Molecular epidemiology of *Mansonella perstans* on Bioko Island: identification of risk factors, co-infection with malaria and *Loa loa* and impact in pregnant women

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Summary

Sensitive, specific, robust, accurate and cost efficient diagnostic tools for parasite detection are essential for guidance and success of parasite control programs. Filariasis is an infectious disease belonging to the group of neglected tropical diseases that are caused by nematodes including *Onchocerca volvulus* (Onchocerciasis), *Wuchereria bancrofti Brugia spp* (Lymphatic filariasis) and *Loa loa* (Loaisis). Molecular diagnostic methods like polymerase chain reaction (PCR) based techniques have shown high sensitivity and specificity to detect filariasis causing parasites - even in people that seem to be amicrofilaremic when diagnosed by microscopy. Large scale implementation over extended time periods of filariasis monitoring in affected populations remains a challenge for low-income countries because of logistics and costs, including collection, transportation and preservation of biological samples and running of analytical tests. The highly neglected Mansonellosis caused by *Mansonella perstans* and Loaisis gained recently attention since both parasites could interfere with mass drug administration regimen based elimination programs of Onchocerciasis and Lymphatic filariasis.

The overall goal of this PhD thesis is to contribute to development and implementation of novel molecular based diagnostic approaches for monitoring highly neglected filariasis causing parasites. Specifically, we aimed to develop and evaluate a novel high-throughput procedure based on extraction of nucleic acids (NA) from malaria rapid diagnostic tests (mRDT) collected in the yearly conducted malaria monitoring programs on Bioko Island. Here, we used mRDT collected in 2018 and extracted NA to monitor the rate of false-positive and false-negative malaria diagnosis based on mRDT when compared to the qPCR based testing as gold standard. Recent malaria treatment resulted in a significant increase of false-positive mRDTs outcomes. Next, extracted NAs were used to detect gene fragments specific for *Plasmodium* spp., *M. perstans* and *L. loa* by qPCR. Results were used to identify and map populations suffering from either mono- or co-infections of malaria and filarial parasites. Risk factors for *M. perstans* infections were identified by making use of the extensive

metadata available from each mRDT collectedand artificial intelligence based tools. We identified cacao farmers as the sub-populations in rural settings that is mostly affected by *M. perstans* infections. The predictions of high risk groups for *M. perstans* infections were reconfirmed in an independent mRDT sample set collected during the malaria indicator survey in 2019 on Bioko Island. Lastly, we explored the presence of co-infections between *Plasmodium* spp., *M. perstans* and *L. loa* in pregnant women, a highly neglected and particularly vulnerable sub-population on Bioko Island with their potential impact on the anemia status.

Manuscript 1: Molecular malaria surveillance using a novel protocol for extraction and analysis of nucleic acids retained on used rapid diagnostic tests

This manuscript describes a high-throughput method to extract nucleic acid (NAs) from the small blood volume retained on nitrocellulose strips of used malaria rapid diagnostic tests (mRDTs). mRDTs used were collected in the field, stored and shipped at room temperature. The co-extraction of DNA and RNA molecules from mRDTs allows detection and quantification of *Plasmodium falciparum* from clinically healthy volunteers with calculated parasite densities as low as 1 parasite/µL of blood. This approach increased the sensitivity for detection of *Plasmodium* spp. parasites independent of storage temperature and storage time. Based on this extraction protocol named ENAR (Extraction of Nucleic Acids from RDTs), a larger scale molecular based screening of malaria infection prevalence was realized using mRDTs collected during the malaria indicator survey conducted in 2018 on Bioko Island, Equatorial Guinea. A total of 2,690 mRDTs were extracted by ENAR and analyzed for *Plasmodium* spp. parasites by RT-qPCR. Additionally, sequence polymorphisms in the propeller region of the *kelch 13* gene among *Plasmodium falciparum* strains circulating on the Island are described.

Manuscript 2: Analysis of nucleic acids extracted from rapid diagnostic tests reveals a significant proportion of false positive test results associated with recent malaria treatment

Based on the ENAR approach, we implemented a RT-qPCR assay for high-throughput identification of *Plasmodium falciparum* strains carrying *pfhrp2* and *pfhrp3* gene deletions that are limiting the usefulness of commonly deployed mRDT. Extracted NAs from 1,800 malaria negative and 1,065 malaria positive RDTs collected in 2018 on Bioko Island were included. Results showed that 4.7% of mRDT reported a false-negative result when compared with the by RT-qPCR based assessment which is mostly associated with low asexual blood stage parasite density infections. 28.4% of malaria positive mRDTs were identified as malaria negative by RT-qPCR and therefore considered to be false-positive mRDTs. This result could be best explained by persistence of circulating PfHRP2 antigen after malaria treatment that occurred within 2 weeks before mRDT collection. Our study supports the need of well-integrated quality control procedures for malaria larger scale testing to continuously assess extend and impact of potentially reduced sensitivity and specificity of mRDTs based on PfHRP2 detection.

Manuscript 3: Characterising co-infections with *Plasmodium* spp., *Mansonella perstans* or *Loa loa* in asymptomatic children, adults and elderly people living on Bioko Island using nucleic acids extracted from malaria rapid diagnostic tests

We repurposed the blood preserved on mRDTs that have been deployed during the MIS conducted during 2018 on Bioko Island. Building on our previous work (Manuscript 1), we extracted NAs from 3214 mRDT followed by multiplex qPCR based amplification and specific detection of *Mansonella perstans* and *Loa loa*. We identified a positivity rate for *M. perstans* and *L. loa* of 6.6 % and 1.5%, respectively, in populationanalysed. *M. perstans* infection positivity rate was higher among older, male participants and in the population living in rural areas. The socio-economic status of participants strongly influenced the infection rate. No increased risk of being co-infected with *Plasmodium* spp.

and these filarial nematodes was observed among the different age groups. Interestingly, the infection intensity of *M. perstans* but not *L. loa* as reflected by the Cq values obtained by qPCR measurements seem to be significantly higher in the population older than 40 years of age. We have also developed the first maps of geographical distributions of these infectious diseases detected by sensitive and specific molecular testing. Our study demonstrates that employing mRDTs probed with finger pricked blood represents a versatile tool to preserve and ship NAs at room temperature to laboratories for molecular, high-throughput diagnosis and genotyping of blood-dwelling nematode filarial infections. Using this approach, rural populations can be reached and surveyed at low cost for infectious diseases beyond malaria.

Manuscript 4: Understanding determinants of *Mansonella perstans* infections in Bioko Island's at-risk population using machine learning classifiers

Our work has highlighted potential risk factors that may contribute to *M. perstans* infection in Bioko Island. To address this question further, we used Machine Learning of six classifiers to identify strong predictors driving the distribution of this infection based on individual socio-economic, geographical and environmental information collected during the MIS conducted in 2018 combined with the infection status assessed by molecular tools and environmental data sets of the island. Here, we found that environmental and geographical factors are the major factors that favor considerably the transmission of *M. perstans* in Bioko Island. Furthermore, agriculture or fisheries as occupational sector represented 44% chance to be infected. Identification of risk factors for *M. perstans* infection will support improved and better targeted clinical and interventional studies including novel drug and vector control interventions tailored for the population most in need.

Manuscript 5: Molecular epidemiology of filariasis and malaria in pregnant women on Bioko Island, Equatorial Guinea

Pregnant women are usually excluded in mass drug administration programs to control lymphatic filariasis and onchocerciasis. Therefore the epidemiology of filarial worms and the consequences for the health of mother and baby remain understudied. This study enabled for the first time to characterize the positivity rate of *M. perstans* infection among 293 pregnant women based on the approaches described in Manuscript 3. mRDTs collected from pregnant women participating in the MIS 2018 and MIS 2019 were included. Apart from the molecular testing for *Plasmodium* spp and *M. perstans*, insecticide treated bed net use and the anaemia status was recorded for these pregnant women. 3.7 % of pregnant women were found to be infected with *M. perstans*. The majority of *M. perstans* infected pregnant women were likely to live in rural areas and had a lower socioeconomic status. Malaria and Loaisis were found in this population with a positivity rate of 8.8% and 1.4%, respectively. Few cases of co-infection were observed between *L. loa* and either *Plasmodium* spp. or *M. perstans*. Self-reported use of bed nets did not seem to prevent *M. perstans* infection confirming that bed nets are not a suitable prevention measure against these vector borne nematode infections. Mild to moderate anaemia was highly prevalent in these women with no obvious link to *M. perstans* infection but to malaria infection.

