in the rainy season as compared with the dry season, suggesting higher overall parasitemia in the rainy season.

Analysis of hemoglobin levels shows that median hemoglobin levels in non-infected study participants were significantly higher than median hemoglobin levels in infected participants. In correlation analyses, there is a positive correlation of hemoglobin with *Plasmodium falciparum* Ct levels in both Gabon and Cameroon, indicating a generally negative effect on hemoglobin levels with higher *P. falciparum* parasitemia.

For children and adolescents below the age of 18 on the other hand, statistical analyses show a significant effect of *Plasmodium malariae* infection on hemoglobin levels, which are consistently lower in infected children than in those who are not infected with *P. malariae*.

4 Discussion

There are three main findings in this study concerning the so far understudied pathogen *Plasmodium malariae*.

The first and most important point are the findings about the epidemiological characteristics of *Plasmodium malariae* in Gabon and Cameroon. Highly sensitive qPCR shows that there is a much higher prevalence of submicroscopic *Plasmodium malariae* infections than previously expected, with *Plasmodium malariae* mono-infections occurring significantly more often in individuals over the age of 60 years. This high burden of infection has been unknown so far. Secondly, this study found a significant negative effect of *Plasmodium malariae* infection on hemoglobin levels in children and adolescents under the age of 18. This might lead to a change in epidemiological strategy concerning the fight against malaria. And third, in mixed infections with *Plasmodium falciparum*, the Ct levels of *P. malariae* are higher than in mono-infections, indicating a possible interaction between the two pathogens.

It was the main goal of this thesis to elucidate the actual prevalence and epidemiological characteristics of *Plasmodium malariae* in Gabon and Cameroon. The results, as mentioned above, not only show its epidemiology but also give us an idea about how this pathogen might affect individuals in chronic, submicroscopic infections. Another aim was to find out more about possible mechanisms of its interaction with *Plasmodium falciparum*, and also for this question, the results hold some answers.

To put the findings of this study into a bigger context, an in-depth literature research was performed to review what has previously been discovered and discussed about the neglected parasite that is *Plasmodium malariae*. The following discussion therefore not only aims to discuss findings from the laboratory research of the present study, but to look beyond those results, place them into the bigger frame of the epidemiology and biology of *Plasmodium malariae*, and to give an idea of the current knowledge and the many open questions that remain.

4.1 What does a positive qPCR result mean?

Its more slowly advancing life cycle, and the mysteries surrounding the biology of *Plasmodium malariae* have already been mentioned in the introduction. The first question to ask about the high amount of subclinical *P. malariae* infections that were found in this study is: what does a positive qPCR result mean? Does it imply an actual infection, possibly one in the prepatent period? Or can the qPCR yield positive results when an infection has already been cleared by the immune system?

Previous studies, as the one by Smith et al. in 2011 on *Plasmodium falciparum* have suggested that “PCR positivity may persist for prolonged periods of time (months to years) and in endemic settings in particular its presence may not reflect current infection” (Smith *et al.*, 2011). However, the study by Smith et al. was referring to a PCR run on DNA, not on RNA. DNA can indeed persist for longer periods of time, while RNA, mRNA (messenger RNA) or rRNA (ribosomal RNA) is normally quickly degraded within hours to days of its production. rRNA, which was used for qPCR in this study, is the more stable form of RNA that forms part of the protein producing ribosome of eu- and prokaryotic cells.

A number of studies have already successfully used qPCR on *P. falciparum* RNA to effectively quantify the parasite load within humans (Murphy *et al.*, 2012; Imwong *et al.*, 2014). Furthermore, Murphy et al. have shown that the 18S rRNA of *Plasmodium* sporozoites, after their initial infection of the host, remains detectable within the bloodstream for a maximum amount of a few hours (Murphy *et al.*, 2018). Chang et al. discuss the use of qRT PCR on *P. falciparum* mRNA and conclude, that although they cannot be certain that “RNA transcripts represent viable parasites” they were able to show dynamics in the parasite clearance after treatment of the *P. falciparum* infection, indicating that most RNA is not stable for a longer period of time after the host cell has perished (Chang *et al.*, 2016).

Considering the current knowledge of RNA, it seems unlikely that *Plasmodium* RNA would persist for longer than a few days after the parasites have been

cleared from the hosts' system. Indeed, it is very likely that it would be degraded quickly by the ubiquitous ribonucleases, the RNA-degrading enzymes of the body, once set free from its original cell.

Considering these different points, the infections that were detected using highly sensitive qPCR on rRNA in this study will be regarded as reflecting an actual infection of the human host by *Plasmodium malariae* or *falciparum*. The detected infections could either be chronic subclinical infections or prepatent infections.

4.2 *P. malariae* is highly prevalent in Gabon & Cameroon and occurs mostly in mixed infections with *P. falciparum*

The main goal of this study was to find out more about the cryptic epidemiology of *Plasmodium malariae*. While it was already suspected that the prevalence of this parasite is much higher than previously detected, the numbers found by highly sensitive qPCR are still surprisingly high, with a subclinical prevalence of *P. malariae* of 38% in Gabon and 52% in Cameroon. *P. falciparum* was found in 77% of samples from Gabon and in 93% of samples in Cameroon.

Previous studies have described various numbers when it comes to the prevalence of *Plasmodium malariae*.

The first relevant large cross-sectional study on *Plasmodium* prevalence and incidence was the Garki project, a study aiming to assess the baseline epidemiology of *Plasmodium* species in the Garki district in Northern Nigeria, including thick blood film surveys conducted in 8 village-clusters every ten weeks for one and a half years (Molineaux et al., 1980). These data were collected before any medical intervention to treat malaria was pursued in the Garki district. The study found a seasonally varying prevalence of *Plasmodium falciparum* and *Plasmodium malariae*. The highest *P. falciparum* rate was around 70% of all samples tested in the rainy season, while the prevalence of this parasite never dropped below 40% in the roughly 5000 persons that were sampled per survey. *P. malariae* reached its peak prevalence in the dry season

with an infection rate of almost 20%. However, even the lowest *P. malariae* prevalence did not drop below 10% of the sampled survey participants. Overall, the Garki study found a ratio of *P. falciparum* to *P. malariae* of 4:1.

Another interesting finding is the “obvious and systematic excess of double infections with *P. falciparum* and *P. malariae*” (Molineaux et al., 1980). The authors go on to explain that with a higher parasitemia of *P. falciparum*, the chance of finding a co-infection with *P. malariae* is higher.

In 2012 a large cross-sectional study in Angola used blood smears and nested PCR for rRNA amplification on extracted DNA from filter papers, as described by Snounou et al. (Snounou, Viriyakosol, Xin Ping Zhu, *et al.*, 1993). This study found 1.2% of all tested samples to be positive for *Plasmodium malariae*, while 15.8 % of all 3,316 samples were tested positive for *Plasmodium falciparum* (Fançony *et al.*, 2012). The study further reported that most of the detected *P. malariae* infections were part of co-infections with other *Plasmodium* species, mainly with *P. falciparum*.

A study that screened Ugandan schoolchildren for malaria infections in 2013, also by blood smear and nested PCR on DNA extracted from filter papers, found 21% of all children (57 of 270) to have an infection with *P. malariae* (Dinko *et al.*, 2013). 53 of these *P. malariae* infections, 93%, were mixed infections of *P. malariae* with *P. falciparum*. The same study by Dinko et al. found 207/270 samples, 77%, to be positive for *Plasmodium falciparum*.

In 2020 a study was published which had assessed the prevalence of different *Plasmodium* species in schoolchildren aged 10-19 years in Nigeria in 2016, using blood smears, RDTs and PCR, targeting the mitochondrial CoxIII gene (Abdulraheem *et al.*, 2022). This study showed a 9% prevalence of subclinical *Plasmodium* infection by microscopy, a 47% prevalence by RDT and an 80% prevalence by PCR. Of all samples screened by PCR, 53% contained a *P. malariae* infection, mostly occurring as a mixed infection with *P. falciparum* and sometimes as a triple infection with *P. falciparum* and *P. ovale*. These numbers are very similar to the ones from the neighboring Cameroon detected in the

current study and suggest that PCR targeting the CoxIII gene is a very sensitive way of detecting malaria infections.

A different recent study from Nguiffo-Nguete et al. from Cameroon looked at the prevalence of malaria pathogens in the same region as this study, by microscopy, RDTs and PCR of rDNA on dried blood spots. It found a 43% prevalence of *P. falciparum*, a 17% prevalence of mixed infections of *P. falciparum* and *P. malariae*, and a 2.5 % prevalence of *P. malariae* mono-infections. Furthermore, it detected 2.6% of mixed infections that included *P. ovale* (Nguiffo-Nguete *et al.*, 2023). These numbers are again much lower as the ones detected by this study and suggest that PCR on rDNA extracted from dried blood spots might not be sensitive enough to get a full epidemiological picture of the actual *Plasmodium* prevalence.

For Gabon, recent data comes from Woldearegai et al., based on a cross-sectional study which screened 834 blood samples with an ultra-sensitive RT-qPCR for *Plasmodium* infection and then performed singleplex, nested PCR on DNA to identify the species (Woldearegai *et al.*, 2019). The authors found a prevalence of *P. malariae* of 23%, and a prevalence of *P. falciparum* of 66%. Only 1% of all *P. malariae* infections were identified as mono-infections, the rest was found to be mixed infections with *P. falciparum* alone or together with *P. ovale*. These results are a little lower than what the present study found, but overall similar to the results from this study. As the current study did not screen for *P. ovale* infections it remains unclear how many samples contain this additional pathogen.

A meta-analysis pooling results from prevalence studies from the years 2000 until 2020 found an average prevalence of *Plasmodium malariae* of 2.01%, with a high heterogeneity of findings across the 102 studies included (Hawadak, Dongang Nana and Singh, 2021).

The huge heterogeneity in findings of the *P. malariae* prevalence can be explained both with the tendency of the parasite to form submicroscopic infections, the varying intensity of transmission as well as the fact that it is very likely that different participants in cross-sectional studies will have a different

level of acquired immunity. Furthermore, some studies were performed using only blood smears, others used RDTs, and the newer ones mostly used PCR on DNA. This plethora of different methods of detection, each with its own sensitivity and specificity, makes it hard to compare the resulting prevalence of *P. malariae*. It is likely that most previously used methods are not sensitive enough to detect the actual burden of *Plasmodium malariae*.

However, general trends can well be compared. For instance, the numbers of infections detected in the samples from Gabon and Cameroon seem to corroborate the trend for *P. malariae* infections to happen mainly as mixed infections with another *Plasmodium* parasite, in our case *P. falciparum*. Indeed, 79% of all *P. malariae* infections in Gabon were mixed with *P. falciparum*, while 92% of samples from Cameroon infected with *P. malariae* also included *P. falciparum*. As we did not test for *P. ovale* and *P. vivax*, we cannot say how many mixed infections there might have been that included an infection with those malaria parasites.

For this study, it is important to emphasize that the detected infections are all subclinical infections, as symptomatic individuals were not included in the study. They are most probably either subclinical chronic infections or prepatent infections. The notion of a chronic, low-level infection with *P. malariae* will be discussed further on.

4.3 Factors involved with high *Plasmodium* prevalence and a change in *P. falciparum* to *P. malariae* ratio over time

In both Gabon and Cameroon, the samples that contain *Plasmodium falciparum* are about double in number when compared to the samples that contain *Plasmodium malariae*.

Figures 16 & 20 in the Results section show visually that the overall amount of *Plasmodium falciparum* infections (counting both mono- and co-infections), which covers about 77% of all samples in Gabon and 93% of all samples in Cameroon, is almost exactly double the amount of *Plasmodium malariae*

infections which is present in about 38% of samples in Gabon and in 52% of samples in Cameroon. This creates a ratio of almost 2:1.

When comparing these numbers to the findings from the Garki study from 1980 Nigeria, a longitudinal study that sampled malaria prevalence using thick blood films, it appears that at the time there was a ratio of 4:1 (*P. falciparum* : *P. malariae*) (Molineaux et al., 1980).

Considering these numbers and keeping in mind that at the time the study was done by screening thick blood films and not by qPCR, it seems like the absolute amount of *Plasmodium malariae* infections might have increased in Africa. This has also been shown in a study from Burkina Faso which sampled children from 2007 to 2010 by examining blood slides. The study showed a constant increase in *P. malariae* prevalence from 0.9% in 2007 to 13.2% in 2010 (Gnémé et al., 2013). Similar trends of an increase in the proportion of *P. malariae* cases have been described in Tanzania, Uganda and in Benin (Betson *et al.*, 2018; Yman *et al.*, 2019; Agonhossou *et al.*, 2022).

This trend could among others be due to the fact that eradication campaigns from the past decades have been focused solely on *P. falciparum*. As recent studies have shown, once the prevalence of *P. falciparum* drops, for instance through mass drug administration, the prevalence of *P. malariae* increases (Betson *et al.*, 2018; Yman *et al.*, 2019; Oriero *et al.*, 2021). There seems to be an ecological niche that opens up for *P. malariae* once *P. falciparum* is successfully treated. Why *P. malariae* seems often to persist or relapse despite antimalarial treatment will be discussed further on.

There are several factors that are relevant to the much larger number of *P. falciparum* positive samples in comparison to samples containing *P. malariae*. First, *P. falciparum* has much shorter incubation and multiplication cycles, both within the human and within the mosquito, than *P. malariae*. In any infection, it would simply grow faster than *P. malariae* and therefore would develop gametocytes earlier and profit off a longer period of infectivity for mosquitoes. Furthermore, it has been found that in mixed infections of *P. falciparum* and *P. malariae* there is a higher production of gametocytes of *P. falciparum* than when

it occurs in mono-infections (Bousema *et al.*, 2008). This might give the parasite another advantage when it comes to transmissibility.

It remains unclear however, if and how exactly a mixed infection actually affects the transmission to and from mosquitoes. In a study by Tang et al. from 2020 including rodent malaria strains, there have been mixed results. Firstly, in no experiment was there an increased transmission of any *Plasmodium* to a mosquito. For *P. yoelii*, oocyst burden in infected mosquitoes remained the same, and therefore its transmission was not altered in co-infections with another *Plasmodium*. The transmission of *P. vinckei* and *P. chabaudi* however was reduced in mixed infections as compared to mono-infections of these parasites (Tang *et al.*, 2020).

On the other hand, in an experimental transmission study including blood samples from asymptomatically infected individuals in Gabon, with *P. malariae* mono-infections and mixed infections with *P. falciparum*, the median parasite count of *P. malariae* was lower in mixed infections, while its median gametocyte count was higher in mixed infections (Pinilla *et al.*, 2021). The authors found that from the mixed infection samples, none transmitted *P. falciparum* to mosquitoes while *P. malariae* was transmitted in a number of cases.

The issue of transmissibility and vectorial factors in mixed infections remains to this date not well studied and should be part of future research endeavors.

It remains at this stage most probable that most of the higher overall prevalence of *P. falciparum* is due to within-host competition with *P. malariae*. Immunological factors that might play a role will be discussed further on.

4.4 Mixed infections - possible mechanisms of interaction between *Plasmodium malariae* and *falciparum*

4.4.1 Seasonal aspects in interactions of mixed Plasmodium infections

As already mentioned above, the Garki study from 1980 Nigeria found that a *Plasmodium falciparum* infection increased the likelihood of being concomitantly infected with *Plasmodium malariae*. Furthermore, they found that the higher the *P. falciparum* parasitemia was, the higher the chance of finding a co-infection with *P. malariae* (Molineaux et al., 1980). The present study found a higher *P. falciparum* parasitemia in mixed infections with *P. malariae* than in mono-infections of *P. falciparum*, pointing towards a similar trend as the Garki results.

Additionally, the findings of the Garki study pointed into the direction of a suppression of *P. malariae* by *P. falciparum* in mixed infections. It was observed that in the collected blood smears, *P. falciparum* peaked in the rainy season while *P. malariae* infections were reduced in the rainy season. However, non-immune children showed a peak of *P. malariae* infections in the rainy season. This led Molineaux et al. to suppose a suppression of *P. malariae* by the rising number of *P. falciparum* infections during the rainy season, rather than attributing this dynamic to factors related to transmissibility (Molineaux et al., 1980). It is important to note that the Garki study only found patent infections, which means infections that were detectable microscopically by blood smear.

Possible reasons for a suppression of *Plasmodium malariae* by *Plasmodium falciparum* during the rainy season can be found in the biology of the parasites in the human host and in the vector. For instance, *P. falciparum* has much shorter hepatic and erythrocytic cycles than *P. malariae* and therefore multiplies faster within the human host. Its parasitemia could certainly outnumber an existing *P. malariae* chronic infection easily within a few days of infection. Furthermore, once gametocytes are transmitted to an *Anopheles* mosquito, the maturation cycle within the mosquito, to make the mosquito infectious for humans, will take around 9 days for *P. falciparum*, while it takes at least 14 days for *P. malariae*. On the contrary to what has been stipulated by the authors of the Garki study, higher transmissibility of *P. falciparum*, by a larger number of

vectors, certainly plays a role in the higher amount of detected *P. falciparum* infections during the rainy season. This multitude of features pertaining to the biology of *P. falciparum* and its vectors, in close relation to the climate conditions, could explain the higher number of patent infections during the rainy season.

Interestingly, Maitland et al. found a similar pattern on the island nation of Vanuatu in 1996. They screened children and detected, by thick and thin blood smears, a pattern of high *P. vivax* prevalence in the dry season and high *P. falciparum* prevalence in the wet season (Maitland *et al.*, 1996; Maitland, Williams and Newbold, 1997). The authors explained this phenomenon by hypothesizing that the seasonally stronger *P. falciparum* suppressed already existing *P. vivax* infections during the wet season, mainly by competition for the hosts' red blood cells. They further stipulated that as a result, the "disease due to *P. vivax* increases in the dry season when transmission pressure from *P. falciparum* is reduced" (Maitland, Williams and Newbold, 1997). Unfortunately, their study only screened children under the age of 10 years, not giving an insight for all age groups in Vanuatu.

In the current study the detected prevalence of *P. malariae* and *P. falciparum* in Gabon did not vary much by season. In the dry season 33.3% of all samples were tested positive for *P. malariae* and 75.7% for *P. falciparum*. In the rainy season those numbers were 42% for *P. malariae* and 76.9% for *P. falciparum*. There seemed therefore to be an increase of *P. malariae* infections during the rainy season. Interestingly, 84% of all *P. malariae* infections occurred as mixed infections during the rainy season, while in the dry season it was only 73%, with a higher number of mono-infections during the dry season.

When comparing median Ct values, both parasites have lower median Ct values during the rainy season, invoking a higher parasitemia in infections during this time of year. This can be explained by the larger number of vectors that are present during the rainy season, increasing the inoculum and the exposure of humans to *Plasmodia*. In all samples, *P. malariae* has higher median Ct values than *P. falciparum*, suggesting a lower overall level of

parasitemia and pointing towards the previously invoked chronic low level parasitemia that seems to be characteristic for *P. malariae*. In the findings from this study there is one month, however, May 2021, the beginning of the long dry season in Gabon, in which there is a fall in median *P. falciparum* parasitemia and a rise in median *P. malariae* parasitemia. This could possibly point towards a similar effect as evoked by Maitland et al. as mentioned above: the reduced transmission pressure of *P. falciparum* in the beginning of the dry season leading to better transmission of *P. malariae*.

To sum up this first part, the current study was unable to reproduce the same findings as the Garki study. It is however very difficult, if not impossible, to compare the results, as the Garki study found only microscopically patent infections and did not exclude clinically symptomatic patients, while this study mainly found submicroscopic infections in asymptomatic individuals. An important factor is also the population that was sampled: in Garki the population had never been widely treated for malaria before the study was conducted, and therefore showed the parasites in an epidemiologically naïve state, while in Gabon, there are many programs for malaria prevention and the local population is frequently treated for malaria. This regular use of antimalarial drugs alters the ecology of malaria and could contribute to the creation of an ecological niche for *P. malariae*, as mentioned above.

Lastly, all sampling sites in Gabon are situated very close to rivers, meaning that a possible breeding reservoir of *Anopheles* mosquitos is available year-round, and a perennial transmission of malaria is highly likely. It has been shown in a study in Mali that malaria prevalence rates don’t vary much by season when a breeding source for mosquitoes is nearby (Koita et al., 2012).

A possible suppression of *P. malariae* by *P. falciparum* in the rainy season can therefore neither be confirmed nor contradicted by the current study.

4.4.2 Microbiological trends in mixed infections: higher P. falciparum parasitemia and lower P. malariae parasitemia

When not specifically looking for seasonal trends, but having an overall look at mixed infections, a clear trend can be observed in the findings from Gabon and Cameroon. In both countries there is a significant difference in median Ct values in mono- versus co-infections.

As it can be seen in the results, the median Ct values of *Plasmodium malariae* are lower in mono-infections and higher in co-infections. This suggests a higher parasitemia of *P. malariae* in mono-infections than when it occurs in mixed infections with *Plasmodium falciparum*. For *P. falciparum* the exact opposite can be observed. *Plasmodium falciparum* has significantly lower median Ct values in mixed infections with *P. malariae*, suggesting a higher overall parasitemia in co-infections, while its parasitemia seems to be lower in comparison when it occurs in mono-infections.

These findings suggest that there is an interaction when both parasites infect the same host, leading to a higher *P. falciparum* parasitemia and a lower parasitemia of *P. malariae*.

Possible reasons for the higher parasitemia of *P. falciparum* in mixed infections can be found in the differing life cycles of both parasites. These have been discussed above and relate to a within-host competition. Factors pertaining to an interaction of the two *Plasmodium* species can also be found in the hosts' immune system. Culleton et al. recently highlighted that according to them "acquired immunity is highly species specific, and thus, unlikely to contribute significantly to interactions between concurrent *Plasmodium* species" (Culleton, Pain and Snounou, 2022). They postulate, however, that once *P. falciparum* enters the host as a second *Plasmodium* species, there might be some kind of "*P. falciparum*-induced non-specific immune response" that leads the hosts' immune system not only to target *P. falciparum* but also the already prevalent *P. malariae*, which would therefore be further reduced in its parasitemia. This mechanism could explain the lower parasitemia of *P. malariae* in mixed infections that was found in the present study.

A second theory from the same authors proposes a tropism of both parasites for reticulocytes, and therefore a competition for host cells, which goes in favor of *P. falciparum* as it multiplies faster than *P. malariae* (Culleton, Pain and Snounou, 2022). The predilection of *P. malariae* for certain forms of red blood cells will be discussed further below.

To sum up, factors influencing this dynamic of a reduced *P. malariae* parasitemia and increased *P. falciparum* parasitemia in mixed infections pertain to the life cycles of both parasites, their preference for certain red blood cells and possible influences of either parasite on the hosts' immune system which remain yet to be elucidated.

4.4.3 Insights on attenuating effects of P. malariae on P. falciparum in mixed infections

In previous studies, the complex interactions of *Plasmodium* species in mixed infections have been discussed in different ways.

Tang et al. showed in 2020 in a mouse model, with rodent *Plasmodium* species, that mixed versus single infections can have a relevant impact on disease severity. They found out that a mixed infection with a *Plasmodium* species which targets normocytes (normally aged red blood cells) together with a species targeting reticulocytes (young red blood cells) is much more lethal than any single infection (Tang *et al.*, 2020). This could be due to less within-host competition which would lead to a higher parasite burden of both species within the body and therefore a higher level of disease. This dynamic however, has only been shown for rodent malaria models.

When considering human malaria pathogens, several studies indicate the opposite: that in mixed infections, "the severity of [the] *P. falciparum* infection is attenuated by the presence of a less severe malaria species" (Smith *et al.*, 2011), like *P. vivax* or *P. malariae*.

This was already postulated in several studies by Maitland and Luxemburger in the late 1990s, who studied mixed infections of *Plasmodium vivax* and

Plasmodium falciparum in Thailand and Vanuatu (Luxemburger *et al.*, 1997, 1999; Maitland, Williams and Newbold, 1997). Luxemburger showed in her study that the occurrence of severe malaria "was 4.2 times less common in patients with mixed *Plasmodium falciparum* and *P. vivax* infections than in those with *P. falciparum* alone, suggesting that *P. vivax* may attenuate the severity of *P. falciparum* malaria" (Luxemburger et al., 1997).

A study conducted in Sri Lanka showed that subjective symptoms of a clinical *P. falciparum* episode were less severe when added to a known infection with *P. vivax* (Gunawardena, Carter and Mendis, 1994).

Maitland et al. discussed in 1997, based on these findings, and next to the notion of within host-competition, a degree of cross-species immunity. This would be a type of immune reaction conferred from an existing *P. vivax* infection, leading to an immune response against *P. falciparum*, and therefore to less severe disease and symptoms (Maitland, Williams and Newbold, 1997).

There have been similar, albeit few, observations when it comes to mixed infections of *P. falciparum* and *P. malariae*.

Black et al. screened children in a village in Ivory Coast by blood smear and PCR and found 0% of children with clinical malaria to have a mixed infection, while asymptomatic yet microscopically malaria positive children had mixed infections in 27% of the cases (Black *et al.*, 1994). They concluded that "*P. malariae*, which is associated with notoriously lengthy subclinical infections, might be responsible for the maintenance of a down-regulation of the cytokine cascade" (Black *et al.*, 1994), therefore attenuating the severity of the concomitant *P. falciparum* infection.

In 1999, Collins and Jeffery reviewed data from neurosyphilis treatment studies in the US from 1940 to 1963. They specifically looked at clinical and microbiological findings of patients who had been previously exposed to a non-falciparum *Plasmodium* (which was the standard treatment for neurosyphilis at the time) and were at the time of the study reinfected and 'treated' with *Plasmodium falciparum*. The patients who had been previously exposed to *P.*

malariae, as opposed to *P. vivax* or *P. ovale*, had fewer episodes of fever, and a lower overall parasite count of *P. falciparum*, with fewer episodes of high-density parasitemia (Collins and Jeffery, 1999). The authors concluded that "*P. falciparum* and *P. malariae* share common antigens that are able to induce parasitologic and clinical protection when infection with *P. falciparum* follows that with *P. malariae*". These results could indeed speak for some degree of cross-species immunity against *P. falciparum*, conferred by an infection with *P. malariae*.

In a cross-sectional study assessing malaria prevalence in southwestern Nigeria in 2016, based on blood smears, RDTs and PCR, Abdulraheem et al. describe that in asymptomatic participants with a malaria infection they detected 62% of mixed infections, mostly co-infections of *P. falciparum* and *P. malariae* (Abdulraheem *et al.*, 2022). In symptomatic patients however, 91% of all infected patients had a mono-infection with *P. falciparum* and only 9% of samples were mixed infections with *P. falciparum* and either *P. malariae* or *ovale*. The same study conducted genetic analyses of *P. falciparum* and found specific haplotypes that only occurred in mixed infections with *P. malariae*, while there were different and fewer genetic haplotypes in *P. falciparum* mono-infections. The authors hypothesize that "the overgrowth of particular *P. falciparum* clones leads to the competitive exclusion of other clones, and other species in the infection" (Abdulraheem *et al.*, 2022).

Based on their findings, the authors stipulate that a possible overgrowth of *P. falciparum* clones within a mixed infection might lead to an increased activation of the immune system in certain hosts, leading to the eradication of *P. malariae* from the hosts system and to a clinical infection with severe symptoms, through increased cytokine production. This would explain the predominance of mono-infections with *P. falciparum* in clinically symptomatic study participants in the study conducted by Abdulraheem et al.

These mostly cross-sectional studies all seem to show that in mixed infections with *P. malariae*, *P. falciparum* is less virulent and leads to either an asymptomatic chronic infection or a non-severe episode of clinical malaria.

A longitudinal study conducted in Papua New Guinea in the early 1990s tried to assess the interactions of the three prevalent parasites in this location, *P. falciparum*, *P. vivax* and *P. malariae*. The authors found that a previous infection with *P. vivax* confers a protection against the severity of *P. falciparum* episodes, while a previous infection with *P. malariae* seems to have a generally protective effect, not only against *P. falciparum* disease severity but also against any kind of morbidity or reason to visit the health center in this tropical setting (Smith *et al.*, 2001). The authors argue that *P. vivax* may be protective against *P. falciparum* through a mechanism of cross-reactive immunity, while *P. malariae* might have an effect on the regulation of cytokines. Indeed, a down-regulation of cytokines might lead to a less pronounced reaction of the immune system to any aggressor and lead to a reduction in clinical symptoms. As Smith et al. relevantly point out however, “there remains the possibility that genetic heterogeneity in host responses is the explanation for the *P. malariae* effect”, as it was also proposed by the authors of the Garki study (Molineaux et al., 1980; Smith et al., 2001).

In a review article from 1999, considering data from 19 studies on mixed *Plasmodium* infections, McKenzie and Bossert conclude that “it seems clear that some mechanisms of heterologous, cross-species immunity exist, but their significance awaits further investigation” (McKenzie and Bossert, 1999).

It has to be highlighted that in the subclinical infections detected in this study, both pathogens are much more prevalent in mixed infections than in single infections, turning up the question of a possible survival benefit for both pathogens in co-infections. Indeed, if both malaria parasites are more stable in a chronic, low parasitemia-mixed infection, they increase their species’ chance of survival by sustaining an infectious, yet subclinical, reservoir for mosquitoes. This seems to be supported by the findings by Bousema et al., who show that the amount of *P. falciparum* gametocytes increases when *P. falciparum* occurs as a mixed infection with *P. malariae* (Bousema *et al.*, 2008). This had previously also been stipulated by McKenzie et al. who reviewed data from malariotherapy patients from the previous century and found that “gametocyte production was enhanced in *P. falciparum* by prior or concurrent *P. malariae*

infection" (McKenzie, Jeffery and Collins, 2002). Bousema et al. stipulate that this could be due to cross-reactive antibodies, produced through the hosts' immune systems' encounter with *P. malariae* that stress *P. falciparum* to produce more sexual stages in order to increase its transmission to mosquitoes (Bousema *et al.*, 2008). A more recent study from Gabon did, however, not find an increase in *P. falciparum* gametocytemia in mixed infections (Woldearegai *et al.*, 2019). Unfortunately, no studies exist on *P. malariae* gametocyte density in mixed infections as no molecular methods of gametocyte detection for *P. malariae* have been reported so far.

These findings support the hypothesis that in mixed infections of both pathogens there is a survival benefit, at least for *P. falciparum*, through cross-reactive immunity and possible effects on the immune response like the downregulation of the cytokine cascade. More research is needed, however, to answer the question whether *P. falciparum* gametocytemia is indeed increased in mixed infections with *P. malariae*, and whether this increases transmission probability or not.

4.4.4 Conclusion on parasite interactions with each other and the hosts' immune system

In this study an increase in *P. falciparum* parasitemia and a decrease in *P. malariae* parasitemia was observed in mixed infections of the two parasites. To explain this dynamic, factors of within-host competition, like the parasites' life cycles and predilection for certain forms of erythrocytes, were mentioned. Furthermore, factors pertaining to the immune response of the host, like cross-reactive-immunity, reduction of the body's cytokine response and a non-specific immune response triggered by a *P. falciparum* super-infection were discussed and might play an important role in the interaction of *P. malariae* with *P. falciparum*.

Any effect of clinical attenuation of *P. malariae* on *P. falciparum* could not be studied as only asymptomatic study participants were recruited. However, the very high number of mixed infections in these asymptomatic study participants

could suggest an attenuating effect of *P. malariae* on *P. falciparum*, as it is likely that clinically symptomatic patients would have a higher number of *P. falciparum* mono-infections in comparison. This question, however, remains to be studied in the future.

It remains unclear what factors are at play in the interaction of multiple *Plasmodia* within a host and with the hosts' immune system. We do not know for certain whether there is an actual attenuating effect of *P. malariae* on *P. falciparum*, or whether there are individual, heterogenous immune system factors involved when it comes to the severity of disease. It is likely that both mechanisms might play a role.

Another open question pertains to possible cellular changes induced by one or the other *Plasmodium* species, for example at a receptor or pathway level. For instance, there could be molecular changes on the cellular level induced by *Plasmodium malariae*. These could lead to less cytoadherence of *P. falciparum*-infected red blood cells, reducing its disease severity. Or they could make the host cells more susceptible to an infection with *Plasmodium falciparum*, explaining its 'enhancement' in co-infections with *P. malariae*.