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Table of Contents

List of acronyms and abbreviations

ALB	Albendazole
An	*Anopheles*
ANC	Antenatal care
BIMCP	Bioko Island Malaria Control Project
Breg	Regulatory B cell
CDC	Center for Disease Control and Prevention
C.	*Chrysops*
DALY	Disability Adjusted Life Year
DEC	Diethylcarbamazine
ELISA	Enzyme-linked immunosorbent assay
ENAR	Extraction of Nucleic Acids from RDTs
Ig	Immunoglobulin
IL	Interleukin
ITNs	Insecticide-treated mosquito nets
ITS	Internal transcribed spacer
IVM	Ivermectin
LAMP	Loop-mediated isothermal amplification
LF	Lymphatic Filariasis
LIPS	Luciferase immunoprecipitation system assay
L .loa	*Loa loa*
L3	third-stage larvae
M. perstans	*Mansonella perstans*
MDA	Mass drug administration
ML	Machine learning
Mf	Microfilariae
Mf+	Microfilaremic
Mf-	amicrofilaremic
MIS	Malaria Indicator Survey
mRDT	Malaria RDT
NA	Nucleic acid
NTD	Neglected tropical disease
P.	*Plasmodium*
PfHRP2/3	*P. falciparum* specific histidine rich protein 2/3
pLDH	Pan-*Plasmodium* spp. enzymes lactate dehydrogenase
qPCR	Quantitative polymerase chain reaction
RDT	Rapid Diagnostic Test
Swiss TPH	Swiss Tropical and Public Health Institute
SDG	Sustainable Development Goals
Treg	regulatory T cell
W.bancrofti	*Wuchereria bancrofti*
WHO	World Health Organisation
O. volvulus	*Onchocerca volvulus*
qPCR	Quantitative polymerase chain reaction
RDT	Rapid Diagnostic Test
Swiss TPH	Swiss Tropical and Public Health Institute
SDG	Sustainable Development Goals
Treg	regulatory T cell
W. bancrofti	*Wuchereria bancrofti*
WHO	World Health Organisation

Chapter I

Introduction

1.1 Background

Neglected tropical diseases (NTDs) are a group of diverse infectious diseases that are widespread in tropical and Sub-tropical regions of Africa, Asia and America (Engels and Zhou, 2020). Although mortality is low (Boutayeb, 2007), morbidity is usually high with more than one billion people suffering from reduction in their quality and productivity of life leading to considerable negative socio-economic impact (Sun and Amon, 2018, Engels and Zhou, 2020) with an estimated 47.9 million disability-adjusted life years (DALYs) calculated in 2014 (Molyneux et al., 2017). NTDs are predominantly found in poor socio-economic environments where access to adequate housing, education, sanitation, protection from disease vectors and healthcare are limited (Sun and Amon, 2018, Engels and Zhou, 2020). In such areas, infections with at least one NTD are frequently contributing to chronic morbidity, associated with stigmatization and impoverishment among the affected population (Sun and Amon, 2018). The World Health Organization (WHO) recognizes currently 20 NTDs as depicted in Table 1, which are targeted for control and elimination efforts (Sun and Amon, 2018, Engels and Zhou, 2020, WHO, 2020a). This global attention has led to inclusion of these NTDs into the Sustainable Development Goals (SDG) that aims to end the "epidemics of AIDS, Tuberculosis, Malaria and NTD" by 2030 (WHO, 2020a, Engels and Zhou, 2020). Thus, since the London Declaration on NTDs in 2012, international global health actors including developmental organizations, governments of disease endemic countries, pharmaceutical companies and a variety of other private or public partners have themselves committed to work together to control, eliminate and finally eradicate NTDs (WHO, 2020a).

Table 1. List of the 20 neglected tropical diseases recognized by WHO (Sun and Amon, 2018).

WHO Recognized NTDs	Common features of these diseases
• Buruli ulcer • Chagas disease • Dengue and chikungunya • Dracunculiasis (guinea-worm disease) • Echinococcosis • Foodborne trematodiases • Human African trypanosomiasis • Leishmaniasis • Leprosy (Hansen's disease) • Lymphatic filariasis • Mycetoma • Onchocerciasis (river blindness) • Rabies • Schistosomiasis • Soil-transmitted helminthiases • Taeniasis/cysticercosis • Trachoma and yaws • Chromoblastomycosis and other deep mycoses • Scabies (and other ectoparasites) • Snakebite envenoming	• Being a proxy for poverty and disadvantage • Affecting populations with low visibility and little political voice • Having a relatively stable endemic foci • Often overlapping geographically • Causing stigma and discrimination, especially for girls and women • Having an important impact on morbidity and mortality • Being relatively neglected by research • Can be controlled, prevented, and possibly eliminated using simple, effective, and feasible solutions

Human filariasis is an infectious disease that occurs in Africa, Asia and South-America and includes Onchocerciasis, Lymphatic filariasis (LF), Loaisis and Mansonellosis (Ta et al., 2018, Mathison et al., 2019). Filariasis is caused by vector-borne nematodes that use humans as their primary, definitive hosts and include *Onchocerca volvulus* (Onchocerciasis), *Wuchereria bancrofti* and *Brugia spp* (LF), *Loa loa* (Loaisis) and *Mansonella perstans*, *M. streptocerca*, *M. ozzardi* (Mansonellosis) (Ta et al., 2018). The life-cycle of filarial worms is alternating between the human host and a distinct group of insect vectors (Bennuru et al., 2018). Adult female worms release microfilariae (mf) into the bloodstream or the skin - depending on the filarial species - and the mf are thereafter ingested by the transmitting insect vectors (intermediate host). Mf taken up during the blood meal will undergo further developmental steps until the infective L3 stage is reached that initiates the transmission to the human host (Knopp et al., 2012, Bennuru et al., 2018). One of the characteristics of filarial nematodes is the dwelling of the adult worm in specific body locations that characterize different diseases (Ta et al., 2018). It is estimated that filarial parasite infections affect more than 150 million people around the world (Knopp et al., 2012). Commonly not lethal, the chronic morbidity associated with these diseases can lead to serious physical, psychological and economic impairments for the patients and their families. Among all of these diseases, onchocerciasis and LF are of greatest public health concerns and hence their inclusion in the list of the 20 NTDs by WHO (Bakowski and McNamara, 2019). However, the different Mansonella species and *Loa loa* (*L. loa*) gained recently increased attention because of the disease burden attributable to these parasites and their negative impact on control programs for LF and onchocerciasis in co-endemic areas. The following section presents an overview of Mansonellosis and Loiasis, touching upon epidemiology, diagnostic approaches, pathology, treatment options and public health relevance.

1.2 Mansonellosis

Mansonellosis is caused by three species belonging to the genus Mansonella, namely *Mansonella ozzardi*, *M. streptocerca*, and *M. perstans* (Mediannikov and Ranque, 2018). Widespread in Sub-Saharan Africa and certain parts of Latin America, the developmental cycle of all three species is similar, but they differ in distinct aspects including their biology, geographical distribution and clinical features. Mansonellosis is considered to be the most neglected among human filarial disease and therefore the least investigated (Ta-Tang et al., 2018). In Africa, *M. perstans* and *M. streptocerca* are prevalent but they differ by location of their mf in the host, which are either found mainly in the bloodstream (*M. perstans*) or in the skin (*M. streptocerca*).

1.2.1 Parasite biology

People are infected during the blood meal of an infected midge belonging to the genus *Culicoides* that introduces third-stage larvae (L3) into the host (Figure 1).

After the entry of L3 larvae into human skin, the larvae reach the body cavities, mainly the peritoneal cavity, where they develop into adult female and male worms (Ta-Tang et al., 2018). The size of the male worm is 45mm in length and 60µm in diameter while the larger female worm is 70 to 80mm long and 120µm in diameter. The development from L3 larvae until the adult stage is not well characterized based on the absence of suitable animal models to maintain this life cycle (Simonsen et al., 2011). Recent studies estimated that L3 larvae can reach the L5 stage in 21 to 30 days in *in vitro* cultures (Njouendou et al., 2017). Mature female worms release mf which move towards the bloodstream and circulate at any time of day and night (aperiodic). However, Asio and colleagues (Asio et al., 2009a) found that mf intensity was higher in the first 12 hours of the day than in the afternoon with a peak around 08:00AM.

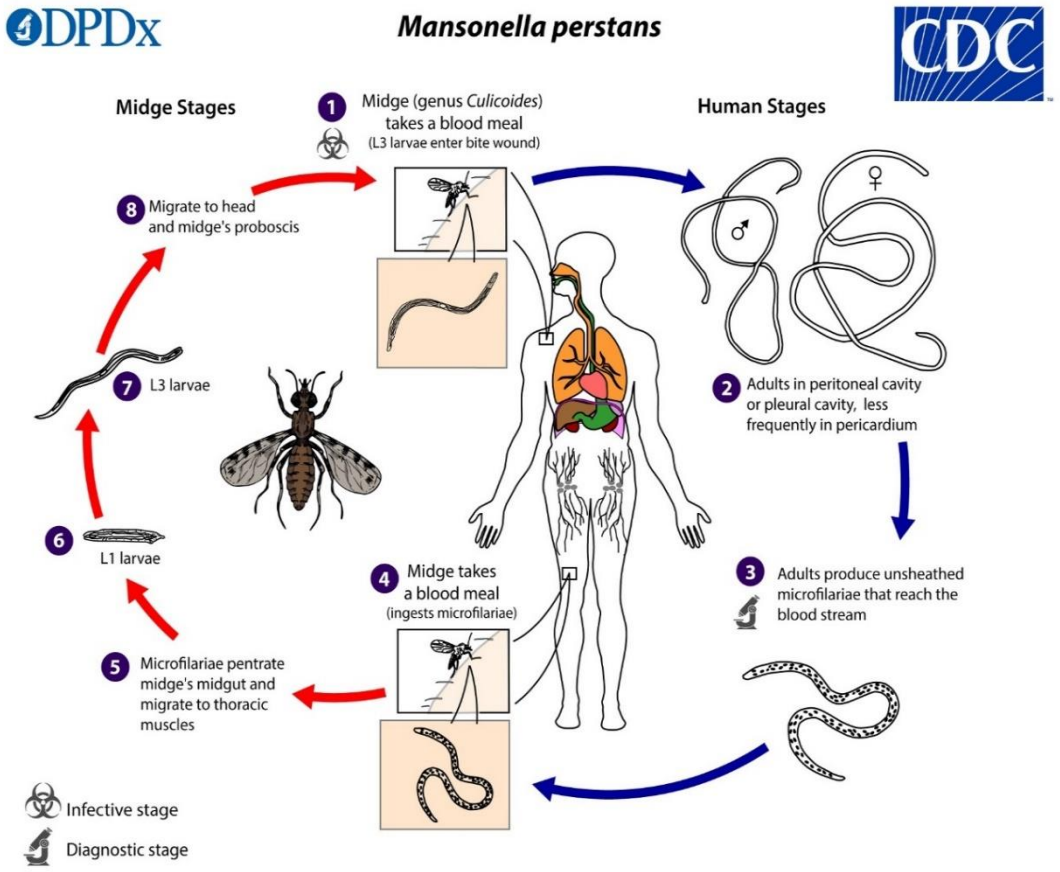

Figure 1: Life cycle of *Mansonella perstans* (picture taken from Center for Disease Control and Prevention, available on: https://www.cdc.gov/dpdx/mansonellosis/index.html, accessed 20th May 2021).