Understanding complex interactions between two species of parasites is further complicated by the complex ecosystem they are a part of. Factors pertaining to the anopheline vectors of *Plasmodia*, like the local microclimate, the presence of open water sources and the practice of mass drug treatment campaigns as well as the use of bednets and spraying all impact the transmission of the parasites to human hosts. As Holzschuh et al. point out, the "heterogeneity in exposure to infectious mosquitoes poses a further challenge to understanding between-species interactions" (Holzschuh et al., 2022).

Finally, also the previous individual history of infection of the human hosts and their level of acquired immunity to different malaria pathogens and haplotypes play a role in the course of any infection, the symptoms, and the parasitemia.

To summarize, there may well be an interplay between the two species of parasites, that certainly needs to be investigated further. It is also likely that the

interaction of the individual parasites with the specific host's immune system might be more important than previously thought and has an impact on the development of either chronic subclinical disease or acute clinical disease.

4.5 Trends and correlations of age and *Plasmodium*-infections

The qPCR data from Gabon suggests, that there are more *P. malariae* mono-infections the older the infected individual gets, with the highest number of infections found in adults over the age of 60, and the lowest in children. Figure 36 shows that this finding is statistically significant. The results from this study correlate with the data from Woldearegai who conducted a cross-sectional study based on RTqPCR in Gabon in 2018 and also found that the mean age of individuals infected with a *P. malariae* mono-infection is significantly higher than for other kinds of infection (Woldearegai *et al.*, 2019). By microscopy on the other hand, most *P. malariae* infections were found in younger participants, suggesting that in children, who have not yet developed a certain level of acquired immunity, *P. malariae* reaches a higher parasitemia than in adults and is therefore easier to detect microscopically. Additionally, there is a low degree of positive correlation for Ct values with age in *P. malariae* mono-infections in Gabon, confirming that the increased number of infections in older adults occurs at lower levels of parasitemia and indicating some level of acquired immunity.

These findings, with a surprising rise of the number of *P. malariae* cases with age as detected by qPCR, could lead us to believe that this parasite indeed has a survival strategy that is mostly based on chronic subclinical infections as it has been suggested by Sutherland in 2016 (Sutherland, 2016). These infections, being at a low level of parasitemia, would mostly not be detectable by light microscopy.

An inverse trend can be shown for *P. falciparum* mono-infections in Gabon. They occur mostly in children up to the age of 11 years and then decrease the older the study participants become. By light microscopy, the highest amount of patent *P. falciparum* infection is found in children aged 5-11 and 12-19, also decreasing in samples of older adults. The same trends were found by the

recent cross-sectional study in Gabon already cited above (Woldearegai et al., 2019).

The youngest children, 1-4 years old, were those that were least infected by *Plasmodia*, suggesting less exposure to the parasites which might be due to existing safety measures and increased awareness by the parents.

This data shows the vulnerability especially of adolescents for *P. falciparum* infections, who are often not targeted by specific prevention measures, unlike younger children who are often the focus group of interventions.

The overall lower amount of *P. falciparum* infections in older participants can most likely be attributed to an increasing level of acquired immunity against this most virulent malaria parasite.

As the individuals' age increases in Gabon, so does their rate of infection with mixed *P. malariae – P. falciparum* infections. Mixed infections were almost solely detected by qPCR. Microscopical findings showed only 9 samples to be positive with a mixed infection in Gabon. Of these 9 samples, one was found to be negative by qPCR, 2 showed only a *P. falciparum* mono-infection by qPCR and 2 resulted in a *P. malariae* mono-infection by qPCR, making the sensitivity of microscopical detection of subclinical co-infections very low at 4/9. As this study did not screen for *P. ovale* species it remains impossible to say whether these infections might have been indeed mixed infections, containing *P. ovale*. As it has been reported that also microscopically, *P. ovale* is frequently misidentified as another *Plasmodium* species (Lover *et al.*, 2018), this might be an interesting target for future studies with high performing molecular detection methods.

Interestingly, only the Ct values of *P. falciparum* occurring in mixed infections significantly correlate with age in Gabon, suggesting that there is an effect of acquired immunity that increases with age. The Ct values of *P. malariae* in mixed infections, however, do not correlate with age. It remains unclear what this dynamic could signify. If a certain mechanism of cross-reactive immunity with an attenuation of *P. falciparum* in mixed infections with *P. malariae* exists,

the reduced parasitemia of *P. falciparum* in mixed infections in older individuals could be a consequence.

Of note are the samples that have been found to be negative by qPCR. Their number decreases with age. The trend towards less negative samples the older the participants get suggests a higher rate of chronic sub-clinical *Plasmodium* infections in adults. This also encompasses possible chronic low-level infections with *P. falciparum*. It has previously been reported that *P. falciparum*, like *P. malariae*, can form very low-density chronic infections, that can relapse up to 13 years after the last exposure (Ashley and White, 2014).

The chapter outlining the results shows that the infection rates obtained by qPCR and microscopy are different in Cameroon. Of all 489 included samples only 20 contain a *Plasmodium malariae* mono-infection. This makes statistical analyses of *P. malariae* mono-infections difficult. When looking at the numbers of these infections there does not seem to be a pattern of increasing *P. malariae* mono-infections with age in Cameroon.

Furthermore, in Cameroon, trends in older participants are difficult to evaluate, as only 17 out of the 480 included study participants were older than 60 years. Indeed, the sampled population in Cameroon was substantially younger than in Gabon, with a median age of 14 (IQR 7 – 28), while the median age was 20 (IQR 8 – 44) in Gabon.

According to statistical population numbers from both countries for 2022, the median age in Cameroon was 17.6 while the median age in Gabon was 21.7 years (*Gabon Population*, *Worldometer,* 2024; *Cameroon Population*, *Worldometer,* 2024). The group of study participants sampled from Cameroon was therefore not as representative of the general Cameroonian population as it was the case for the study participants from Gabon. As this study only assesses half of the samples from Cameroon however, this data may change when the full dataset from Cameroon becomes available.

Among the studied set of samples in Cameroon there is an overwhelming amount of *P. falciparum* mono-infections and a large number of mixed infections

of the two parasites. Microscopically and by qPCR there are more *P. falciparum* mono-infections in children which then decline with increasing age. As in Gabon, the highest amount of patent *P. falciparum* infections can be found in the age groups of 5-11 and 12-19, suggesting a higher burden of infection in these age groups.

In Cameroon, 49 mixed infections were detected microscopically. Of these, highly sensitive qPCR found 2 to be *P. malariae* mono-infections, 16 *P. falciparum* mono-infections and confirmed 31 to be indeed mixed *P. falciparum* / *P. malariae* co-infections. None were negative by qPCR. This makes microscopy more sensitive for mixed infections in Cameroon than in Gabon, but these results might also be due to the higher number of overall infections and the higher patency of infections in Cameroon. Again, as this study did not test for *P. ovale*, the amount of actual mono-infections remains unknown.

Microscopically the rate of *P. falciparum* mono-infections declines further in older adults while by qPCR it rises again in the group of 20–60-year-olds, possibly reflecting a high rate of chronic sub-microscopic P. falciparum infections. Mixed infections of *P. falciparum* and *P. malariae* make up a much higher proportion of all infected samples in Cameroon than in Gabon. Microscopically they are mostly detected in children and then decline with age. By qPCR however it becomes obvious that about 35% of all samples in the youngest age group represent co-infections, with this rate rising up to 60% in adolescents. It declines again in adults and stabilizes to make up 40-45% of all processed samples in adults. These results again suggest that the largest amount of co-infections is indeed sub-microscopical and in most samples both *P. falciparum* and *P. malariae* are present in their human hosts at a very low level, chronic parasitemia.

When correlating Ct values of infections with age in Cameroon, all Ct values of the different kinds of infections correlate positively with age, except for *P. malariae* mono-infections for which not enough samples were available for a meaningful statistical analysis. This means that the median Ct values are rising with higher age, indicating less parasitemia in older individuals and very likely

reflecting an increased degree of acquired immunity with age in the population of this area where malaria is highly endemic and prevalent. Together with the results from microscopy which found more microscopically patent infections in Cameroon than in Gabon, these results indicate a different epidemiological situation for malaria infections in the sampled individuals in Cameroon as compared to the sampled population in Gabon. In Cameroon there are more patent infections, with acquired immunity clearly playing a role as age increases. In Gabon, there are more submicroscopic infections and a less marked effect of acquired immunity with age. Whether very low-density parasitemia has a lesser effect on the acquisition of immunity, as suggested by this data, remains to be studied.

To sum up, in Gabon *P. malariae* mono-infections occur significantly more often in older individuals than any other kind of malaria infection, confirming the findings by Woldearegai et al. that were discussed above. This is not the case in Cameroon. However, the very small number of *P. malariae* mono-infections detected as well as the low number of older individuals sampled in Cameroon makes it difficult to statistically analyze this small set of data.

The highest prevalence of *Plasmodium* infections, as well as of mixed infections, with 88% and 98% respectively, was found in both Gabon and Cameroon in the age group of 12–19-year-old adolescents (see Tables 12 & 14). It seems that in the population that was sampled for this study, this age group is the most susceptible to malaria infections, possibly because they have not yet acquired a sufficient level of immunity but do not benefit of all the protective measures as children do anymore. By comparison, in previous studies from Gabon and Cameroon the highest overall prevalence of malaria was found in the age group of 6–10-year-olds, with the group of 11–15-year-olds coming second (Woldearegai *et al.*, 2019; Nguiffo-Nguete *et al.*, 2023). Woldearegai et al. found a 91% prevalence of *Plasmodium* infections in 6-10-year-olds in Gabon, as compared to 82% in 5-11-year-olds in the present study. In 11-15-year-old adolescents they found a prevalence of 80%, while this study found a prevalence of 88% in 12-19-year-old adolescents (Woldearegai *et al.*, 2019). For Cameroon Nguiffo-Nguete determined the highest prevalence of

Plasmodium infections, 64%, to be present in children aged 6-10 years (Nguiffo-Nguete *et al.*, 2023), as compared to a 95% prevalence in children aged 5-11 years found by the present study. Interestingly, the study from Gabon used qPCR based on RNA while the study from Cameroon used nested PCR on DNA. The determined results, compared with the prevalence found in the present study, show the increased sensitivity of using qPCR on RNA instead of DNA.

Overall, both age groups, older children and adolescents, seem to be left vulnerable to malaria infections and to all the consequences that might ensue. Recurring severe infections and chronic anemia can especially impact attendance and concentration in school and therefore scholarly achievements. This could have actual consequences on the lives of the affected children and adolescents. For instance, it might lead to the students having less academic success, with a reduced chance of finding a well-paying job and supporting a family later in life.

The data from both Gabon and Cameroon furthermore suggest the existence of a large pool of individuals who are carriers of low-level *Plasmodium* parasitemia. These asymptomatic carriers probably make up the largest part of the *Plasmodium* reservoir in malaria endemic countries. It has been reported, and confirmed by molecular methods, that in *P. falciparum* infections even individuals with no microscopical gametocytemia can be infectious to mosquitoes (Bousema and Drakeley, 2011; Ashley and White, 2014). In a recent longitudinal study from Uganda, Andolina and colleagues determined that the biggest infectious reservoir in an endemic setting is indeed constituted by asymptomatic yet microscopically detectable infections and asymptomatic infections that are only detectable by PCR (Andolina *et al.*, 2021). Surprisingly, symptomatic malaria infections only made up 0.6% of the infectious human reservoir in their study. The data from the present study further points towards a large pool of asymptomatic yet infectious *Plasmodium* carriers, for both *P. malariae* and *P. falciparum*. This knowledge is an important steppingstone for further action to eliminate malaria.

4.6 Differentiating results from qPCR and light microscopy

The discrepancies that can be found when comparing the results obtained by qPCR with the results by light microscopy further point into the direction of a high number of submicroscopic, low-level chronic infections. This is also supported by the median Ct values in qPCR-positive but microscopically negative samples that are significantly higher in both countries than when the samples are detected to be positive both microscopically and by qPCR. This suggests a higher parasitemia in the samples that are identified as an infection by both methods, and a lower parasitemia for the samples which are only found to be positive by qPCR.

Additionally, the fact that some samples were found to be negative by qPCR but positive by light microscopy, could indicate that *P. falciparum* and *P. malariae* are indeed not the only *Plasmodium* species that are prevalent in Gabon and Cameroon. It is very likely that *P. ovale* is prevalent, too, and there is also a chance of some *P. vivax* infections, as it has been shown in recent years that *P. vivax* is, in fact, prevalent in African countries and even originates from Sub-Saharan Africa (Culleton and Carter, 2012; Liu et al., 2014; Twohig et al., 2019).

To sum up this first part of the discussion, it seems that the theory of there being a high amount of “chronic, low-density, multi-species, asymptomatic infections” as an adaptation of *Plasmodium* for better survival as suggested by Sutherland (Sutherland, 2016) can be confirmed by our findings, that show a much higher rate of infections detected by qPCR than by microscopy. This adaptation could be explained by it giving the parasites a better chance of long-term transmission from human to mosquito hosts. Along the same line of argument, Culleton suggested recently that it is likely that transmission of *P. malariae* occurs throughout the whole year “at low levels, which would ensure a constant pool of infective mosquitoes throughout the transmission season” (Culleton, Pain and Snounou, 2022).

4.7 High rates of anemia and its causes

With 45% in Gabon and 64% in Cameroon, a high number of the individuals participating in this study were anemic to some degree. The majority of cases of anemia were found in children and adolescents.

There is a multitude of reasons for anemia in populations in Sub-Saharan Africa. Chronic infections like HIV and tuberculosis, but also parasitic infections like malaria, helminths, leishmaniasis and trypanosomiasis can lead to anemia, while especially in the SSA populations there is a predilection for hemoglobinopathies like sickle-cell-anemia, thalassemia and genetic variants like glucose-6-phosphate dehydrogenase (G6PD) deficiency (Crawley, 2004; Langston and Bales, 2021). The groups that are most at risk of suffering from anemia are children and pregnant women. Helminth infections, like schistosomiasis and hookworms, and malaria infections, are among the most common causes in these high-risk groups (Mwangi *et al.*, 2021). Malnutrition also plays a very important role, leading to iron and vitamin B12 deficiencies. It is often related to the educational and socioeconomic status of the affected individual (Ehrhardt *et al.*, 2006). Anemia is a large cause of morbidity especially in young children, as it can “impair cognitive and motor development, growth, immune function and physical work capacity” (Crawley, 2004).

In the course of a malaria infection, there are several mechanisms that can cause anemia. Hemolysis, the destruction of infected and non-infected red blood cells by the spleen and the immune system, is the most important one (White, 2018; Rivera-Correa and Rodriguez, 2020). Additionally, erythropoiesis, the production of red blood cells in the bone marrow, is reduced in malaria infections, so the body is not able to reproduce the destroyed RBCs (Casals-Pascual *et al.*, 2006; Aguilar *et al.*, 2014). This can lead to significant levels of anemia even after the malaria infection has been treated and subsequently cleared by the body.

4.7.1 Higher levels of anemia in Cameroon than in Gabon

The overall higher rates of anemia in Cameroon versus Gabon seem to be found in the larger number of children in Cameroon who have high rates of anemia, and the study population in Cameroon being significantly younger than the one in Gabon, as mentioned above. While the exact burden of helminths is not clear, there are very high rates of malaria infections in children in Cameroon that could contribute to the high number of anemic children.

There could also be indirect factors linked to this difference, however. For instance, the GDP per capita for Gabon was 8,820.35 USD in 2022, while it was 1,563.49 USD for Cameroon (*GDP per capita (current US$), World Bank - Gabon, Cameroon*, 2024).The health expenditure per capita was 234 USD in Gabon and 64 USD in Cameroon in 2021 (*Current health expenditure per capita (current US$), World Bank - Cameroon, Gabon*, 2024). These large differences in economy and health expenditures indicate a better healthcare system and possibly better prevention and access to health care in Gabon, leading to less anemia and less patent malaria infections in the Gabonese population that was sampled for this study.

4.7.2 The burden of P. malariae: Significant impact of Plasmodium malariae infections on anemia rates in children

As the results from this study have shown, there is a small but significant impact of *Plasmodium falciparum* infections on the hemoglobin levels of study participants from all age groups. While the level of anemia is mostly not as severe as it can be in acute infections with *P. falciparum*, these data demonstrate the insidious nature of chronic *Plasmodium* infections and their effects on the host. The association of anemia with symptomatic *P. falciparum* infections is well documented and researched (Price et al., 2001; Ehrhardt et al., 2006; White, 2018).

In recent years however, the impact of chronic asymptomatic infections has been put into the focus of researchers. As in the present study, a number of other studies have demonstrated that not only acute but also chronic infections

with *P. falciparum* have a significant effect on hemoglobin levels, leading to increased risk of anemia and increased overall morbidity (May *et al.*, 2000; Hayuma *et al.*, 2021; Fogang *et al.*, 2022). In pregnant women for instance, chronic low-level *P. falciparum* infection has been linked to maternal anemia, premature births and low birth weight (Cottrell *et al.*, 2015).

For *Plasmodium malariae,* the primary interest of this study, there is a significant impact on hemoglobin mainly for children and adolescents. Up to this day, there are very few studies on the impact of *P. malariae* infections on hemoglobin levels. Two studies from Southern Papua found that in clinically symptomatic *P. malariae* infections, detected by blood smear, the level of hemoglobin was significantly lower than in any other kind of *Plasmodium* infection (Douglas *et al.*, 2013; Langford *et al.*, 2015).

It remains unclear how the very low-density chronic infections cause these rates of anemia. One theory is that a significant part of the *Plasmodium malariae* biomass could be sequestered, not within the circulation, but within the spleen and the bone marrow, as it has been shown for *P. falciparum* and *P. vivax* (Bousema and Drakeley, 2011; Aguilar *et al.*, 2014; Barber *et al.*, 2015; Langford *et al.*, 2015; Kho S *et al.*, 2021; Zhang and Deitsch, 2022). This could, in long-term chronic infections, contribute significantly to the burden of anemia as it can impair the process of erythropoiesis within the bone marrow.

The findings of this study are the first to determine the relevant impact of chronic sub-clinical *Plasmodium malariae* infections on hemoglobin levels in children and adolescents and open up a bigger picture in the fight against malaria. Not only *P. falciparum* and *P. vivax* should be researched and eliminated, but also *P. malariae*, which reveals itself to be much more prevalent and more impactful than previously thought.

Of course, part of the anemia observed in the study participants could as well have been due to some of the comorbidities which can cause anemia in these settings and which have been mentioned above. These serve as confounding factors and are a limitation of this study, as their impact on the levels of anemia of the study participants cannot be determined at this point.

4.8 Implications of *Plasmodium malariae* infections for clinical practice

As has been discussed above, *Plasmodium malariae* is a pathogen that rarely causes severe malaria but tends to form chronic infections. These have been shown to cause a number of pathologies. Most important is certainly the impact on levels of hemoglobin, causing chronic anemia and increased overall morbidity. Furthermore, *P. malariae* can lead to the deposition of immune complexes in the glomeruli of the kidney and can thus cause structural kidney damage through glomerulonephritis (Hendrickse and Adeniyi, 1979; Collins and Jeffery, 2007). The result is a nephrotic syndrome which has been reported to be difficult to treat (Eiam-Ong, 2003; Langford *et al.*, 2015). This kidney damage and nephrotic syndrome occurs most frequently in children (Ehrich and Eke, 2007) who have less acquired immunity against malaria infections than adults. Lastly, chronic *P. malariae* infections can lead to splenomegaly (Vinetz *et al.*, 1998) and, in rare cases, to an overreaction of the immune system and a subsequent hyperreactive malarial splenomegaly, which has been shown to be lethal in 36% of cases within 3 years of the clinical presentation (Chim CS *et al.*, 2004; Leoni *et al.*, 2015).

In the light of the results from this study, it seems that the group most affected by chronic *P. malariae* infections are indeed children and adolescents, who have a higher risk of becoming anemic and possibly suffer renal damage.

To further investigate this, it would be interesting to routinely screen children not only for malaria, but specifically for anemia and proteinuria. Following this, it would be possible to treat *P. malariae* infections in a timely manner, thus avoiding consequences like developmental delays or impaired concentration which can in turn impact educational success.

4.8.1 Clinical implications of mixed infections

The possible interactions of *P. malariae* and *P. falciparum* in mixed infections and their effects have been discussed above. Clinically relevant is probably mostly the notion that *P. malariae* seems to have the potential to attenuate the disease severity of *P. falciparum*. This could lead to more patients with

symptomatic mono-infections consulting health centers, as they would be more symptomatic than individuals harboring chronic mixed infections. This is a suspicion corroborated by Bruce et al. who found, in a cross-sectional study in Malawi in 2008, that in those individuals who had clinical malaria (defined as a temperature of > 37.5°C and parasite density of >5000/µl) there were more *Plasmodium* mono-infections and less mixed infections (Bruce *et al.*, 2008). Whether mixed infections of different *Plasmodium* species predispose to a higher risk of anemia, renal damage and splenomegaly remains unclear.

This opens up the question of whether regular screening-tests for certain parts of the population might make sense from a public health point of view, as we have seen that chronic infections, both with one or several species of *Plasmodium*, can lead to significant morbidity.

This is especially important because malaria infections cannot only be co-occurring with other malaria species, but frequently serve as comorbidities to other major health problems like HIV or tuberculosis infections, increasing the risk of a severe course of disease (Hochman and Kim, 2009; Bijker *et al.*, 2023).

For the groups that are already at risk for anemia through malaria, namely children and pregnant women, a co-infection with hookworms was shown to increase this anemia even further, necessitating an “integrated approach to malaria and helminth control” (Brooker *et al.*, 2007).

It becomes clear again that there are groups that are more at risk from suffering increased morbidities from any kind of co-infection with malaria. These are children and pregnant women who should be specifically targeted by public health programs.

4.9 Factors to consider for the elimination of *P. malariae*

Global eradication of malaria has long been a goal of humankind, and accordingly, the WHO’s Global Malaria Programme published a ‘Framework for malaria elimination’ in 2017. One of the goals of this framework is to “reduce malaria incidence globally compared with 2015” by at least 90% by the year

2030 (*A framework for malaria elimination*, 2017). This is a very ambitious goal, as the elimination of malaria is a complex issue, with 5 *Plasmodium* species infecting humans, “at least 10 intermediate host/reservoir species globally, and more than 40 major anopheline vector species“ (Lover *et al.*, 2018).

Interestingly, the report still identifies *P. falciparum* and *P. vivax* as the most important threats, and mentions, but overall neglects the other *Plasmodium* species that infect humans. While those two pathogens certainly cause more deaths and should indeed be tackled by elimination efforts, it becomes more and more clear that malaria elimination can only happen as a concerted effort against all of the species infecting humans in parallel, as suggested by Lover and colleagues in 2018 (Lover *et al.*, 2018). As it has been shown by the present study, the prevalence of *P. malariae* in Gabon and Cameroon is surprisingly high, and it can well be suspected that this is the case for many other malaria endemic regions of this world. Furthermore, it has recently been suggested that the decrease in *Plasmodium falciparum* infections, brought on by elimination efforts, might lead to the creation of an ecological niche for other species like *Plasmodium malariae* to grow (Yman *et al*., 2019; Oriero *et al*., 2021). Indeed, previous studies in populations with a high prevalence of mixed infections of *P. falciparum* with *P. vivax* have shown a peak in clinical *P. vivax* malaria cases after successful treatment of *P. falciparum* (Looareesuwan *et al.*, 1987; Maitland, Williams and Newbold, 1997). A similar pattern has been observed in Uganda, where an 18-month long longitudinal study of children and their mothers showed a “3-fold rise in *P. malariae* prevalence”, “despite regular artemisinin combination therapy” (Betson et al., 2018).

This means that as the world community progresses on elimination efforts of *P. falciparum* with measures like mass-drug treatment, and most recently the roll-out of vaccines targeting only *P. falciparum* antigens, the prevalence of *Plasmodium malariae* and the other neglected *Plasmodium* species might be on the rise and needs to be monitored.

Another key factor to successful elimination is the question of the sylvatic reservoir of species of *Plasmodium* that can be found within non-human primate

populations around the world. Indeed, there is another *Plasmodium* species, *Plasmodium brasilianum*, known to infect South American primates, which is morphologically indistinguishable from *Plasmodium malariae*. Both parasites can be infectious to primates and humans alike (Lalremruata *et al.*, 2015; Plenderleith *et al.*, 2022). Genetic studies have revealed that the two parasites are genetically almost identical, sharing 99.7% of the same genome (Talundzic *et al.*, 2017), therefore hypothesizing that the two parasites are indeed part of the same species and not two distinct forms of *Plasmodium* (Culleton, Pain and Snounou, 2022). Or, as some authors suggest, they are actually one and the same (Lalremruata *et al.*, 2015; Rutledge, Böhme, *et al.*, 2017). The fact that quartan malaria might indeed be, as suggested in the same study by Lalremruata et al. a “true anthropozoonosis”, easily switching from human to primate host, will make elimination efforts of this almost ubiquitous parasite extremely difficult, as the large non-human, sylvatic reservoir for the parasite will not be accessible to elimination programs. The case of *P. brasilianum* is not the only one, as there are other zoonotic species of *Plasmodium* that have been shown to spill over to humans, like *P. simium* in the Brazilian Amazon (Brasil *et al.*, 2017). The WHO ‘Framework for Malaria Elimination’ does not mention this huge sylvatic malaria reservoir at all, possibly because most studies on anthropozoonoses and zoonotic spillovers are quite recent.

Considering all of these findings, the way towards elimination of malaria is certainly long, and the goals set by the WHO are ambitious, while the 2017 framework is outdated and lacks the full picture of malaria prevalence. It seems of course judicious to focus on the elimination of symptomatic malaria cases and to thus reduce the immense health, social and economic burden of the disease. However, individuals belonging to high-risk groups such as pregnant women and children should not only be treated for symptomatic infections, but also repeatedly screened for subclinical chronic infections with parasites like *Plasmodium malariae*, which can lead to clinically relevant anemia and kidney injury as we have seen above. Finally, all of the malaria reservoirs have to be recognized in the light of the new prevalence findings of the present study and

other recent studies, and a concertation of experts for the development of new elimination strategies seems crucial to advance in the fight against malaria.

4.10 Remaining questions about *Plasmodium malariae*

As the research community is just getting started on learning more about the less studied species of *Plasmodium* like *P. malariae*, many questions remain.

Open questions include how *Plasmodium malariae* parasites enter liver cells and how they choose which red blood cells to infect. A major burden to better study these mechanisms certainly is the fact that it remains very difficult to put *P. malariae* into a stable and continuous in vitro culture (Culleton, Pain and Snounou, 2022).

In the past years, there has been an increasing number of reports of *P. malariae* relapsing in the months and years after a primary infection had been treated (Franken *et al.*, 2012; Rutledge, Marr, *et al.*, 2017). It is unclear which mechanisms are underlying to this form of relapse, as it had previously been established that *P. malariae* does not form dormant liver stages like *P. vivax* or *P. ovale*. Possible reasons for this will be discussed further on.

4.10.1 The tropism of P. malariae merozoites remains unclear

The preference of *Plasmodium malariae* for aging red blood cells which has been purported widely in the scientific literature, traces, according to McKenzie et. al “back to a single, problematic study by Kitchen (1939), which, he concluded, ‘‘raises the question of whether the predilection of *P. malariae* may not be quite the opposite of that of *P. vivax.*’” (Kitchen, 1939; McKenzie, Jeffery and Collins, 2001). Indeed, a year before Kitchen’s research was published, Hegner had postulated a tropism of *Plasmodium malariae* for mature reticulocytes (Hegner, 1938). Since then there has been a single study, conducted by Chwatt in 1948, on 16 *P. malariae* cases in Nigeria of which 5 contained infected reticulocytes and all cases infected mature red blood cells.

Based on these results, the author argued for a tropism of *Plasmodium malariae* for ageing erythrocytes (Chwatt, 1948) .

Ever since 1948 there have been no other studies that have investigated the exact tropism of *Plasmodium malariae*. However, the selectivity for aging red blood cells has been widely acknowledged in textbooks without clear evicence for this. Simpson et. al already questioned this in 1999, stating that this kind of erythrocyte "selectivity could not account for the range of parasitemias observed in their study" (Simpson *et al.*, 1999, McKenzie, Jeffery and Collins, 2001). They suggest that there must be other factors that limit uncontrolled parasite growth in *P. malariae* infection (Simpson *et al.*, 1999). As Hang et. al describe, it has been difficult in the past to establish stable *Plasmodium malariae* cultures in vitro and "the difficulties in establishing continuous parasite culture hinder the identification of the receptor-ligand interactions involved in the merozoite invasion mechanism" (Hang *et al.*, 2021).

It has also been suggested that the infection of RBCs by *P. malariae* merozoites might lead to the faster maturaton of the infected red blood cells (Hang *et al.*, 2021), therefore creating the microscopic impression of *P. malariae* infecting older erythrocytes. Lastly, in 2022 Culleton et al. proposed that *P. malariae* might in fact have a tropism for reticulocytes, which would explain the difficulty establishing a continuous in vitro culture of this parasite (Culleton, Pain and Snounou, 2022).

4.10.2 Chronically low parasitemia and late stage relapses of P. malariae infections

The parasitemia of *P. malariae* often does not reach the high amounts that *P. falciparum* or *P. vivax* reach. This has led to an underestimation of this malaria parasite in the past (Snounou, Pinheiro, *et al.*, 1993; Zhou *et al.*, 1998; McKenzie, Jeffery and Collins, 2001). The chronically low parasitemia of *P. malariae* has been attributed to the longer maturation period in the liver, the longer cycle of 72 hours to release new merozoites and the purported preference of *P. malariae* parasites for older red blood cells (Collins and Jeffery,

2007) which might indeed be an incorrect assumption as discussed above. The number of merozoites released by *P. malariae*, with 6-14 merozoites released per infected red blood cell every 72h, is relatively low compared to other *Plasmodium* species (Collins and Jeffery, 2007; Hang et al., 2021). This reduced growth rate as compared to other kinds of *Plasmodium* infections leads to a different balance of parasite growth versus clearance by the hosts' immune system and could therefore partially explain the low level parasitemia of *P. malariae*.

Yet we still don't know why and how *Plasmodium malariae* can persist for years, sometimes for life in its human host. No dormant liver stages, hypnozoites, have been found so far. So far, the persistence of *Plasmodium malariae* has been attributed to a continuous erythrocytic cycle in the blood. There have however, been multiple reports of patients who have either been treated for malaria (with *P. falciparum* or *P. malariae*) and had a recurring infection of *P. malariae* or developed a *P. malariae* infection decades after traveling to an endemic region (Siala *et al.*, 2005; Müller-Stöver *et al.*, 2008; Hedelius *et al.*, 2011; Smith *et al.*, 2011; Franken *et al.*, 2012; Rutledge, Marr, *et al.*, 2017; Grande *et al.*, 2019; Tournoy *et al.*, 2022; Rizzo *et al.*, 2023; Utpat *et al.*, 2024).

A possible reason for treatment failure and recrudescence might have been the long asexual life cycle of 72h, for which a standardized treatment with artemether lumefantrine for 3 days might not have been enough, as several researchers have suggested (Smith *et al.*, 2011; Rutledge, Marr, *et al.*, 2017). Another reason might be the very long prepatent period of *P. malariae* during which the parasite will not be susceptible to treatment, as most current treatment options target the blood-stages of *Plasmodium* species. This applies especially to the returning traveler who is treated for acute *P. falciparum* malaria after returning from an endemic region and has a secondary infection with *P. malariae* which has not reached the erythrocytic stage yet and therefore is not covered by the treatment of *P. falciparum*.