The development of mf in the insect vector takes about 6 to 12 days to reach the infective L3 stage (length 750–900µm) fit for transmission to the next human host (Simonsen et al., 2011, Mediannikov and Ranque, 2018). The lifespan of adult *M. perstans* worms in humans is unknown while mf persist at least for 3 months in the bloodstream (Simonsen *et al*., 2011).

1.2.2 Epidemiology of *Mansonella perstans*

M. perstans is thought to be the most widespread parasite among all filarial species in Sub-Saharan Africa (Mediannikov and Ranque, 2018). It is widely spread across Sub-Saharan Africa (Figure 2), except in Western (Eritrea, Ethiopia, Djibouti, Somalia), Southern (Botswana, Swaziland, Lesotho, Namibia, South Africa), and Northern (Algeria, Mauritania, Egypt, Libya, Morocco, Tunisia) Africa, from which local cases have not been reported (Simonsen et al., 2011). *M. perstans* has been reported in 33 countries in Sub-Saharan Africa, where more than 114 million people were estimated to be infected (Wanji et al., 2016b) and 600 million people are at risk for *M. perstans* infection (Simonsen et al., 2011, Ta-Tang et al., 2018).

The prevalence of *M. perstans* infection can be very high ranging from 80 to 100% in certain populations (Knopp et al., 2012, Mediannikov and Ranque, 2018). *M. perstans* infection is usually found in poor, rural populations and infections can be found even in children below 5 years of age (Asio et al., 2009b). The prevalence and intensity of the infection increase gradually with age and elderly men are more likely to be infected with higher mf intensities (Asio et al., 2009b, Simonsen et al., 2011).

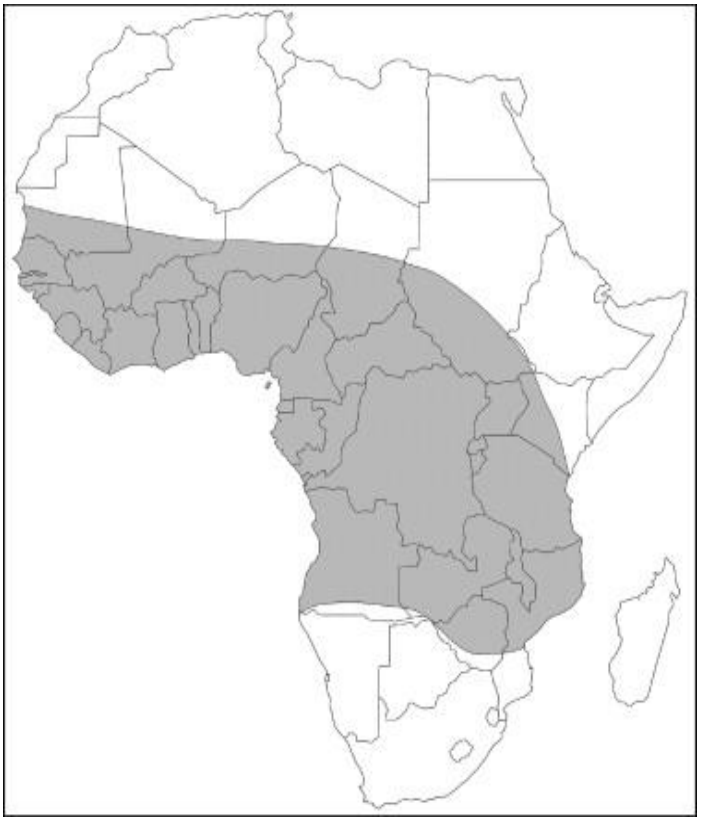

Figure 2: Geographic distribution of *M. perstans* in Sub-Saharan Africa (Simonsen et al., 2011).

Prevalence of *M. perstans* can vary strongly within endemic countries encompassing regions reported as free of infections and areas with higher number of people carrying mf. Knowledge about environmental factors that account for this differential distribution is limited although rain forest regions seem to favor *M. perstans* infections (Simonsen et al., 2011). Current studies suggest a strong link between the presence of banana or plantain plantations and *M. perstans* infection intensity and prevalence in affected populations (Ta-Tang et al., 2018).

1.2.3 Vector biology

M. perstans is transmitted by biting midge belonging to the genus *Culicoides* (Order Diptera: Family Ceratoponidae) (Simonsen et al., 2011, Wanji et al., 2019b), which is one of the most abundant hematophagous insect worldwide (Wanji et al., 2019b). This insect measures 1-4mm in length and its development takes 2 to 6 weeks depending of environmental conditions and follows along the egg, larva and pupa stage to finally reach the adult stage (Simonsen et al., 2011). Culicoides biting midge have a number of hosts including humans, livestock and wildlife for their blood meal but only the female midge takes blood for maturation of eggs (Sick et al., 2019). Humidity is a key factor in the development of the larval stage and therefore their distribution and abundance depend on the availability of moisture-rich breeding sites. Potential breeding sites include ponds, marshes, streams, bogs, beaches, swamps, banana or plantain stems, irrigation pipe leaks, soil, animal dung and rotting fruits (Wanji et al., 2019b, Sick et al., 2019). The presence of cattle, horses and sheep also seem to drive focal abundance of *Culicoides* spp. (Wanji et al., 2019b).

1.3 Loaisis

1.3.1 Parasite biology

Loaisis is caused by the filarial parasite *Loa loa* (*L. loa*) which is transmitted by biting flies of the genus *Chrysops* spp. *L. loa* infections are acquired by the human host when infective L3 stages actively migrate out of the mouthparts of the fly and enter the biting wound during the blood meal (Figure 3) (Pedram et al., 2017). When mature, approximately within 9 to 20 days after inoculation (Boussinesq, 2006, Whittaker et al., 2018), the adult worms reside freely in the subcutaneous tissues of humans where the female parasite can produce daily 12 000 to 39 000 sheated mf in the absence of reproductive constraints (Whittaker et al., 2018).

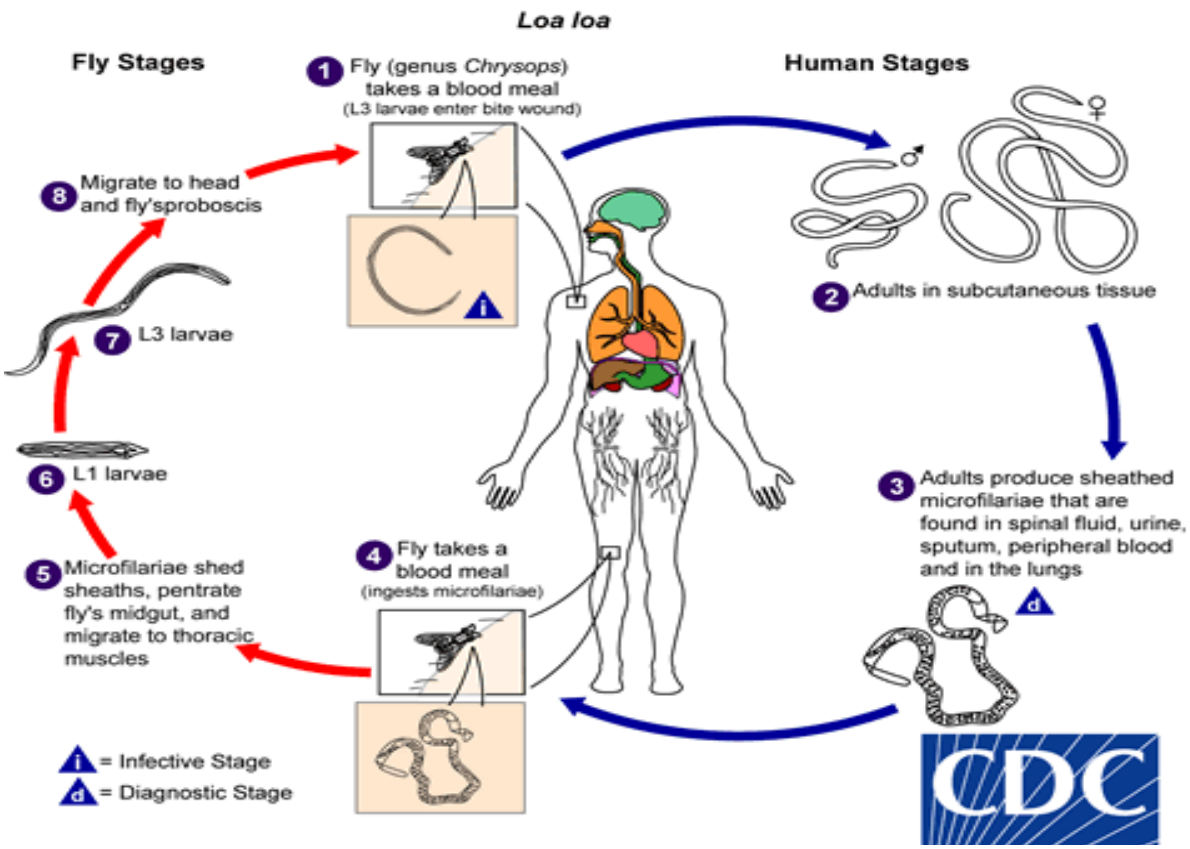

Figure 3: Life cycle of *L. loa* (Picture taken from Center for Disease Control and Prevention, available on: https://www.cdc.gov/parasites/loiasis/biology.html, accessed 20th May, 2021).

Despite this, mf are not detected in the peripheral blood until five to six months later (Whittaker et al., 2018), due to some delay in either their production (possibly caused by the time taken for adult stage sexual maturation and mating) or their release into the peripheral bloodstream from the lungs (reservoir of mf before invasion of peripheral blood) (Boussinesq, 2006, Whittaker et al., 2018). When mf circulate in the peripheral blood, they show diurnal periodicity with a peak observed between 10AM and 4PM, and then to decrease to very low mf levels during the night (Boussinesq, 2006, Boussinesq, 2012, Metzger and Mordmuller, 2014). In the vector, mf develop to L3 stage within 10 days and 12 days - depending on the ambient temperature (Whittaker et al., 2018). The life-span of *L. loa* mf is estimated to be around 6 to 12 months (Boussinesq, 2006) whereas the adult worm seems to live more than 15 years in the human host (Knopp et al., 2012, Whittaker et al., 2018).

1.3.2 Epidemiology of *Loa loa*

Loaisis, also known as African eye worm, is restricted to 10 countries in Africa where more than 10 million people are infected (Metzger and Mordmuller, 2014). Furthermore, an estimated 14.4 million people live in high-risk areas where the estimated prevalence of *L. loa* is more than 40%, and 15.2 million people live in intermediate risk regions where the parasite prevalence is around 20% to 40% (Metzger and Mordmuller, 2014). Travelers and immigrants are also at risk of Loasis infection (Padgett and Jacobsen, 2008). Loiasis is endemic in the rainforest and some savannah areas of Western and Central Africa including Nigeria, Cameroon, Equatorial Guinea, Gabon, Angola, Central African Republic, Chad, Democratic Republic of the Congo, Republic of Congo and Sudan (Figure 4) (Metzger and Mordmuller, 2014).

In some endemic regions, loiasis is the second or third most common cause of medical consultations (Metzger and Mordmuller, 2014). Most of infected people are asymptomatic but some people develop clinical symptoms including intense itching, Calabar swelling or a visible eye worm associated with elevated eosinophil granulocyte levels in peripheral blood (Mouri et al., 2019). These symptoms are due to hypersensitivity reactions after release of mf or antigenic material from adult worms, or are based on migration of the worm under the bulbar conjunctiva (Mouri et al., 2019, Whittaker et al., 2018). The long lifespan of adult worms and continuous exposure to infective vectors would mean that individuals not treated might live with their worm infection for the entire life span (Whittaker et al., 2018). *L. loa* infections in human show sex-specific patterns, meaning that the prevalence of mf infection is higher in female than in male populations across all age groups (Whittaker et al., 2018).

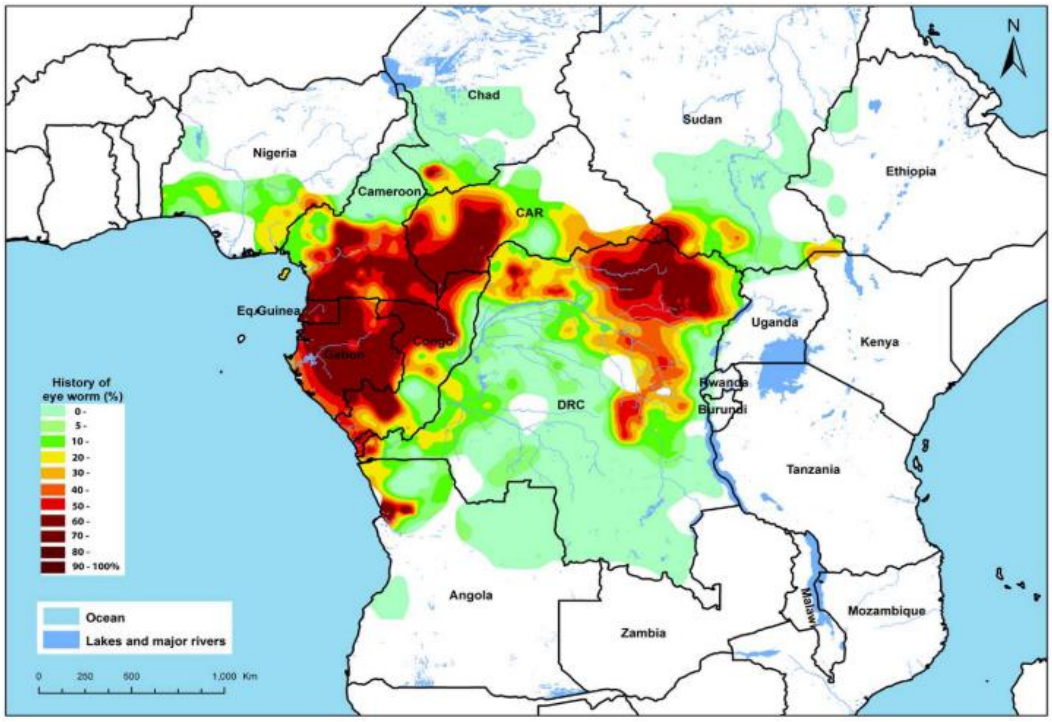

Figure 4. Geographic distribution of Loaisis based on estimated prevalence of eye worm history in Africa (Zouré et al., 2011).

Moreover, loiasis prevalence has been associated with factors including environmental temperature, rainfall, ground water, tree height, forest coverage and/or intensity of light that were identified as important factors driving vector biting behavior and infections rates (Badia-Rius et al., 2019). The epidemiology of loaisis has been primarily investigated because of severe adverse events observed among individuals that are carrying high numbers of mf and are simultaneously treated with the drugs ivermectin or diethylcarbamazine (Whittaker et al., 2018).

1.3.3 Vector biology

Chrysops spp. are small bloodsucking flies that live in and around forested and muddy areas, on the edges of water reservoirs and in dying or rotting vegetation. *Chrysops silacea* and *C. dimidiata* (Order Diptera: Family Tabanidae) are the two tabanid flies responsible for the transmission of *L. loa* (Badia-Rius et al., 2019). The adult female fly (5 to 20mm in length) requires a blood meal to produce eggs

within 5 to 6 days for the duration of the gonotrophic cycle (Whittaker et al., 2018). Eggs are deposited near water sources and hatch within 5 to 7 days. The larvae fall into water or soil for the next developmental stages (Padgett and Jacobsen, 2008). People mostly exposed are those living and working in such environments (Knopp et al., 2012). *C. silacea* and *C. dimidiata* display strong diurnal biting preferences, with the greatest biting activity during the morning (9 to 11AM) and late afternoon (2 to 4PM), coinciding with the appearance of mf in the blood of infected humans (Whittaker et al., 2018). Recent studies show that the spatial distribution and ecological niche of both species depend on factors including annual mean temperature (24.6°C and 24.1°C, respectively), annual rain precipitation (1848.6mm and 1868.8mm, respectively), altitude (368.8m and 400.6m, respectively) and tree canopy height (22.4m and 25.1m, respectively) (Badia-Rius et al., 2019). *C. silacea* is more dominant in cleared forested areas (tree canopy coverage not less than 34%), while *C. dimidiata* is more closely associated with forested areas (tree canopy coverage more than 74%) (Badia-Rius et al., 2019). Moreover, both species are strongly attracted by wood fire smoke, which increases biting activity 11 fold and 4 to 5 fold for *C. silacea* and *C. dimidiate*, respectively (Whittaker et al., 2018).

1.4 Diagnosis

1.4.1 Clinical diagnosis

M. perstans infections are commonly reported to remain asymptomatic (Simonsen et al., 2011). Some studies have associated the presence of adult worms living in serous body cavities with some disease manifestations (Simonsen et al., 2011). These symptoms include itching of the skin, severe pain in the abdomen and liver regions, joint or muscle pain, neurological and psychological symptoms and lymphedema in face and limbs like Calabar swellings (Asio et al., 2009b, Simonsen et al., 2011, Mediannikov and Ranque, 2018, Ta-Tang et al., 2018). A high level of blood eosinophilia has been reported as manifestation of *M. perstans* infection (Mediannikov and Ranque, 2018, Ta-Tang et al.,

2018). However, these clinical symptoms are not suitable to diagnose specifically Mansonellosis (Ta-Tang et al., 2018). Furthermore, most of knowledge relating to *M .perstans* arose from studies on other human filariasis (*W. bancrofti*, *L. loa* and *O. volvulus*) where *M. perstans* had co-infected some patients or from case reports of expatriates or travelers returning from filariasis endemic areas to Europe (Asio et al., 2009b, Mourembou et al., 2015).

Main clinical manifestations of *L. loa* infection (Zouré et al., 2011, Akue et al., 2018, Puente et al., 2020b) include the Calabar swelling and ocular passage of the adult worm (also known as eye worm) under the conjunctiva. In addition, *L. loa* infection has started to become associated with presentation of arthritis, cardiomyopathy, encephalopathy, lymphangitis, peripheral neuropathy, retinopathy and an increased risk of death (Chesnais et al., 2017) indicating that it might not be as benign as previously assumed . Encephalitis has been observed also after treatment of LF and onchocerciasis with ivermectin or diethylcarbamazine in people co-infected with *L. loa* that have more than 30,000 mf/mL of blood - posing a serious risk to LF and onchocerciasis control programs (Kelly-Hope et al., 2017).