Recent studies exploring chronic, low-level *Plasmodium falciparum* infections propose another intriguing mechanism for these kinds of infection. *P. falciparum*

is a parasite that causes the infected red blood cells to stiffen to a certain degree, leading to their elimination by the spleen. To avoid this passage through the human spleen and therefore avoid elimination, *P. falciparum* expresses a surface protein on the RBC, called *Plasmodium falciparum* erythrocyte membrane protein one (PfEMP1), that allows the infected erythrocytes to adhere to the walls of small capillaries (Baruch *et al.*, 1995). This mechanism contributes most to why *P. falciparum* infections can become so severe, as they sometimes lead to occlusion of the small blood vessels of different organs by cytoadherence of infected RBCs. This leads to local inflammation and hypoxia and can cause severe organ damage. The problem for the parasite is that the hosts' immune system rapidly develops antibodies against the expressed surface protein and can, again, eliminate the infected red blood cells. To avoid this, *P. falciparum* is capable of changing the surface protein that helps it to cytoadhere (Smith *et al.*, 1995; Staalsoe *et al.*, 2002). These changes of expressed surface antigen, to which no antibodies have been formed so far, have been linked to cyclical bouts of fever and disease in the active infection with *Plasmodium falciparum*, as the switch to a protein that is not yet recognized by the hosts' immune system allows the parasite to replicate faster and cause more pathology (Miller *et al.*, 2002; Zhang and Deitsch, 2022).

Recent studies suggest that in order to persist in chronic infections, *P. falciparum* can downregulate the expression of PfEMP1. This leads to less cytoadherence and therefore an increased clearance of infected red blood cells by the spleen, leading to a low level of parasitemia in the hosts' body which is invisible to existing antibodies (Andrade *et al.*, 2020; Zhang and Deitsch, 2022). This mechanism has been proposed to be the way that *P. falciparum* survives within the host during the dry season, waiting for the rainy season with more vectors to be transmitted again (Bejon, 2020). It has also been shown that some cells, especially those containing the sexual forms of the parasite, gametocytes, but also some ring stages, can sequester in the bone marrow of the host, leading to a reservoir of parasites outside of the blood circulation (Bousema and Drakeley, 2011; Aguilar *et al.*, 2014; Zhang and Deitsch, 2022). In 2021, Kho and colleagues proved that in asymptomatic individuals, a large part of the

biomass of *P. falciparum* and *P. vivax* is located in the spleen, as opposed to the circulation (Kho S *et al.*, 2021).

How and if there is a mechanism of cytoadherence in *P. malariae* and whether it sequesters in bone marrow and spleen remains unclear and has not been studied yet. Cytoadherence has, however, been shown to exist for *P. vivax* and *P. knowlesi*, parasites which can both cause severe malaria through this mechanism (Carvalho *et al.*, 2010; Fatih *et al.*, 2012; Lee *et al.*, 2022). Studies on this phenomenon in *P. malariae* will certainly be helpful to better understand why it occurs most often as a clinically less severe, chronic infection. It would seem logical for *P. malariae* to have a less strong mechanism of cytoadherence, leading to the chronic low level parasitemia that has been discussed above. Furthermore, adding to the discussion point of interaction in mixed infections, it would be interesting to find out whether the presence of *P. malariae* in a co-infection with *P. falciparum* might impact or even reduce the expression of PfEMP1 in *Plasmodium falciparum* and through this mechanism possibly make it less virulent.

On the other hand, while it was previously thought that *Plasmodium malariae* does not form dormant liver stages, this has been challenged by Franken and colleagues who reported that a patient was treated for *P. falciparum* malaria and relapsed 4 months later with *P. malariae* (Franken *et al.*, 2012).

Richter et al. propose that all *Plasmodium* species might cause relapses, and that the much-cited *P. vivax* is just the parasite that relapses the most frequently (Richter *et al.*, 2016). Culleton et al. further open up this discussion by invoking two theories from the 20th century that haven't been investigated further for a long time: the notion of a secondary erythrocytic cycle, which means the re-infection of hepatocytes by circulating merozoites, and the existence of quiescent erythrocytic forms, for example in the lymphatic system (Culleton, Pain and Snounou, 2022). Both theories seem, in theory, to be biologically possible, and further studies are needed to investigate the mechanism of *P. malariae* relapse.

Lastly, it has been suggested that some artemisinin-based combination therapies do not fully eliminate *P. malariae* infections. Several schoolchildren in Ghana and Uganda were tested to be *P. malariae* positive after a full course of dihydroartemisinin-piperaquine and artemether-lumefantrine, respectively (Dinko et al., 2013; Betson et al., 2014; Sutherland, 2016). As Sutherland pointed out recently, “it is important to remember that coendemicity with *P. falciparum* and *P. vivax* across its geographical range means that populations of *P. malariae* will have experienced substantial drug pressure since the widespread deployment of quinine began in the 1820s” (Sutherland, 2020).

In conclusion, additionally to parasite-inherent factors like the life cycle, possible extra-circulatory reservoirs, and mechanisms of cytoadherence, there might be a notion of drug-resistance adding to the issue of chronic, relapsing *P. malariae* infections. Lastly, the interactions between the parasites and the hosts’ immune system certainly also play an important role, which remains poorly understood so far.

4.11 Limitations

4.11.1 Non-specific Plasmodium falciparum primers and their effects

It was already explained in the results section, that a number of *P. falciparum* PCR curves, while being clearly positive, did not rise high like the control sample curve, and seemed to be “flattened”.

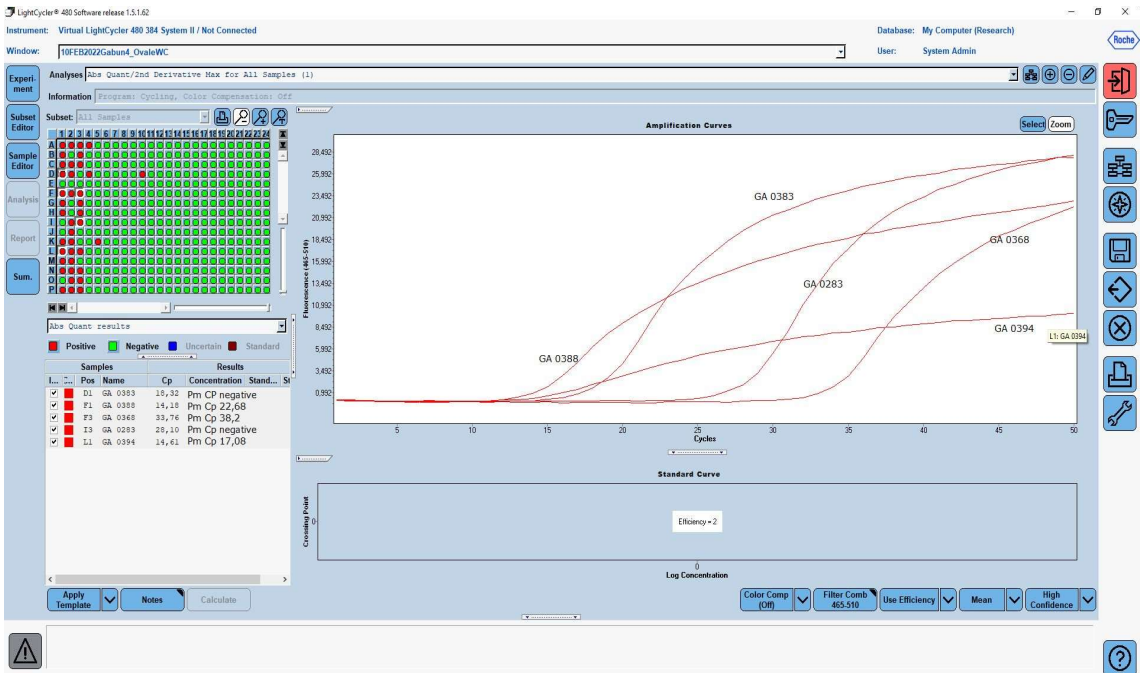

Figure 70 – Example of PCR curves from Gabon samples showing flattened and regular curves in positive samples

As a reason for this issue, it was suspected that the non-specificity of the primers of the *Plasmodium falciparum* assay that were used might be the cause. Indeed, the *Plasmodium falciparum* primers used in this study are primers that are non-specific to *P. falciparum* and bind to all *Plasmodium* DNA. While the probe that was used is highly specific for *P. falciparum*, and will only amplify *P. falciparum* RNA/DNA, the non-specific primers might cause this problem in samples with mixed infections, and lead to less material being amplified and therefore flattened curves. As this issue was observed in a number of samples, both in clearly detected *P.falciparum/P.malariae* co-infections and ‘*P. falciparum*-monoinfections’, which could very well also be mixed infections, for instance with *Plasmodium ovale*, it was decided to

establish a new *P. falciparum* assay for the analysis of all other, up to this point non-treated, samples.

Repetition-qPCR trials with a new *P. falciparum* assay that were performed after the data for this study was collected suggest that the flattening of the curve makes indeed no difference for the resulting Ct value, as the initial rise, which counts for the Ct value, is the same in both the initially performed qPCRs and in the new qPCRs with a more specific *P. falciparum* primer.

The only samples that were excluded for this study were the ones with curves that did rise a little, suggesting some kind of infection, but were so flat that any kind of inclination and therefore Ct value measurement was impossible to obtain. These samples will be repeated with the new qPCR assay in the future and included for further analyses into the data that is yet to be analysed.

4.11.2 Variations in sample quality in PaxGene® tubes

The correct use of the PaxGene® tubes is essential for the extraction process to yield high-quality RNA samples. In some of the frozen PaxGene® tubes, variations of blood volume were detected. Indeed, some tubes were not filled with the required amount of 2.5mL of venous blood. This might have led to the extraction of less RNA and less genetic material for the qPCR, possibly affecting its sensitivity. However, as the qPCR has been shown to be extremely sensitive, detecting a parasitemia of as low as 5-10 P. malariae parasites per mL of blood (Borrmann 2021, unpublished data), this variation in sample quality was accepted for the already shipped samples. The field samplers from the respective countries were subsequently again informed about the importance of the correct sampling volume for sample quality. This led to an increase in samples which contained the right amount of venous blood.

Another limitation regarding sample quality is that the samples were collected in different countries by many different samplers. It is, retrospectively, difficult to establish whether the sampling protocol was followed systematically in the

same way throughout the whole sampling process or whether there might have been variations in sample collection and treatment.

4.11.3 Further limitations

As the qPCR was conducted as an epidemiological tool, only a single well was used per sample. This means that there is no way of saying whether the Ct values that were obtained for every sample are exact. At this point in time, because there are no normalized curves yet for the samples from Gabon and Cameroon, it is not important to have the most exact Ct value. However, should there be a more detailed analysis on the exact parasitemia of the collected samples, at least 3 PCR-wells should be used to get robust Ct value data. Furthermore, the fact that no normalized curves exist for the *Plasmodium malariae* samples yet means that the qPCR Ct values cannot be interpreted regarding exact parasitemia. This study can only conclude to whether the individual parasitemia was higher or lower in a certain sample.

As already discussed above, there was a very low case number of *P. malariae* mono-infections in Cameroon which makes it difficult to interpret correlations and statistical tests for these samples as they have a high chance of being skewed.

Furthermore, as only the samples collected during the dry season in Cameroon were sent to Tübingen so far, it is at this point not possible to analyze seasonal data for *Plasmodium* infections in Cameroon. Due to the Nagoya protocol, many further samples remain in Cameroon. It will hopefully be possible in the future, once these samples have been analyzed by our Cameroonian colleagues or sent to Tübingen, to get better statistical analyses both for seasonality aspects and *Plasmodium malariae* mono-infections.

Finally, our data remains cross-sectional: it is merely an “instant photography” of the epidemiological situation in one moment in time. It does not tell us anything about length and development of infection or disease over time.

It remains as Leonard Bruce-Chwatt already framed it over 50 years ago: “The usual single, cross-section malariometric survey gives about as much information of the natural history of malaria in an endemic area as a few ‘frames’ taken out at random from a reel of film would of its plot” (Bruce-Chwatt, 1963).

4.12 Outlook on avenues for future research

First and most importantly, these results only represent the first data that was collected as part of the COMAL work package 1. Samples from Benin, Cameroon and Congo remain to be fully extracted and analyzed. With these data, there will be an even better idea of the prevalence of *Plasmodium malariae* in Western Africa.

Once these data are available, new insights into the effects of *P. malariae* on hemoglobin levels in children as well as the interaction of *P. malariae* and *P. falciparum* in mixed infections will be gained.

In the future it will be very important to detect cases of *Plasmodium malariae* and observe them over time. Longitudinal studies are needed to better understand the course of disease and to observe what biological and immunological effects there are in mixed infections with other *Plasmodium* species, mechanisms that we can only speculate about at this point.

Additionally, seeing how high the real prevalence of *Plasmodium malariae* is in Gabon and Cameroon, future studies should study the prevalence of all *Plasmodium* species. Especially the study of *Plasmodium ovale* seems important, as it remains largely understudied and therefore underestimated. In the past it has been difficult to determine the true prevalence of *P. ovale*, as this malaria parasite species tends to have a lower parasitemia, and often a patent period of less than two weeks, making it difficult to detect by light microscopy (Mueller, Zimmerman and Reeder, 2007).

It might furthermore be interesting to test for *Plasmodium vivax*. This malaria pathogen has been found in Africa in multiple studies, even though the majority

of the African population does not have the Duffy antigen receptor for chemokines (DARC) that *P. vivax* needs to infect red blood cells (Culleton and Carter, 2012; Twohig *et al.*, 2019). Indeed, Liu et. al have shown that all existing *Plasmodium vivax* strains are related to one common ancestor who escaped out of Africa and that this common ancestor has led to the natural selection of the Duffy-negative mutation that is now very common in sub-Saharan African populations (Liu et al., 2014).

To better understanding the species of *Plasmodium malariae* and its biology an interesting tool for future analyses could be next generation sequencing, which has not been applied widely to this pathogen yet. In-depth genetic analyses of *P. malariae* and its vectors is already planned as part of the COMAL work package 3.

Lastly, the development of methods to put *Plasmodium malariae* into a stable in vitro culture would certainly advance the research on this neglected malaria parasite and would pave the way to demystifying relevant factors regarding its life cycle and its interaction with human cells.

4.13 Conclusion: The high burden of *P. malariae* in Gabon and Cameroon

This study has shown that there is a high number of chronic, low-parasitemia infections with *Plasmodium malariae* in Gabon and Cameroon which mostly occur as a mixed infections with *P. falciparum*. As chronic parasitemia can often be very low, this study shows the importance of using highly sensitive detection methods for epidemiological research, to get an accurate picture of the actual prevalence of relevant *Plasmodium* species.

Most affected by *P. falciparum* and *P. malariae* were school children and adolescents. Together with the relevant impact of *P. malariae* infections on the hemoglobin levels in children, the data from this study point toward the high, so far underestimated burden that constitutes the infection with this parasite. This burden is especially high for children and adolescents, who can be severely

affected by the morbidity related to chronic *P. malariae* infections, which may ultimately lead to reduced educational and economic success.

Furthermore, both the data from Gabon and Cameroon show that in the general, asymptomatic population, there is a large amount of chronic, low-parasitemia infections which constitute a huge transmission reservoir for *P. falciparum* and *P. malariae*. This needs to be taken into account for further actions to reduce the burden of malaria on the societies and economies of these countries.

In the light of these findings, an integrated public health approach, with updated elimination strategies focusing on all prevalent species of *Plasmodia*, needs to be developed. Future research is needed to shed light on the cryptic biology, immunological processes and parasite-vector-interactions involving *P. malariae* which have not yet been well understood, but are paramount for developing elimination strategies.

5 Summary

5.1 English summary

Background

Malaria remains one of the deadliest infectious diseases in the world causing 250 million cases annually. Elimination efforts, focusing mostly on the species with highest morbidity and mortality, *P. falciparum* and *P. vivax*, have been stalling for some years. Scientific evidence of the last decades points to the importance of the other human malaria pathogens. One of these pathogens, *Plasmodium malariae*, causes mostly chronic infections and has not been studied intensively so far. The COMAL study is a comprehensive study package aiming at investigating the cryptic burden of *Plasmodium malariae*. This dissertation focuses on elucidating the epidemiology and the burden of this parasite in Gabon and Cameroon and sheds light on possible interactions of *P. malariae* and *P. falciparum* in mixed infections.

Methods

As a part of the COMAL work package 1 household based cross-sectional studies were conducted in Gabon and Cameroon, in both dry and rainy seasons. During these surveys, venous blood, mosquitoes and anthropometric data were collected from 1,584 clinically asymptomatic individuals. After sampling, microscopy was performed on all blood samples to identify possible patent *Plasmodium* infections. At the Institute of Tropical Medicine in Tübingen, RNA was extracted from the blood collected into PaxGene® tubes using a magnetic-beads-based approach. On the eluate a singleplex qRT-PCR was run, to detect *P. falciparum* and *P. malariae* in the samples at a sensitivity of 5-10 parasites per mL. 1,557 samples were included into the final analysis.

Results

This study has three main findings concerning *Plasmodium malariae*.

The first and most important finding is the new knowledge acquired about the epidemiological characteristics of *P. malariae* in Gabon and Cameroon. Highly sensitive qPCR revealed much higher prevalence rates than were detected by light microscopy, indicating the presence of a large number of chronic infections with low-level submicroscopic parasitemia. In blood samples from Gabon this study found 8% *P. malariae* mono-infections and 30% *P. falciparum – P. malariae* co-infections. 47% of individuals showed a *P. falciparum* mono-infection, and only 16% of samples were tested negative. In Cameroon, 4% of all samples were identified as *P. malariae* mono-infections and 48% as *P. falciparum – P. malariae* co-infections. Almost half of all samples, 45%, were shown to be *P. falciparum* mono-infections, and only 4% of the samples were found to be negative. In both countries *Plasmodium* prevalence was highest in adolescents aged 12-19 years. In Gabon, *P. malariae* mono-infections occurred significantly more often in individuals older than 60 years. Overall, the epidemiological situations in the two countries differed from one another: in Cameroon the sampled population was found to have a higher prevalence of malaria infections with more microscopically detectable yet subclinical infections and a clear correlation of age with Ct values, pointing towards rising levels of acquired immunity with age. In Gabon the prevalence was slightly lower than in Cameroon, with more submicroscopic infections and less impact of age on Ct values.

The second main finding of this study is the impact of *Plasmodium malariae* infections on hemoglobin levels in children and adolescents. Indeed, hemoglobin levels in children infected with *P. malariae* were significantly reduced compared to children not infected with *P. malariae*, adding a clinical significance to the previously unknown high burden of *Plasmodium malariae*.

Thirdly, in mixed infections of the two parasites, higher median *P. malariae* Ct values were found as compared to mono-infections. This indicates a lower

parasitemia in mixed infections and might point towards a possible interaction between *P. malariae* and *P. falciparum*.

Conclusion

This study reveals the large amount of subclinical and submicroscopic *P. malariae* infections in Gabon and Cameroon. With a relevant impact on hemoglobin levels in children, this pathogen has thus proven that it constitutes a high burden in affected countries that has to be taken seriously. It is time to stop neglecting and to start studying *Plasmodium malariae* more intensively. For any success in the elimination efforts against malaria it is of utmost importance to further study this pathogen, its biology and its vectors. Finally, the overall large amount of subclinical chronic infections of both *P. malariae* and *P. falciparum* constitutes an enormous reservoir of transmission for malaria and needs to be taken into account for future elimination strategies.

5.2 German Summary: Zusammenfassung

Hintergrund

Malaria ist mit 250 Millionen Fällen jährlich eine der tödlichsten Infektionskrankheiten der Welt. Die Bemühungen zur Ausrottung des Malaria-Parasiten, die bisher hauptsächlich auf die Arten mit der höchsten Morbidität und Mortalität, *P. falciparum* und *P. vivax*, konzentriert waren, geraten seit einigen Jahren ins Stocken. Die Forschungsergebnisse der letzten Jahrzehnte zeigen jedoch, wie wichtig die Erforschung weiterer Malariaerreger ist. Einer dieser Erreger, *Plasmodium malariae*, verursacht meist chronische Infektionen und war bislang kaum Gegenstand von Untersuchungen. In einem umfassenden Studienpaket untersucht nun die COMAL-Studie die bisher unerkannte Krankheitslast, die durch *Plasmodium malariae* Infektionen entsteht.

Die vorliegende Arbeit konzentriert sich auf die Untersuchung der Epidemiologie dieses Parasiten in Gabun und Kamerun und analysiert mögliche Interaktionen von *P. malariae* und *P. falciparum* bei Mischinfektionen.

Methoden

Im Rahmen des COMAL-Arbeitspakets 1 wurden in Gabun und Kamerun haushaltsbezogene Querschnittsstudien durchgeführt, sowohl in der Trocken- als auch in der Regenzeit. Bei diesen Erhebungen wurden venöses Blut, Stechmücken und anthropometrische Daten von 1.584 klinisch beschwerdefreien Personen gesammelt. Nach der Probeentnahme wurden alle Blutproben mikroskopisch untersucht, um mögliche *Plasmodium*-Infektionen zu identifizieren. Im Institut für Tropenmedizin in Tübingen wurde die RNA aus den in PaxGene®-Röhrchen abgenommenen Blutproben mit Hilfe eines auf Magnetkügelchen basierenden Verfahrens extrahiert. Mit dem Eluat wurde eine Single-Plex-qRT-PCR durchgeführt, um *P. falciparum* und *P. malariae* in den Proben mit einer Sensitivität von 5-10 Parasiten pro mL nachzuweisen. 1.557 Proben wurden in die finale Analyse aufgenommen.

Ergebnisse

In dieser Studie konnten drei wesentliche Erkenntnisse über *Plasmodium malariae* herausgearbeitet werden.

Das erste und wichtigste Ergebnis sind neue Erkenntnisse zur epidemiologischen Situation von *P. malariae* in Gabun und Kamerun. Mit der Methode der hochsensitiven qPCR ergaben sich deutlich höhere Prävalenzraten des Parasiten als durch Mikroskopie. Dies weist auf die Präsenz einer großen Anzahl chronischer Infektionen mit geringer und submikroskopischer Parasitämie hin. In Gabun wurden unter den Studienteilnehmern 8 % *P. malariae*-Monoinfektionen und 30 % *P. falciparum*-*P. malariae*-Koinfektionen festgestellt. 47 % wiesen eine *P. falciparum*-Monoinfektion auf, und nur 16 % der Proben wurden negativ getestet. In

Kamerun wurden 4 % aller Proben als *P. malariae*-Monoinfektionen und 48 % als *P. falciparum* - *P. malariae*-Koinfektionen identifiziert. Fast die Hälfte aller Proben, 45 %, erwiesen sich als *P. falciparum*-Monoinfektion, und nur 4 % der Proben waren in der qPCR negativ. In beiden Ländern war die *Plasmodium*-Prävalenz bei Jugendlichen im Alter von 12-19 Jahren am höchsten. In Gabun traten zudem *P. malariae*-Monoinfektionen signifikant häufiger bei über 60-Jährigen auf. Insgesamt war die epidemiologische Situation in den beiden Ländern unterschiedlich: In Kamerun wies die untersuchte Bevölkerung eine höhere Prävalenz von Malaria-Infektionen auf. Diese waren häufiger mikroskopisch nachweisbar, blieben jedoch subklinisch und wiesen eine deutliche Korrelation zwischen Alter und Ct-Werten auf. Dies deutet auf eine mit dem Alter zunehmende erworbene Immunität hin. In Gabun war die Prävalenz etwas niedriger als in Kamerun, mit mehr submikroskopischen Infektionen und einem geringeren Einfluss des Alters auf die festgestellten Ct-Werte.

Das zweite wichtige Ergebnis dieser Studie ist die Auswirkung von *Plasmodium malariae*-Infektionen auf den Hämoglobinwert bei Kindern und Jugendlichen. Die Hämoglobinwerte bei Kindern, die mit *P. malariae* infiziert waren, waren im Vergleich zu Kindern, die nicht mit *P. malariae* infiziert waren, statistisch signifikant reduziert. Dies verleiht dem bisher wenig untersuchten Parasiten *Plasmodium malariae* eine neue klinische Bedeutung.

Drittens wurden bei Mischinfektionen mit den beiden Parasiten im Vergleich zu Monoinfektionen höhere mediane *P. malariae* Ct-Werte festgestellt. Dies deutet auf eine geringere Parasitämie bei Mischinfektionen hin und könnte ein Hinweis auf eine mögliche Interaktion zwischen *P. malariae* und *P. falciparum* sein.

Schlussfolgerung

Diese Studie verdeutlicht die große Zahl subklinischer und submikroskopischer *P. malariae*-Infektionen in Gabun und Kamerun. *Plasmodium malariae*, ein Erreger der Hämoglobinwerte bei Kindern signifikant reduziert, stellt in den betroffenen Ländern eine größere Belastung dar als bisher angenommen. Es ist

an der Zeit, *Plasmodium malariae* zum Gegenstand weiterer Studien zu machen. Um wirkungsvollere Strategien zur Eliminierung von Malaria zu entwickeln, ist es von größter Bedeutung diesen Erreger, seine Biologie und seine Vektoren eingehender zu untersuchen. Darüber hinaus stellt die große Zahl subklinischer chronischer Infektionen mit *P. malariae* und *P. falciparum* auch ein enormes Übertragungsreservoir für Malaria dar, das bei künftigen Eliminierungsstrategien berücksichtigt werden sollte.

6 References

A framework for malaria elimination (2017). Geneva. Available at: https://www.who.int/publications/i/item/9789241511988 (Accessed: 13 June 2024).

Abdulraheem, M.A. *et al.* (2022) 'High prevalence of Plasmodium malariae and Plasmodium ovale in co-infections with Plasmodium falciparum in asymptomatic malaria parasite carriers in southwestern Nigeria', *International Journal for Parasitology*, 52(1), pp. 23–33. Available at: https://doi.org/10.1016/j.ijpara.2021.06.003.

Adams, M. *et al.* (2015) 'An ultrasensitive reverse transcription polymerase chain reaction assay to detect asymptomatic low-density Plasmodium falciparum and Plasmodium vivax infections in small volume blood samples', *Malaria Journal*, 14(1), pp. 1–5. Available at: https://doi.org/10.1186/s12936-015-1038-z.

Agonhossou, R. *et al.* (2022) 'Surveillance of Plasmodium malariae infection among inhabitants of rural areas in Ouidah–Kpomasse–Tori Bossito health district, Benin', *Parasitology Research*, 121(1), pp. 275–286. Available at: https://doi.org/10.1007/s00436-021-07398-z.

Aguilar, R. *et al.* (2014) 'Severity of anaemia is associated with bone marrow haemozoin in children exposed to Plasmodium falciparum', *British journal of haematology*, 164(6), p. 877—887. Available at: https://doi.org/10.1111/bjh.12716.

Andolina, C. *et al.* (2021) 'Sources of persistent malaria transmission in a setting with effective malaria control in eastern Uganda: a longitudinal, observational cohort study', *The Lancet Infectious Diseases*, 21(11), pp. 1568–1578. Available at: https://doi.org/10.1016/S1473-3099(21)00072-4.

Andrade, C.M. *et al.* (2020) 'Increased circulation time of Plasmodium falciparum underlies persistent asymptomatic infection in the dry season', *Nature Medicine*, 26(12), pp. 1929–1940. Available at: https://doi.org/10.1038/s41591-020-1084-0.

Ashley, E.A. and White, N.J. (2014) 'The duration of Plasmodium falciparum infections', *Malaria Journal*, 13(1). Available at: https://doi.org/10.1186/1475-2875-13-500.

Barber, B.E. *et al.* (2015) 'Parasite Biomass-Related Inflammation, Endothelial Activation, Microvascular Dysfunction and Disease Severity in Vivax Malaria', *PLOS Pathogens*, 11(1), pp. e1004558-. Available at: https://doi.org/10.1371/journal.ppat.1004558.

Baruch, D.I. *et al.* (1995) *Cloning the P. falciparum Gene Encoding PfEMP1, a Malarial Variant Antigen and Adherence Receptor on the Surface of Parasitized Human Erythrocytes*.

Battle, K.E. *et al.* (2019) ‘Mapping the global endemicity and clinical burden of Plasmodium vivax, 2000–17: a spatial and temporal modelling study’, *The Lancet*, 394(10195), pp. 332–343. Available at: https://doi.org/10.1016/S0140-6736(19)31096-7.

Bejon, P. (2020) ‘Malaria parasites hide in plain sight in the dry season’, *Nature Medicine*, 26(12), pp. 1816–1818. Available at: https://doi.org/10.1038/s41591-020-01171-y.

Berzosa, P. *et al.* (2018) ‘Comparison of three diagnostic methods (microscopy, RDT, and PCR) for the detection of malaria parasites in representative samples from Equatorial Guinea’, *Malaria Journal*, 17(1). Available at: https://doi.org/10.1186/s12936-018-2481-4.

Betson, M. *et al.* (2014) ‘Detection of persistent Plasmodium spp. infections in Ugandan children after artemether-lumefantrine treatment’, *Parasitology*, 141(14), pp. 1880–1890. Available at: https://doi.org/10.1017/S003118201400033X.

Betson, M. *et al.* (2018) ‘Emergence of Nonfalciparum Plasmodium Infection Despite Regular Artemisinin Combination Therapy in an 18-Month Longitudinal Study of Ugandan Children and Their Mothers’, in *Journal of Infectious Diseases*. Oxford University Press, pp. 1099–1109. Available at: https://doi.org/10.1093/infdis/jix686.

Bijker, E. *et al.* (2023) ‘Malaria and tuberculosis co-infection—a review’, *Oxford Open Immunology*, 4. Available at: https://doi.org/10.1093/oxfimm/iqad008.

Black, J. *et al.* (1994) ‘Mixed Infections with Plasmodium falciparum and P malariae and fever In malaria’, *The Lancet*, 343, p. 1095.

Bousema, J.T. *et al.* (2008) *Increased Plasmodium falciparum Gametocyte Production in Mixed Infections with P. malariae*. Available at: www.controlled-trials.com/ISRCTN31291803.

Bousema, T. and Drakeley, C. (2011) ‘Epidemiology and infectivity of Plasmodium falciparum and Plasmodium vivax gametocytes in relation to malaria control and elimination’, *Clinical Microbiology Reviews*, pp. 377–410. Available at: https://doi.org/10.1128/CMR.00051-10.

Boussougou-Sambe, S.T. *et al.* (2022) ‘Assessment of malaria transmission intensity and insecticide resistance mechanisms in three rural areas of the Moyen Ogooué Province of Gabon’, *Parasites and Vectors*, 15(1), pp. 1–13. Available at: https://doi.org/10.1186/s13071-022-05320-9.

Brasil, P. *et al.* (2017) ‘Outbreak of human malaria caused by Plasmodium simium in the Atlantic Forest in Rio de Janeiro: a molecular epidemiological investigation’, *The Lancet Global Health*, 5(10), pp. e1038–e1046. Available at: https://doi.org/10.1016/S2214-109X(17)30333-9.

Brooker, S. *et al.* (2007) ‘Epidemiology of plasmodium-helminth co-infection in Africa: populations at risk, potential impact on anemia, and prospects for combining control.’, *The American journal of tropical medicine and hygiene*, 77

6 Suppl, pp. 88–98. Available at: https://api.semanticscholar.org/CorpusID:1398859.

Bruce, M.C. *et al.* (2008) 'Effect of transmission setting and mixed species infections on clinical measures of malaria in Malawi', *PLoS ONE*, 3(7). Available at: https://doi.org/10.1371/journal.pone.0002775.

Bruce-Chwatt, L.J. (1963) 'A Longitudinal Survey of Natural Malaria Infection in a Group of West African Adults. Part II.', *West African Medical Journal*, 12, pp. 199–217.

Cameroon Population (2024) *Worldometer*. Available at: https://www.worldometers.info/world-population/cameroon-population/ (Accessed: 10 June 2024).

Carvalho, B.O. *et al.* (2010) 'On the Cytoadhesion of Plasmodium vivax–Infected Erythrocytes', *The Journal of Infectious Diseases*, 202(4), pp. 638–647. Available at: https://doi.org/10.1086/654815.

Casals-Pascual, C. *et al.* (2006) 'Suppression of erythropoiesis in malarial anemia is associated with hemozoin', *Blood*, 108, pp. 2569–2577. Available at: https://doi.org/10.1182/blood-2006-05-018697.