1.4.2 Parasitological diagnosis

The current gold standard of diagnosis of filarial worm infections is primarily based on detection and morphological identification of mf of *L. loa* and *M. perstans* by light microscopy (Figure 5). (Kelly-Hope et al., 2018a). Depending on the location of the mf in the skin or blood, different methods are followed to detect these filarial worms (Mathison et al., 2019).

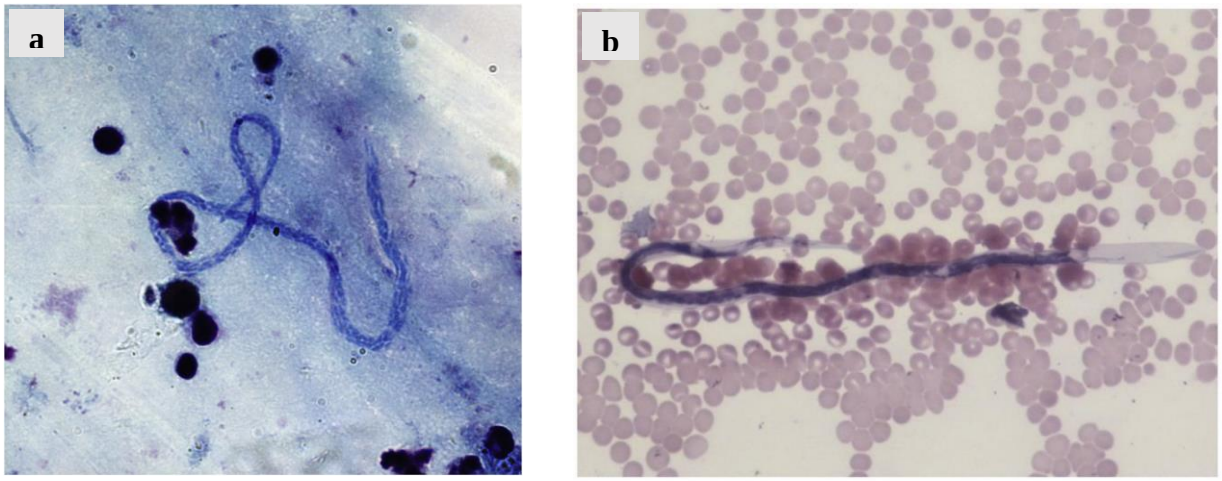

Figure 5. Filarial worms morphological identification: a) *M. perstans* mf in stained thick blood film (Giemsa straining, ×1000) (Mediannikov and Ranque, 2018). B) *L. loa* mf in a thin blood film stained with Giemsa and Dellafield stain, which depicts the mf sheath clearly (Miller and Bain, 2015).

When mf are present in high numbers in the blood of patients, they can be observed by examining blood smears (20 to 60µl finger prick blood) stained with Giemsa or hematoxylin and identified through their distinct morphologic features (Table 2) (Boussinesq, 2012, Mathison et al., 2019). To increase diagnostic sensitivity when microfilaraemia is low, larger volumes of blood of approximately 1ml can be collected followed by concentration techniques such as the Knott's filtration technique (Boussinesq, 2006, Mathison et al., 2019). Blood for diagnostic purposes can be collected at any time of the day for *M. perstans* detection (Ta-Tang et al., 2018) while for *L. loa* infections, blood needs to be drawn between 10AM and 4PM (Boussinesq, 2012). *M. perstans* mf are typically short and thin (190–200µm × 4.0–4.5µm), unsheathed, and the nuclei extend to the tip of the blunt tail (Figure 5a) (Ta-Tang et al., 2018, Mediannikov and Ranque, 2018). In contrast, *L. loa* mf are 230 to 300µm long and 6 to 8µm in diameter, sheathed with the body nuclei extend continuously to the tip of the tail and mf display a hooked tail at one end (Figure 5b) (Padgett and Jacobsen, 2008, Mathison et al., 2019). In Table 2, an overview is provided of the morphological features and geographical distribution of mf causative agents of human filariasis.

Although microscopy of stained blood smears is relatively simple and inexpensive to perform, accurate differential diagnosis requires a skilled and experienced microscopist who can identify mf of *M. perstans* – a task that can be challenging because of their small size (Simonsen et al., 2011). In addition, the diagnosis of *L. loa* mf is difficult because it depends on the time of blood collection and more than 50% of infected people seem to harbor adult worms without detectable mf in the blood stream at any time (Metzger and Mordmuller, 2014, Akue et al., 2018). Recently, two research groups described two novel and sensitive techniques for *L. loa* mf quantification based on either a cell phone microscope with software that can determine mf abundance through pixel changes in two sequential recordings (D'Ambrosio et al., 2015, Geary, 2016) or by using flow cytometry based methods allowing automated mf quantification in whole blood (Mouri et al., 2019).

Table 2: Features for morphological detection and discrimination of human filarial worms (Mathison et al., 2019)

Species	**Geographic Distribution**	**Preferred specimen type**	**Periodicity**	**Length (µm)**	**Key diagnostic morphologic features**
W. bancrofti	Tropics and subtropics of Africa, Asia, South Pacific, South America, Caribbean	Blood	Nocturnal	244–296*a*	Usually colorless sheath (Giemsa), anucleated tail, short headspace, relatively loose nuclear column
B. malayi	Southeast Asia, including India, Philippines, Vietnam, Malaysia, Indonesia, South Korea	Blood	Nocturnal	177–230*a*	Usually hot-pink sheath (Giemsa), terminal and sub-terminal tail nuclei separated by large gaps, long headspace
B. timori	Lesser Sunda Archipelago	Blood	Nocturnal	310 (avg)*a*	Usually colorless sheath (Giemsa), terminal and sub-terminal tail nuclei separated by large gaps, long headspace
L. loa	West Central Africa, south of the Sahara	Blood	Diurnal	231–250*a*	Usually colorless sheath (Giemsa), tail nuclei randomly distributed to the tip of the tail, short headspace, relatively dense nuclear column
M. perstans	Sub-Saharan tropical Africa, Central and South America, Caribbean	Blood	None	190–200*a*	Sheath never present, compact nuclear column, tail nuclei densely packed to tip of blunt tail
M. ozzardi	Central and South America, Caribbean	Blood	None	163–203*a*	Sheath never present, compact nuclear column, anucleated tail, tail tapered and pointed
M. streptocerca	Tropical sub-Saharan Africa	Skin snips	None	180–240*b*	Sheath never present, hooked tail with nuclei arranged to the tip of the tail
O. volvulus	Western and central Africa south of the Sahara, Yemen, Central and South America	Skin skips	None	304–315*b*	Sheath never present, tail tapered and often flexed, anucleated

a: stained blood films
b: skin snips

1.4.3 Immuno-diagnostic approaches

Immunological methods are based on detection of either antibodies or antigens in the human host. In filarial infection, the immunodiagnostic assays are based on detecting immunoglobin (Ig)G4 isotype because these are significantly elevated in microfilaremic people (Ta-Tang et al., 2018). It was found that some immunodiagnostic assays developed for onchocerciasis and LF disease-control programs would show some degree of cross-reactivity with *Mansonella* spp. and *L. loa* (Simonsen et al., 2011, Wanji et al., 2016a, Ta-Tang et al., 2018). Up to now, there is no suitable immunological assay for sensitive and specific detection of *M. perstans* available and the sero-diagnostic tests for *L. loa* is currently not standardized (Ta-Tang et al., 2018). A protein (LOAG-16297) circulating in human urine was identified as biomarker specific to *L. loa* and used to develop a test to quantify circulating antigen through the luciferase immunoprecipitation system (LIPS) assay (Drame et al., 2016). The LOAG-16297 antigen LIPS assay has a sensitivity and specificity of 76.9% and 96%, respectively (Drame et al., 2016, Akue et al., 2018). Furthermore, a recombinant antigen, the *L. loa* derived SXP-1, was tested as diagnostic marker using sera from monkeys experimentally infected with *L. loa* in an enzyme-linked immunosorbent assay (ELISA)-IgG4 that showed low sensitivity (56%) but high specificity (98%) (Klion et al., 2003). Recently, an *L. loa*-SXP-1 based lateral flow rapid diagnostic test (RDT) was developed and it was found to be more sensitive (94%) with 82 to 100% specificity - depending on the antigen control panel included in the experiment (*L. loa*, *W. bancrofti*, *M. perstans*, *O. volvulus*, *Strongyloides stercoralis*) (Pedram et al., 2017). Gobbi and colleagues showed possible positivity with RDT in case of past *L. loa* infection, so the test cannot be used for post-treatment follow up (Gobbi et al., 2020). Nevertheless, the RDT could be recommended for mapping *L. loa* prevalence in endemic countries (Gobbi et al., 2020).

1.4.4 Molecular diagnosis

The best technique currently available for diagnosis of *Mansonella* spp. and *L. loa* is based on NA-based molecular diagnosis including PCR or Loop-mediated Isothermal Amplification (LAMP) that amplify specific DNA target sequences (Alhassan et al., 2015, Akue et al., 2018, Ta-Tang et al., 2018, Poole et al., 2019, Sandri et al., 2020). The PCR-based target mostly deployed is the internal transcribed spacer (ITS) regions located between the conserved ribosomal genes 18S, 5S-5.8S and 28S of filarial DNA enabling to distinguish filarial nematodes (Alhassan et al., 2015, Poole et al., 2019). The size of this region varies among filarial worms that yield amplicons of different sizes for each species (Tang et al., 2010). Thus, PCR assays are able to differentiate *M. perstans* from other Mansonella species (Tang et al., 2010, Ta-Tang et al., 2018). In addition to ITS gene based detection, the LLMF72 gene sequence was found to represent a *L. loa*-specific DNA target in human blood (Fink et al., 2011, Alhassan et al., 2015) which enables to perform a highly sensitive and specific qPCR assay (Fink et al., 2011). PCR-based diagnostic methods generally require better trained personnel and relatively expensive equipment and consumables that might constitute a limitation to research facilities working under mobile field conditions or in low-income settings (Poole et al., 2019).

The LAMP method represents an alternative to PCR assays since it amplifies in a one-step reaction the target DNA with high specificity, efficiency and speed under isothermal conditions (Fernandez-Soto et al., 2014). This assay have been developed for other human filarial nematodes including *Brugia malayi*, *Brugia timori*, *W. bancrofti* and *O. volvulus* (Poole et al., 2019). A LAMP assay was described for *L. loa* and *M. perstans* targeting the LLMF72 gene (Drame et al., 2014) and the *M. perstans* Mp 419 repeat, respectively (Poole et al., 2019). Its simplicity without the need of expensive equipment offers considerable advantages compared to PCR assays (Poole et al., 2019). Limitations might be variability in the target gene sequences across regions or over time as result of selective pressures as has been observed in the *L. loa*-LAMP assay (Drame et al., 2014). Although highly

sensitive and specific, molecular based-diagnostics remain difficult to implement as point-of-care test (Mouri et al., 2019) and are not yet integrated in clinical routine assessments in health posts or hospitals (Gobbi et al., 2020).

1.5 Drug treatment of Mansonellosis and Loaisis

The drugs used to treat human filariases include Albendazole (ALB), Ivermectin (IVM) and Diethylcarbamazine (DEC). These drugs show however limited efficacy against *M. perstans* infections with a high proportion of treatment failures (Batsa Debrah et al., 2019). In fact, *M. perstans* seem to be one of the most difficult filarial disease to treat since it likely exists as distinct genetic strains - some of which lack the endosymbiont Wolbachia resulting in variability of drug treatment outcomes (Ta-Tang et al., 2018, Sandri et al., 2021, Sandri et al., 2020). However, a regimen of anthelminthic drugs including Mebendazole alone (100 mg/12 hours for 30 days) or in combination with DEC (200mg/12 hours then 100 to 200mg/day for 21 days) proved to show good efficacy against *M. perstans* (Bregani et al., 2006, Ta-Tang et al., 2018). The antibiotic doxycycline (200mg/day for 6 weeks) has proven to be effective and safe in treatment of *M. perstans* strains harboring the endosymbiont Wolbachia (Ta-Tang et al., 2018, Batsa Debrah et al., 2019, Sandri et al., 2020).

In loiasis, adult *L loa* worms can be surgically extracted from the eye which necessitates the anesthesia of the infected eye followed by extraction of the worm using a forceps through a small incision in the conjunctiva (Knopp et al., 2012). However elimination of mf need more precaution because of the risk of adverse effects directly correlated to mf density in the patient (Mouri et al., 2019). Currently followed strategies for loiasis treatment include the following: 1) standard treatment (DEC 8 to 10mg/kg per day for 3 weeks) if mf density < 2000 mf/mL; 2) if mf density >2000mf/mL and <8000mf/mL, it is recommended to administer IVM (a single dose of 150µg/kg). Mild side effects similar to those observed with DEC can occur; 3) if mf density >8000 and <30,000mf/mL, IVM can be given but close surveillance with hospitalization during the first three to four days after treatment

is needed; 4) If mf density >30,000 mf/ mL, ALB alone (2 x 200mg/day for 21 days) is recommended until reduction of mf levels is achieved (Knopp et al., 2012, Boussinesq, 2012, Mouri et al., 2019).

1.6 Host immune interaction with *M. perstans* and *L. loa*

The prototypical host immune response to helminth infections in humans is characterized as Type-2 (or (Th2) response and it involves: 1) secretion of cytokines like Interleukin (IL)-4, IL-5, IL-9, IL-10 and IL-13; 2) induction of antigen-specific IgG1, IgG4 and IgE isotypes and 3) increased proportions of eosinophile granulocytes in peripheral blood and presence of alternatively activated macrophages. This immune response occurs primarily at the time of patency, when egg laying or mf release from adult females occurs (Maizels et al., 2009). Its initiation requires interaction with many different cell types, most notably dendritic cells and macrophages, eosinophils, mast cells and basophils, dermal cells, epithelial cells and innate lymphoid cells (Nutman, 2015). Filarial parasites commonly modulate the host immune response to ensure their long-term survival (Njambe Priso et al., 2018), thereby maintaining asymptomatic infections with elevated plasma levels of parasite antigen specific IgG4 (Maizels and Yazdanbakhsh, 2003), regulatory T cells (Treg), regulatory B (Breg) cells and alternatively activated macrophages with high production capacity of IL-10 (Girgis et al., 2013, Nutman, 2015). Parasite secretions also appear to play an important role in host immune modulation (Nutman, 2015). The phosphorylcholine, present in the excretory/secretory products of helminths contains ES-62, a molecule that inhibits effector T and B cells. Furthermore, some protease inhibitors (cystatin and serpin) are involved in the production of the regulatory cytokine IL-10, inhibit neutrophil proteinases and also participate in the regulation of T cells, B cells and the complement system (Nutman, 2015). This shift to a immune regulatory milieu leads to suppression of T cell proliferation and decreased interferon-γ (IFN-γ) and IL-2 production in response to filarial antigens as well as to bystander antigens (Ricciardi and Nutman, 2021). Despite residence in tissues and migration through blood, *M. perstans* infection seldom causes detectable pathology which might be

based on the strong immune-modulating effect of the parasite (Hillier et al., 2008). Recent studies showed that microfilaremic individuals had significantly reduced cytokine (IL-4, IL-6 and IL-12p70) and chemokine levels (IL-8 and RANTES), but significant higher MIP-1β and increased *M. perstans*-specific IgG4 levels compared to amicrofilaremic individuals. Moreover, analysis of immune cell in peripheral blood from microfilaremic individuals revealed increased Th2 cells response, natural killer cells, regulatory B cells and Treg subsets but decreased type 1 regulatory T cells (Ritter et al., 2018, Bottieau et al., 2021).

Immunological studies conducted in *L. loa* infections found polyclonal IgE responses and elevated levels of antigen-specific IgG4 which is similar to observations in other filarial worm infections (Pinder et al., 1994, Akue et al., 1994, Njambe Priso et al., 2018). Furthermore, some studies suggested that Th1 and Th2 immune responses (Baize et al., 1997) and eosinophil-associated cytokines (Herrick et al., 2015) play a major role during *L. loa* infections (Chunda et al., 2020). Interestingly, *L. loa* can induce immune cell memory which might contribute to immunity and protection against reinfection (Chunda et al., 2020).

1.7 *Mansonalla perstans* and *Loa loa* infections as public health problem

The prevailing perspective of mansonellosis and loiasis as benign parasitic infections with little or no direct effect on patients' well-being has left these diseases somewhat out of focus of the public health community (Knopp et al., 2012). However, loiasis can represent the second most stated reason for medical visits, after malaria, in endemic areas (Akue et al., 2011). In Cameroun, an estimated 14.5% of mortality was attributed to loiasis which is higher than the estimated 5% mortality across West Africa caused by onchocerciasis (Whittaker et al., 2018). The impact of *M. perstans* and *L. loa* on public health is certainly significant in the presence of other pathogens in co-endemic areas. Loaisis is a major impediment for the treatment of onchocerciasis and LF by mass chemotherapy, using IVM

and DEC due to the risk of fatal severe adverse events - notably encephalopathy and death (Badia-Rius et al., 2019). This side effects are commonly observed in individuals with mf concentrations up to 30,000mf/mL (Kelly-Hope et al., 2018b). *L. loa* infections can negatively affect the accuracy of immunological tests commonly used for detection and mapping of LF based on antibody cross-reactivity on the filarial strip test (Wanji et al., 2019a). *M. perstans* is also implicated in the impairment of LF and onchocerciasis control programs based on difficulties in differential diagnosis using microscopy. In areas where different filarial worms are co-endemic, misclassification of infection by microscopy can be problematic (Wanji et al., 2019a). *M. perstans* mf have been reported in the skin which is commonly thought of being the location of *O. volvulus* mf (Moya et al., 2016, Ta-Tang et al., 2018). Some studies demonstrated cross-reactivity of *M. perstans* proteins with recombinant ov33 and ov20/ovS1 antigens used to detect *O. volvulus* (Simonsen et al., 2011). Furthermore, *M. perstans* might cause an increased susceptibility and worsened disease course of tuberculosis, HIV and malaria (Batsa Debrah et al., 2019) and a lower efficacy of vaccine induced protection (Ta-Tang et al., 2018). Some studies showed that *M. perstans* infection during pregnancy might induce poorer responses to the tuberculosis vaccine Bacillus Calmette Guerin and tetanus toxoid in the offspring (Blackwell, 2016, Ta-Tang et al., 2018). In malaria endemic areas, *M. perstans* co-infection during pregnancy seem to affect the burden of malaria morbidity among children (Ndibazza et al., 2013, Blackwell, 2016).