Chai, V. *et al.* (2005) 'Optimization of the PAXgene^{TM} blood RNA extraction system for gene expression analysis of clinical samples', *Journal of Clinical Laboratory Analysis*, 19(5), pp. 182–188. Available at: https://doi.org/10.1002/jcla.20075.

Chang, H.H. *et al.* (2016) 'Persistence of Plasmodium falciparum parasitemia after artemisinin combination therapy: Evidence from a randomized trial in Uganda', *Scientific Reports*, 6(October 2015), pp. 1–8. Available at: https://doi.org/10.1038/srep26330.

Chwatt, L.J. (1948) 'Infection of Reticulocytes by Plasmodium Falciparum and Plasmodium Malariae in Hyperendemic Indigenous Malaria', *Annals of Tropical Medicine & Parasitology*, 42(1), pp. 101–112. Available at: https://doi.org/10.1080/00034983.1948.11685353.

Collins, W.E. and Jeffery, G.M. (1999) 'A Retrospective Examination of Sporozoite- and Trophozoite-Induced Infections with Plasmodium Falciparum in Patients Previously Infected with Heterologous Species of Plasmodium: Effect on Development of Parasitologic and Clinical Immunity', *The American Society of Tropical Medicine and Hygiene*, 61(1_Supplement), pp. 36–43. Available at: https://doi.org/10.4269/tropmed.1999.61-036.

Collins, W.E. and Jeffery, G.M. (2007) 'Plasmodium malariae: Parasite and disease', *Clinical Microbiology Reviews*, pp. 579–592. Available at: https://doi.org/10.1128/CMR.00027-07.

Cottrell, G. *et al.* (2015) 'Submicroscopic Plasmodium falciparum Infections Are Associated With Maternal Anemia, Premature Births, and Low Birth Weight', *Clinical Infectious Diseases*, 60(10), pp. 1481–1488. Available at: https://doi.org/10.1093/cid/civ122.

Cox, F.E. (2010) ‘History of the discovery of the malaria parasites and their vectors’, *Parasites and Vectors*, 3(1), pp. 1–9. Available at: https://doi.org/10.1186/1756-3305-3-5.

Crawley, J. (2004) ‘Reducing the burden of anemia in infants and young children in malaria-endemic countries of Africa: from evidence to action’, *The American journal of tropical medicine and hygiene*, 71(2 Suppl), p. 25—34. Available at: https://doi.org/10.4269/ajtmh.2004.71.25.

Chim CS *et al.* (2004) ‘Concurrent hyperreactive malarial splenomegaly and quartan malarial nephropathy - Plasmodium malariae revisited’, *Haematologica*, 89(7), pp. ECR21–ECR21. Available at: https://doi.org/10.3324/%x.

Culleton, R. and Carter, R. (2012) ‘African Plasmodium vivax: Distribution and origins’, *International Journal for Parasitology*, pp. 1091–1097. Available at: https://doi.org/10.1016/j.ijpara.2012.08.005.

Culleton, R., Pain, A. and Snounou, G. (2022) ‘Plasmodium malariae: the persisting mysteries of a persistent parasite’, *Trends in Parasitology* [Preprint]. Available at: https://doi.org/10.1016/j.pt.2022.11.008.

Current health expenditure per capita (current US$) - Cameroon, Gabon (2024) *World Bank*. Available at: https://data.worldbank.org/indicator/SH.XPD.CHEX.PC.CD?locations=CM-GA&name_desc=true (Accessed: 12 June 2024).

Delacre, M., Lakens, D. and Leys, C. (2017) ‘Why psychologists should by default use welch’s t-Test instead of student’s t-Test’, *International Review of Social Psychology*, 30(1), pp. 92–101. Available at: https://doi.org/10.5334/irsp.82.

Dinko, B. *et al.* (2013) ‘Persistent detection of Plasmodium falciparum, P. malariae, P. ovale curtisi and P. ovale wallikeri after ACT treatment of asymptomatic Ghanaian school-children’, *International Journal for Parasitology: Drugs and Drug Resistance*, 3, pp. 45–50. Available at: https://doi.org/10.1016/j.ijpddr.2013.01.001.

Douglas, N.M. *et al.* (2013) ‘Major Burden of Severe Anemia from Non-Falciparum Malaria Species in Southern Papua: A Hospital-Based Surveillance Study’, *PLoS Medicine*, 10(12), pp. 1–17. Available at: https://doi.org/10.1371/journal.pmed.1001575.

Ehrhardt, S. *et al.* (2006) *Malaria, Anemia, and Malnutrition in African Children-Defining Intervention Priorities*. Available at: https://academic.oup.com/jid/article/194/1/108/792926.

Ehrich, J. and Eke, F. (2007) ‘Malaria-induced renal damage: Facts and myths’, *Pediatric nephrology (Berlin, Germany)*, 22, pp. 626–637. Available at: https://doi.org/10.1007/s00467-006-0332-y.

Eiam-Ong, S. (2003) ‘Malarial Nephropathy’, *Seminars in Nephrology*, 23(1), pp. 21–33. Available at: https://doi.org/https://doi.org/10.1053/snep.2003.50002.

Fadel, A.N. *et al.* (2019) ‘A combination of metabolic resistance and high frequency of the 1014F kdr mutation is driving pyrethroid resistance in Anopheles coluzzii population from Guinea savanna of Cameroon’, *Parasites and Vectors*, 12(1), pp. 1–13. Available at: https://doi.org/10.1186/s13071-019-3523-7.

Fançony, C. *et al.* (2012) ‘Various pfcrt and pfmdr1 genotypes of Plasmodium falciparum cocirculate with P. malariae, P. ovale spp., and P. vivax in Northern Angola’, *Antimicrobial Agents and Chemotherapy*, 56(10), pp. 5271–5277. Available at: https://doi.org/10.1128/AAC.00559-12.

Fatih, F.A. *et al.* (2012) ‘Cytoadherence and virulence - the case of Plasmodium knowlesi malaria’, *Malaria Journal*, 11(1), p. 33. Available at: https://doi.org/10.1186/1475-2875-11-33.

Fogang, B. *et al.* (2022) ‘High Prevalence of Asymptomatic Malarial Anemia and Association with Early Conversion from Asymptomatic to Symptomatic Infection in a Plasmodium falciparum Hyperendemic Setting in Cameroon’, *American Journal of Tropical Medicine and Hygiene*, 106(1), pp. 293–302. Available at: https://doi.org/10.4269/ajtmh.21-0316.

Franken, G. *et al.* (2012) ‘Why do Plasmodium malariae infections sometimes occur in spite of previous antimalarial medication?’, *Parasitology Research*, 111(2), pp. 943–946. Available at: https://doi.org/10.1007/s00436-012-2851-8.

Gabon Population (2024) *Worldometer*. Available at: https://www.worldometers.info/world-population/gabon-population/ (Accessed: 10 June 2024).

GDP per capita (current US$) - Gabon, Cameroon (2024) *World Bank*. Available at: https://data.worldbank.org/indicator/NY.GDP.PCAP.CD?locations=GA-CM (Accessed: 12 June 2024).

Gnémé, A. *et al.* (2013) ‘Plasmodium species occurrence, temporal distribution and interaction in a child-aged population in rural Burkina Faso’, *Malaria Journal*, 12(1), pp. 1–9. Available at: https://doi.org/10.1186/1475-2875-12-67.

Goman, M., Mons, B. and Scaife, J. (1991) ‘The complete sequence of a Plasmodium malariae SSUrRNA gene and its comparison to other plasmodial SSUrRNA genes’, *Molecular and Biochemical Parasitology*, 45(2), pp. 281–288. Available at: https://doi.org/10.1016/0166-6851(91)90096-O.

Grande, R. *et al.* (2019) ‘A case of Plasmodium malariae recurrence: Recrudescence or reinfection?’, *Malaria Journal*, 18(1), pp. 1–9. Available at: https://doi.org/10.1186/s12936-019-2806-y.

Gunawardena, D., Carter, R. and Mendis, K. (1994) ‘Patterns of acquired anti-malarial immunity in Sri Lanka’, *Memórias do Instituto Oswaldo Cruz*, 89 Suppl 2, pp. 63–65. Available at: https://doi.org/10.1590/S0074-02761994000600015.

Hang, J.W. *et al.* (2021) 'Zoonotic Malaria : Non- Laverania Plasmodium Biology and Invasion Mechanisms', *Pathogens*, 10, pp. 1–22. Available at: https://api.semanticscholar.org/CorpusID:236002098.

Hawadak, J., Dongang Nana, R.R. and Singh, V. (2021) 'Global trend of Plasmodium malariae and Plasmodium ovale spp. malaria infections in the last two decades (2000–2020): a systematic review and meta-analysis', *Parasites and Vectors*, 14(1), pp. 1–14. Available at: https://doi.org/10.1186/s13071-021-04797-0.

Hayuma, P.M. *et al.* (2021) 'Prevalence of asymptomatic malaria, submicroscopic parasitaemia and anaemia in Korogwe District, north-eastern Tanzania', *Malaria Journal*, 20(1). Available at: https://doi.org/10.1186/s12936-021-03952-3.

Hedelius, R. *et al.* (2011) 'Nephrotic syndrome and unrecognized plasmodium malariae infection in a US navy sailor 14 years after departing Nigeria', *Journal of Travel Medicine*, 18(4), pp. 288–291. Available at: https://doi.org/10.1111/j.1708-8305.2011.00526.x.

Hegner, R. (1938) 'Relative Frequency of Ring-Stage Plasmodia in Reticulocytes and Mature Erythrocytes in Man and Monkey.', *American Journal of Hygiene*, 27(3), pp. 690–718 pp.

Hendrickse, R.G. and Adeniyi, A. (1979) 'Quartan malarial nephrotic syndrome in children', *Kidney International*, 16(1), pp. 64–74. Available at: https://doi.org/https://doi.org/10.1038/ki.1979.103.

Hochman, S. and Kim, K. (2009) 'The Impact of HIV and Malaria Coinfection: What Is Known and Suggested Venues for Further Study', *Interdisciplinary perspectives on infectious diseases*, 2009, p. 617954. Available at: https://doi.org/10.1155/2009/617954.

Holzschuh, A. *et al.* (2022) 'Co-infection of the four major Plasmodium species: Effects on densities and gametocyte carriage', *PLoS Neglected Tropical Diseases*, 16(9). Available at: https://doi.org/10.1371/JOURNAL.PNTD.0010760.

Huang, L.H. *et al.* (2017) 'The effects of storage temperature and duration of blood samples on DNA and RNA qualities', *PLoS ONE*, 12(9), pp. 1–13. Available at: https://doi.org/10.1371/journal.pone.0184692.

Imwong, M. *et al.* (2014) 'High-throughput ultrasensitive molecular techniques for quantifying low-density malaria parasitemias', *Journal of Clinical Microbiology*, 52(9), pp. 3303–3309. Available at: https://doi.org/10.1128/JCM.01057-14.

Kamau, E. *et al.* (2011) 'Development of a highly sensitive genus-specific quantitative reverse transcriptase real-time PCR assay for detection and quantitation of plasmodium by amplifying RNA and DNA of the 18S rRNA genes', *Journal of Clinical Microbiology*, 49(8), pp. 2946–2953. Available at: https://doi.org/10.1128/JCM.00276-11.

Kho S *et al.* (2021) ‘Hidden Biomass of Intact Malaria Parasites in the Human Spleen’, *New England Journal of Medicine*, 384(21), pp. 2067–2069. Available at: https://doi.org/10.1056/NEJMc2023884.

Kitchen, S.F. (1939) ‘The Infection of Mature and Immature Erythrocytes by Plasmodium falciparum and Plasmodium malariae.’, *American Journal of Tropical Medicine and Hygiene*, 19, pp. 47–62.

Koita, O.A. *et al.* (2012) ‘Effect of seasonality and ecological factors on the prevalence of the four malaria parasite species in Northern Mali’, *Journal of Tropical Medicine* [Preprint]. Available at: https://doi.org/10.1155/2012/367160.

Lalremruata, A. *et al.* (2015) ‘Natural infection of Plasmodium brasilianum in humans: Man and monkey share quartan malaria parasites in the Venezuelan Amazon’, *EBioMedicine*, 2(9), pp. 1186–1192. Available at: https://doi.org/10.1016/j.ebiom.2015.07.033.

Langford, S. *et al.* (2015) ‘Plasmodium malariae Infection Associated with a High Burden of Anemia: A Hospital-Based Surveillance Study’, *PLoS Neglected Tropical Diseases*, 9(12). Available at: https://doi.org/10.1371/journal.pntd.0004195.

Langston, S.M. and Bales, B.D. (2021) ‘Anemia In The Tropics’, in K.J. Knoop et al. (eds) *The Atlas of Emergency Medicine, 5e*. New York, NY: McGraw-Hill. Available at: accessmedicine.mhmedical.com/content.aspx?aid=1181051206.

Lee, W.-C. *et al.* (2022) ‘Cytoadherence Properties of Plasmodium knowlesi-Infected Erythrocytes’, *Frontiers in Microbiology*, 12. Available at: https://www.frontiersin.org/journals/microbiology/articles/10.3389/fmicb.2021.804417.

Leoni, S. *et al.* (2015) ‘The hyper-reactive malarial splenomegaly: A systematic review of the literature’, *Malaria Journal*, 14(1). Available at: https://doi.org/10.1186/s12936-015-0694-3.

Liu, W. *et al.* (2014) ‘African origin of the malaria parasite Plasmodium vivax’, *Nature Communications*, 5. Available at: https://doi.org/10.1038/ncomms4346.

Looareesuwan, S. *et al.* (1987) ‘High rate of Plasmodium vivax relapse following treatment of falciparum malaria in Thailand’, *The Lancet*, 330(8567), pp. 1052–1055. Available at: https://doi.org/https://doi.org/10.1016/S0140-6736(87)91479-6.

Lover, A.A. *et al.* (2018) ‘Malaria elimination: Time to target all species’, *American Journal of Tropical Medicine and Hygiene*. American Society of Tropical Medicine and Hygiene, pp. 17–23. Available at: https://doi.org/10.4269/ajtmh.17-0869.

Luxemburger, C. *et al.* (1997) ‘The epidemiology of severe malaria in an area of low transmission in Thailand’, *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 91(3), pp. 256–262. Available at: https://doi.org/https://doi.org/10.1016/S0035-9203(97)90066-3.

Luxemburger, C. *et al.* (1999) 'Treatment of vivax malaria on the western border of Thailand', *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 93(4), pp. 433–438. Available at: https://doi.org/10.1016/S0035-9203(99)90149-9.

Maitland, K. *et al.* (1996) 'The interaction between Plasmodium falciparum and P. vivax in children on Espiritu Santo island, Vanuatu', *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 90(6), pp. 614–620. Available at: https://doi.org/https://doi.org/10.1016/S0035-9203(96)90406-X.

Maitland, K., Williams, T.N. and Newbold, C.I. (1997) 'Plasmodium vivax and P. falciparum: Biological interactions and the possibility of cross-species immunity', *Parasitology Today*, 13(6), pp. 227–231. Available at: https://doi.org/10.1016/S0169-4758(97)01061-2.

Malentacchi, F. *et al.* (2016) 'Effects of Transport and Storage Conditions on Gene Expression in Blood Samples', *Biopreservation and Biobanking*, 14(2), pp. 122–128. Available at: https://doi.org/10.1089/bio.2015.0037.

May, J. *et al.* (2000) *Impact of subpatent multi-species and multi-clonal plasmodial infections on anaemia in children from Nigeria*. Available at: www.who.intlchdlpublications/newslet/dialogl9lana-.

Mbama Ntabi, J.D. *et al.* (2022) 'Prevalence of non-Plasmodium falciparum species in southern districts of Brazzaville in The Republic of the Congo', *Parasites and Vectors*, 15(1), pp. 1–14. Available at: https://doi.org/10.1186/s13071-022-05312-9.

McKenzie, F.E. and Bossert, W.H. (1999) 'Multispecies Plasmodium infections of humans', *Journal of Parasitology*, 85(1), pp. 12–18. Available at: https://doi.org/10.2307/3285692.

McKenzie, F.E., Jeffery, G.M. and Collins, W.E. (2001) 'Plasmodium malariae blood-stage dynamics', *Journal of Parasitology*, 87(3), pp. 626–637.