1.8 Overview on Malaria

Malaria is recognized by WHO as an important public health problem. It is widely spread in 85 countries (WHO, 2021) of the tropical and sub-tropical areas of South-East Asia, the Amazon region and Sub-Saharan Africa (Nascimento et al., 2019). In 2020, an estimated 241 million malaria cases occurred worldwide and 627 000 related death where reported and 77%, the percentage of total malaria deaths occurred among children under 5 years of age (WHO, 2021). The Sub-Saharan African

region is the most malaria affected region carrying more than 90% of total cases and death burden globally (WHO, 2019). Malaria infection is caused by Apicomplexan parasites of the genus *Plasmodium* spp. and the parasite is transmitted by the female *Anopheles* mosquito during a blood meal (Basu and Sahi, 2017). Six species are responsible of the malaria worldwide including *Plasmodium falciparum, P. vivax, P. malariae, P. knowlesi, P. ovale curtisi and P. ovale wallikeri* (Milner, 2018). The parasite has different stages in the human host (Figure 6), which leads to characteristic clinical symptoms during asexual blood stage parasitemia (Meibalan and Marti, 2017, Maier et al., 2019). With timely drug treatment, symptoms subside rapidly in uncomplicated malaria cases and parasites are cleared completely.

However, malaria might lead to significant clinical complications, including cerebral malaria, severe malarial anemia, coma or death (Buck and Finnigan, 2021). The antimalarial therapeutic and chemoprophylactic regimens are guided by the malaria species, geography, drug susceptibility, and patient demographics (Ashley et al., 2018, Buck and Finnigan, 2021). Most malaria attributable deaths and severe diseases are caused by *P. falciparum* (White, 2018). Malaria-associated maternal illness and anemia, preterm birth and low birthweight of newborns are also mostly the result of *P. falciparum* infection (Ndibazza et al., 2013, WHO, 2020b). Overall, about 24 million children were estimated to be infected with *P. falciparum* in 2018 and an estimated 1.8 million of them were likely to have severe anemia (WHO, 2019). Tools currently used to reduce transmission focus on antimalarial combination therapy and vector-control measures such as insecticide treated bed nets and indoor spraying (Ashley et al., 2018). Artemisinin-based combination therapies are currently used as a first-line treatment worldwide (Meibalan and Marti, 2017).

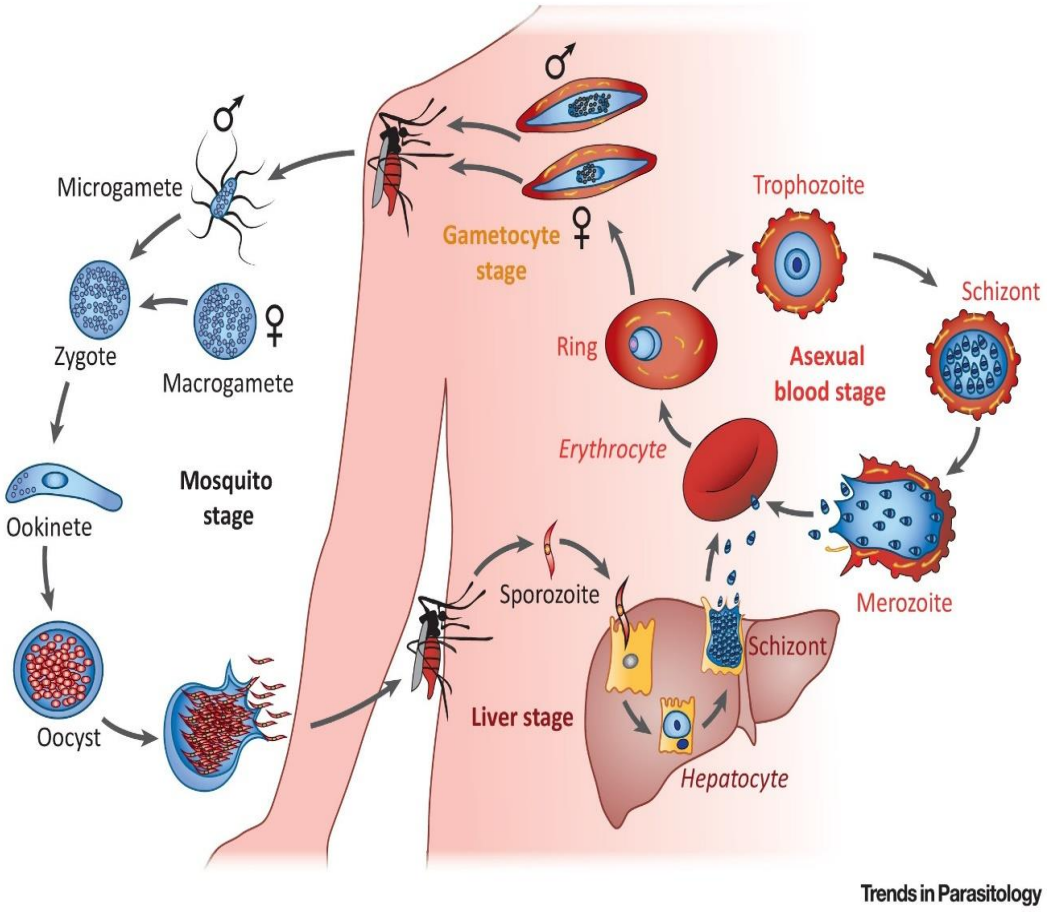

Figure 6: The *Plasmodium falciparum* life cycle (Maier et al., 2019). The human phase of the malaria starts with the inoculation of sporozoites during the bite of an infected female Anopheles mosquito. The parasite undergoes a pre-erythrocytic liver stage which typically lasts for 5- 6 days before the asexual blood stage, where serial cycles of asexual replication produce rising parasite numbers and human disease. A subpopulation of intra-erythrocytic parasites switches to sexual (female and male) development. This stage allows the transmission of malaria to the mosquito via a blood meal. Male gametocytes exflagellate in the mosquito midgut and male and female gametes fuse to form zygotes that transforms into mobile ookinetes that passes through the gut wall. The oocyst releases sporozoites which migrate to the mosquito salivary glands, ready to be transmitted to the human host and completing the life cycle (Meibalan and Marti, 2017).

Confirming the presence of parasites in all malaria cases ensures species-specific antimalarial treatment (Ashley et al., 2018) that favor timely therapy enabling to reduce malaria morbidity and mortality primarily in young children. Diagnosis based malaria treatment also prevents unnecessary use of antimalarials thereby reducing threat of emerging drug resistance development (Basu and Sahi, 2017). Rapid diagnostic tests (mRDTs) predominate as first-line *Plasmodium* spp. diagnostic tool (Ashley et al., 2018). Malaria RDTs (mRDTs) detect circulating antigens like the *P. falciparum* specific histidine rich protein 2 (PfHRP2) or histidine rich protein 3 (PfHRP3) and the pan-*Plasmodium* spp. enzymes lactate dehydrogenase (pLDH) or Aldolase (Moody, 2002, Hosch et al., 2022). The limit of detection for mRDTs is around 200 parasites/µL blood, especially PfHRP2-based tests (McMorrow et al., 2011, Mathison and Pritt, 2017). Although mRDTs present great advantages in terms of rapidity, simplicity and ease of handling, it shows limitations including lack of sensitivity in detecting low parasitemia levels and reporting of false-positive results after recent parasite treatment (Ochola et al., 2006, Mathison and Pritt, 2017). mRDT sensitivity decreases also in pregnant women, possibly due to sequestration of antigens in placental circulation (Mathison and Pritt, 2017). Furthermore, a number of reports have highlighted the issue of *P. falciparum* strains carrying *pfhrp*2/*pfhrp*3 gene deletions resulting in false negative PfHRP2-based mRDTs that may have serious consequences for diagnosis and treatment (Schindler et al., 2019, Poti et al., 2020).

1.9 Study site

Bioko Island is part of Equatorial Guinea in West Africa and lies approximately 40 km off the coast of Cameroon. Bioko Island covers an area of 2,017 km2 and is divided into two provinces, Bioko Norte and Bioko Sur (Figure 6). Four districts are delineated on Bioko Island, namely Malabo, the capital of Equatorial Guinea, Baney in Bioko Norte, Luba and Riaba both located in Bioko Sur (Herrador et al., 2018a, Ta et al., 2018). Bioko Island has an estimated population size of 340,000 people (Herrador et al., 2018a) with around 85% living in Malabo (Guerra et al., 2019). The island

shows humid tropical climate with a rainy season from April to October and a dry season from November to March (Herrador et al., 2018a). Mean daily maximum and minimum temperatures range between 29 to 32 °C and 19 to 22 °C, respectively (Hernández-González et al., 2016).

Malaria has historically been hyper-endemic in Bioko Island with annual entomological inoculation rates over 750 and 250 infectious bites per person per year by *Anopheles funestus* and *An. gambiae,* respectively (Bradley et al., 2013). Therefore, the Government of Equatorial Guinea started an intensive malaria control strategy in 2004 (Bradley et al., 2013, Guerra et al., 2019). This strategy is based on combinations of integrated malaria interventions including massive vector control programs, prevention of malaria during pregnancy, behavioral change communications, monitoring and evaluation and operational research (Overgaard et al., 2012). The results of these combined efforts have reduced malaria prevalence by over 75% between 2004 and 2016 (Cook et al., 2018). As part of the monitoring and evaluation plan, a malaria indicator survey (MIS) is carried out yearly on Bioko Island to assess the impact and effectiveness of the malaria control program in a larger number of households including volunteers from a broad age range.

On Bioko Island, filarial worms have been reported including *O. volvulus*, *L. loa*, *M. streptocerca* and *M. perstans* (Ta et al., 2018, Herrador et al., 2018a). A literature search of reports conducted showed that from 1978 to 2020, 20 publications described Mansonellosis, Loasis, Onchocerciasis and LF in Equatorial Guinea (Table 3). Six reports described filarial nematode infections among Equato-Guineans living abroad, in Spain, Singapore or Morocco, while three were case reports of foreigners returning from Equatorial Guinea. Only one smaller scale, cross-sectional study investigated the prevalence of *L. loa* and *M. perstans* on Bioko Island in the local population using qPCR-based diagnostics (Ta et al., 2018).