McKenzie, F.E., Jeffery, G.M. and Collins, W.E. (2002) 'Plasmodium malariae infection boosts Plasmodium falciparum gametocyte production', *American Journal of Tropical Medicine and Hygiene*, 67(4), pp. 411–414. Available at: https://doi.org/10.4269/ajtmh.2002.67.411.

Miller, L.H. *et al.* (2002) 'The pathogenic basis of malaria', *Nature*, 415(6872), pp. 673–679. Available at: https://doi.org/10.1038/415673a.

Molineaux, L. *et al.* (1980) 'A longitudinal study of human malaria in the West African Savanna in the absence of control measures: relationships between different Plasmodium species, in particular P. falciparum and P. malariae.', *The American journal of tropical medicine and hygiene*, 29(5), pp. 725–737. Available at: https://doi.org/10.4269/ajtmh.1980.29.725.

Moody, A. (2002) 'Rapid Diagnostic Tests for Malaria Parasites', *Clinical Microbiology Reviews*, 15(1), pp. 66–78. Available at: https://doi.org/10.1128/cmr.15.1.66-78.2002.

Mueller, I., Zimmerman, P.A. and Reeder, J.C. (2007) 'Plasmodium malariae and Plasmodium ovale - the "bashful" malaria parasites', *Trends in Parasitology*, 23(6), pp. 278–283. Available at: https://doi.org/10.1016/j.pt.2007.04.009.

Müller-Stöver, I. *et al.* (2008) 'Plasmodium malariae infection in spite of previous anti-malarial medication.', *Parasitology research*, 102(3), pp. 547–550. Available at: https://doi.org/10.1007/s00436-007-0804-4.

Murphy, S.C. *et al.* (2012) 'Real-time quantitative reverse transcription PCR for monitoring of blood-stage Plasmodium falciparum infections in malaria human challenge trials', *American Journal of Tropical Medicine and Hygiene*, 86(3), pp. 383–394. Available at: https://doi.org/10.4269/ajtmh.2012.10-0658.

Murphy, S.C. *et al.* (2018) 'Plasmodium 18S rRNA of intravenously administered sporozoites does not persist in peripheral blood', *Malaria Journal*, 17(1), pp. 1–7. Available at: https://doi.org/10.1186/s12936-018-2422-2.

Mwangi, M.N. *et al.* (2021) 'Iron deficiency anaemia in sub-Saharan Africa: a review of current evidence and primary care recommendations for high-risk groups', *The Lancet Haematology*. Elsevier Ltd, pp. e732–e743. Available at: https://doi.org/10.1016/S2352-3026(21)00193-9.

Nguiffo-Nguete, D. *et al.* (2023) 'Plasmodium malariae contributes to high levels of malaria transmission in a forest–savannah transition area in Cameroon', *Parasites & Vectors*, 16(1), pp. 1–10. Available at: https://doi.org/10.1186/s13071-022-05635-7.

Nilsson, S.K. *et al.* (2015) 'Targeting Human Transmission Biology for Malaria Elimination', *PLoS Pathogens*, 11(6). Available at: https://doi.org/10.1371/journal.ppat.1004871.

Oriero, E.C. *et al.* (2021) 'Plasmodium malariae, current knowledge and future research opportunities on a neglected malaria parasite species', *Critical Reviews in Microbiology*, 47(1), pp. 44–56. Available at: https://doi.org/10.1080/1040841X.2020.1838440.

Pinilla, Y.T. *et al.* (2021) 'Experimental transmission of plasmodium malariae to anopheles gambiae', *Journal of Infectious Diseases*, 223(3), pp. 522–526. Available at: https://doi.org/10.1093/infdis/jiaa382.

Plenderleith, L.J. *et al.* (2022) 'Zoonotic origin of the human malaria parasite Plasmodium malariae from African apes', *Nature Communications*, 13(1). Available at: https://doi.org/10.1038/s41467-022-29306-4.

Price, R.N. *et al.* (2001) 'Factors contributing to anemia after uncomplicated falciparum malaria.', *American Journal of Tropical Medicine and Hygiene*, 65(5), pp. 614–622. Available at: https://doi.org/10.4269/ajtmh.2001.65.614.

Recker, M. *et al.* (2019) 'COMAL Research grant proposal', *Medical Research Council* [Preprint].

Richter, J. *et al.* (2016) ‘Clinical implications of a gradual dormancy concept in malaria’, *Parasitology Research*, 115(6), pp. 2139–2148. Available at: https://doi.org/10.1007/s00436-016-5043-0.

Rivera-Correa, J. and Rodriguez, A. (2020) ‘Autoimmune Anemia in Malaria’, *Trends in Parasitology*. Elsevier Ltd, pp. 91–97. Available at: https://doi.org/10.1016/j.pt.2019.12.002.

Rizzo, A. *et al.* (2023) ‘Multiple episodes of Plasmodium malariae despite antimalarial treatment: “Quartana te teneat”?’, *Travel Medicine and Infectious Disease*, 56(September), p. 102671. Available at: https://doi.org/10.1016/j.tmaid.2023.102671.

Ruiz Cuenca, P. *et al.* (2022) ‘Is there evidence of sustained human-mosquito-human transmission of the zoonotic malaria Plasmodium knowlesi? A systematic literature review’, *Malaria Journal*, 21(1), p. 89. Available at: https://doi.org/10.1186/s12936-022-04110-z.

Rutledge, G.G., Marr, I., *et al.* (2017) ‘Genomic characterization of recrudescent plasmodium malariae after treatment with artemether/lumefantrine’, *Emerging Infectious Diseases*, 23(8), pp. 1300–1307. Available at: https://doi.org/10.3201/eid2308.161582.

Rutledge, G.G., Böhme, U., *et al.* (2017) ‘Plasmodium malariae and P. ovale genomes provide insights into malaria parasite evolution’, *Nature*, 542(7639), pp. 101–104. Available at: https://doi.org/10.1038/nature21038.

Sarathkumara, Y.D. *et al.* (2022) ‘The Effect of Tropical Temperatures on the Quality of RNA Extracted from Stabilized Whole-Blood Samples’, *International Journal of Molecular Sciences*, 23(18). Available at: https://doi.org/10.3390/ijms231810609.

Siala, E. *et al.* (2005) ‘Relapse of Plasmodium malariae malaria 20 years after living in an endemic area’, *Presse medicale (Paris, France : 1983)*, 34(5), pp. 371–372. Available at: https://doi.org/10.1016/s0755-4982(05)83926-0.

Simpson, J.A. *et al.* (1999) ‘Red cell selectivity in malaria: a study of multiple-infected erythrocytes’, *Transactions of The Royal Society of Tropical Medicine and Hygiene*, 93(2), pp. 165–168. Available at: https://doi.org/10.1016/S0035-9203(99)90295-X.

Smith, A. *et al.* (2011) ‘Plasmodium species co-infection as a cause of treatment failure’, *Travel Medicine and Infectious Disease*, 9(6), pp. 306–309. Available at: https://doi.org/10.1016/j.tmaid.2011.09.006.

Smith, J.D. *et al.* (1995) *Switches in Expression of Plasmodium falciparum VW Genes Correlate with Changes in Antigenic and Cytoadherent Phenotypes of Infected Erythrocytes*, *Cell*.

Smith, T. *et al.* (2001) ‘Prospective risk of morbidity in relation to malaria infection in an area of high endemicity of multiple species of Plasmodium’, *American Journal of Tropical Medicine and Hygiene*, 64(5), pp. 262–267. Available at: https://doi.org/10.4269/ajtmh.2001.64.262.

Snounou, G., Viriyakosol, S., Xin Ping Zhu, *et al.* (1993) ‘High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction’, *Molecular and Biochemical Parasitology*, 61(2), pp. 315–320. Available at: https://doi.org/10.1016/0166-6851(93)90077-B.

Snounou, G., Viriyakosol, S., Jarra, W., *et al.* (1993) ‘Identification of the four human malaria parasite species in field samples by the polymerase chain reaction and detection of a high prevalence of mixed infections’, *Molecular and Biochemical Parasitology*, 58(2), pp. 283–292. Available at: https://doi.org/10.1016/0166-6851(93)90050-8.

Snounou, G., Pinheiro, L., *et al.* (1993) ‘The importance of sensitive detection of malaria parasites in the human and insect hosts in epidemiological studies, as shown by the analysis of field samples from Guinea Bissau’, *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 87, pp. 649–653.

Sorof Uddin, M. and Cheng, Q. (2015) ‘Recent application of biotechniques for the improvement of mango research’, *Applied Plant Genomics and Biotechnology*, pp. 195–212. Available at: https://doi.org/10.1016/B978-0-08-100068-7.00012-4.

Staalsoe, T. *et al.* (2002) ‘In vivo switching between variant surface antigens in human Plasmodium falciparum infection’, *Journal of Infectious Diseases*, 186(5), pp. 719–722. Available at: https://doi.org/10.1086/342390.

Sutherland, C.J. (2016) ‘Persistent Parasitism: The Adaptive Biology of Malariae and Ovale Malaria’, *Trends in Parasitology*, 32(10), pp. 808–819. Available at: https://doi.org/10.1016/j.pt.2016.07.001.

Sutherland, C.J. (2020) ‘A New Window on Plasmodium malariae Infections’, *Journal of Infectious Diseases*. Oxford University Press, pp. 864–866. Available at: https://doi.org/10.1093/infdis/jiz103.

Swan, H. *et al.* (2005) ‘Evaluation of a real-time polymerase chain reaction assay for the diagnosis of malaria in patients from Thailand.’, *The American journal of tropical medicine and hygiene*, 73(5), pp. 850–854.

Talundzic, E. *et al.* (2017) ‘First Full Draft Genome Sequence of Plasmodium brasilianum.’, *Genome announcements*, 5(6). Available at: https://doi.org/10.1128/genomeA.01566-16.

Tang, J. *et al.* (2020) ‘The Consequences of Mixed-Species Malaria Parasite Co-Infections in Mice and Mosquitoes for Disease Severity, Parasite Fitness, and Transmission Success’, *Frontiers in Immunology*, 10. Available at: https://doi.org/10.3389/fimmu.2019.03072.

Tournoy, T.K. *et al.* (2022) ‘Plasmodium malariae after successful treatment of P. falciparum malaria with artemether-lumefantrine’, *International Journal of Infectious Diseases*, 119, pp. 56–58. Available at: https://doi.org/10.1016/j.ijid.2022.03.045.

Twohig, K.A. *et al.* (2019) ‘Growing evidence of Plasmodium vivax across malaria-endemic Africa’, *PLoS Neglected Tropical Diseases*, 13(1). Available at: https://doi.org/10.1371/journal.pntd.0007140.

Utpat, S. *et al.* (2024) ‘Antimalarial prophylaxis failure: Malaria in a returning traveler despite mefloquine prophylaxis’, *Tropical Parasitology*, 14, pp. 45–47. Available at: https://doi.org/10.4103/tp.tp_39_23.

Vinetz, J.M. *et al.* (1998) ‘Plasmodium malariae infection in an asymptomatic 74-year old greek woman with splenomegaly’, *New England Journal of Medicine*, 338(6), pp. 367–371.

Weiss, D.J. *et al.* (2019) ‘Mapping the global prevalence, incidence, and mortality of Plasmodium falciparum, 2000–17: a spatial and temporal modelling study’, *The Lancet*, 394(10195), pp. 322–331. Available at: https://doi.org/10.1016/S0140-6736(19)31097-9.

White, N.J. (2018) ‘Anaemia and malaria’, *Malaria Journal*. BioMed Central Ltd. Available at: https://doi.org/10.1186/s12936-018-2509-9.

WHO (2023) *World malaria report 2023*. Available at: https://www.who.int/teams/global-malaria-programme/reports/world-malaria-report-2023 (Accessed: 12 June 2024).

Woldearegai, T.G. *et al.* (2019) ‘Characterization of Plasmodium infections among inhabitants of rural areas in Gabon’, *Scientific Reports*, 9(1), pp. 1–10. Available at: https://doi.org/10.1038/s41598-019-46194-9.

Yman, V. *et al.* (2019) ‘Persistent transmission of plasmodium malariae and plasmodium ovale species in an area of declining plasmodium falciparum transmission in Eastern Tanzania’, *PLoS Neglected Tropical Diseases*, 13(5), pp. 1–16. Available at: https://doi.org/10.1371/journal.pntd.0007414.

Zhang, X. and Deitsch, K.W. (2022) ‘The mystery of persistent, asymptomatic Plasmodium falciparum infections’, *Current Opinion in Microbiology*. Elsevier Ltd. Available at: https://doi.org/10.1016/j.mib.2022.102231.

Zhou, M. *et al.* (1998) ‘High prevalence of Plasmodium malariae and Plasmodium ovale in malaria patients along the Thai-Myanmar border, as revealed by acridine orange staining and PCR-based diagnoses’, *Tropical Medicine and International Health*, 3(4), pp. 304–312. Available at: https://doi.org/10.1046/j.1365-3156.1998.00223.x.

7 Erklärung zum Eigenanteil

Die Arbeit wurde am Institut für Tropenmedizin der Universitätsklinik Tübingen unter Betreuung von Professor Dr. Steffen Borrmann durchgeführt.

Die Konzeption der Studie erfolgte durch Steffen Borrmann, Mario Recker und Ayola Adegnika im Rahmen des COMAL Projektes in Zusammenarbeit mit dem CERMEL Gabon (Leitung A. Adegnika), CRID Cameroon (Leitung C. Wondji), FCRM Congo (Leitung F. Ntoumi) und FORS Benin (Leitung L. Djogbénou).

Die Versuche wurden nach Einarbeitung durch die labortechnische Assistentin Frau Andrea Weierich von mir eigenständig und teils unter Supervision durch Frau Weierich durchgeführt. Die Blutproben sowie die anthropometrischen Daten, die für diese Arbeit verwendet wurden, wurden durch die Kolleginnen und Kollegen des CERMEL in Gabun und des CRID in Kamerun abgenommen und erhoben. Auch die vorangegangene mikroskopische Untersuchung und Bestimmung der Parasitämie der Proben wurde von den Teams vor Ort in Gabun und Kamerun durchgeführt. Die PCR-Primer und Sonden- Sequenzen stammen von Charles Wondji (CRID Kamerun) und Albert Lalremruata (ITM Tübingen).

Die graphische und statistische Auswertung erfolgte durch mich eigenständig und nach wiederkehrender Beratung durch Dr. Mario Recker (Universität Exeter).

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

Tübingen, den 28.07.2025

Felicitas Beger

8 Veröffentlichungen

Eine Veröffentlichung mit dem Titel “The hidden public health burden of *Plasmodium malariae*: a population-based study” von Recker et al. ist aktuell in Vorbereitung. Diese basiert auf der Auswertung der gleichen Blutproben wie diese Studie, inkludiert jedoch noch einige weitere Proben aus Gabun die in dieser Studie noch nicht enthalten sind. Das noch unveröffentlichte Manuskript von Recker et al. befasst sich hauptsächlich mit Daten zur Prävalenz von *P. malariae* und *P. falciparum* sowie mit Hämoglobin-Analysen, die den Ergebnissen dieser Studie entsprechend ähneln.

9 Danksagung

Danken möchte ich allen voran herzlich Professor Dr. Steffen Borrmann für die Aufnahme in seine Forschungsgruppe, die Vergabe des Themas und die Unterstützung bei der Umsetzung der Arbeit. Dr. Mario Recker möchte ich sehr herzlich für die allzeit konstruktive statistische Beratung und Unterstützung danken.

Mein Dank gilt zudem Professor Dr. Peter Gottfried Kremsner für die Aufnahme am Institut für Tropenmedizin in Tübingen.

Frau Andrea Weierich hat mich in der experimentellen Phase meiner Promotion tatkräftig unterstützt und mich in die Laborarbeit eingewiesen, auch ihr gilt mein großer Dank.

Zudem möchte ich dem gesamten Team des Tropeninstituts Tübingen danken. Viele von euch haben mir meine unzähligen Fragen beantwortet, mich in die Geräte eingewiesen oder mir hilfreichen Denkstoff geliefert. Danke für eure Unterstützung!

Ohne den mentalen Support meiner Familie und Freunde, insbesondere meines Mannes Jannis, hätte ich diese Arbeit nicht geschafft. Danke, dass ihr für mich da wart!