Onchocerchiasis has been targeted by the national elimination program that was introduced in 2000 and was based on mass treatment with IVM combined with a large-scale larvicide distribution (temephos) operations using ground-based applications (Traoré et al., 2009).

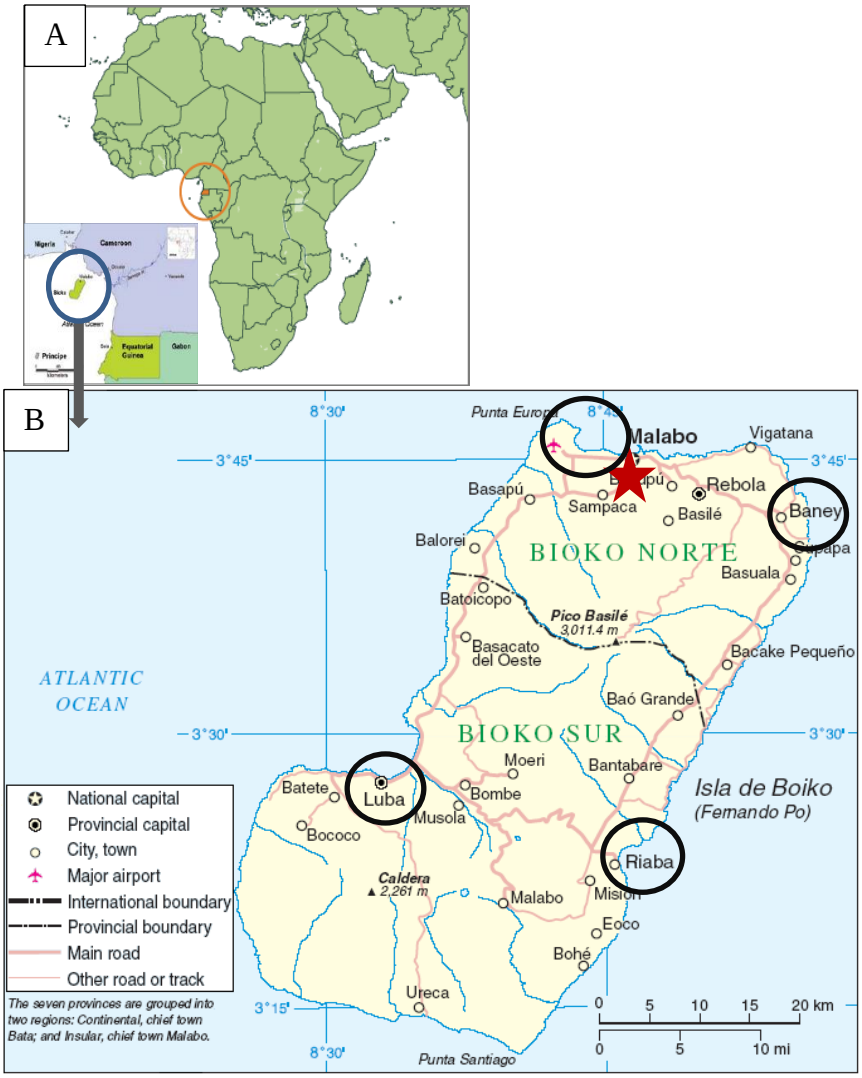

Figure 7. (A) Map of Sub-Saharan Africa with Equatorial Guinea and Bioko Island marked in orange. (B) Map of Bioko Island with the four districts marked in black. (https://fr.wikipedia.org/wiki/Bioko). Accessed on 20th May 2021

Since 2005, the vector for onchocerchiasis, *Simulium yahense*, has been reported as eliminated and the IgG4 seroprevalence for the Onchocerchiasis specific antigen OV-16 was very low in children < 10 years of age in 2014 (Moya et al., 2016). The last mass treatment with IVM was administered during 2012 in urban Malabo and during 2016 elsewhere on the island according to the Ministry of Health (Herrador et al., 2018a).

Table 3: Literature overview of studies carried out on filarial worms in Equatorial Guinea.

Pathogen	Study	Population	Prevalence	Method	Location	Year of investigation	Reference
L. loa,	Retrospective study	Immigrants (94 % Equatoguinean)	131 cases (out of 5700 individuals) 45.8 % only *L.loa* 54.2 % co-infection with (*L. loa, O. volvulus, M. streptocerca, W. bancrofti*)	- Clinical manifestation (eye visualization, eosinophilia, calabar swelling, subcutaneous lesion) - Microscopy	Spain	19 years	(Puente et al., 2020b)
M. perstans	Retrospective study	Immigrants (97.6% Equatoguinean)	503 cases 61,2 % only *M. perstans* 38.8 % co-infection with (*L. loa, O. volvulus, M. streptocerca, W. bancrofti*)	- Clinical manifestation (eye visualization, eosinophilia, calabar swelling, subcutaneous lesion), - Microscopy	Spain	19 years	(Puente et al., 2020a)
L. loa	Case report	Equatoguinean	One case (9 years old girl)	- Clinical manifestation (eye visualization), - Microscopy, - PCR	Spain	2019	(Placinta et al., 2019)
L. loa *O. volvulus* *M. perstans*	Cross-sectional study	Equatoguinean (543 participants)	*M.perstans* (8.8%) *L.loa* (0.7%).	- RT-PCR	Bioko Island	2014	(Ta et al., 2018)
O. volvulus *W. bancrofti*	Cross-sectional study	Equatoguinean (7052 children)	*O. volvulus* and *W.bancrofti* negative by PCR	-Serological (Ov 16 RDT, ELISA) -RT-PCR	Bioko Island	2016-2017	(Herrador et al., 2018b)
O. volvulus	Cross-sectional study	Equatoguinean (150 households)	*O. volvulus*	-Serological (ELISA)	Bioko Island	2014	(Gómez-Barroso et al., 2018)
L. loa	Case report	Immigrant (American) from Bioko Island	One case (25 years old woman)	-Clinical manifestations (swelling the ankle, knee pain, pruritis, eosinophilia) -PCR	USA	2016	(Priest and Nutman, 2017)
O. volvulus	Cross-sectional study	Equatoguinean (544 participants)	Skin PCR result: 11 case *O. volvulus* (one), *L. Loa* (one), *M. perstans* (seven), *M. streptocerca* (two)	-Microscopy (skin snip) -Serology test (ELISA) -RT-PCR	Bioko Island	2014	(Moya et al., 2016)

O. volvulus	Cross-sectional study	Equatoguinean (544 participants)	*O.volvulus* (7.9 %)	-Serology test (ELISA)	Bioko Island	2014	(Hernández-González et al., 2016)
L. loa	Case report	Ecuatoguinean	One (woman)	- Clinical manifestations (eye visualization, eosinophilia)	Equatorial guinea	2013	(Burgués-Ceballos et al., 2014) (Abstract available)
L. loa	Case report	Immigrant (Chinese) from Equatorial Guinea	One (35 years old men)	- Clinical manifestations (swelling of the wrist, eosinophilia) - Nested PCR	China	2012	(Wang et al., 2012)
L. loa	Case report	Equatoguinean	Two (23 years old man and 18-years old woman)	-Clinical manifestation (eye visualization)	Equatorial Guinea	2010	(Lichtinger et al., 2011)
O. volvulus *L. loa* *M. perstans*	Retrospective study	Immigrants (Equatoguinean)	14 cases (children aged between 3 and 15 years old): *O. volvulus* (8), *M. perstans* (8) and *L. loa* (2)	-Clinical manifestation (pruritis, eye visualization, eosinophilia) - Microscopy	Spain	1995-2007	(Cuello et al., 2009) (Abstract available)
O. volvulus	Cross-sectional study	Equatoguinean (1723 individuals in 1989 and 1082 in 1998)	77.1 % in pre-treatment (1989) 35.8 % in post-treatment (1998)	-Microscopy (skin snip)	Bioko Island	1989-1998	(Mas et al., 2006)
L. loa	Case report	Immigrants (Equatoguinean)	Seven (22-43 years old men)	-Clinical manifestations (swelling of the hands and arms, itching and pain in the eyes, eye worms, eosinophilia)	Singapore	2002	(Lee and Paton, 2004)
O.volvulus	Cross-sectional study	Equatoguinean (3218 individuals)	6.8% with visual impairment 3.2 % with blindness	-Clinical manifestations (visual acuity)	Bioko Island	1999	(Moser et al., 2002)
L. loa	Case report	Immigrants (Morrocan) from Equatorial Guinea	Twenty-four	-Clinical manifestations (pruritis, edema, eye worms)	Morocco	2001	(El Haouri et al., 2001) (Abstract available)
O. volvulus	Cross-sectional study	Equatoguinean (1799 participants)	75.2 %	-Clinical manifestations (nodules, dermatitis, blindness, lymphedema) -Microscopy (skin snips)	Bioko Island	1987-1989	(Mas et al., 1995) (Abstract available)
L. loa *M. perstans*	Comparative study	Equatoguinean (829 participants)	Parasitology incidence was 27.1 % for *L. Loa* and 66.3 %. for *M. perstans*	-Microscopy	Continental area in Equatorial Guinea	1990	(Vila Montlleo, 1990) (Abstract available)
O. volvulus	Case report	Immigrant (American) from Equatorial Guinea	One case in woman	-Microscopy	USA	1987	(Joyce and Pearson, 1987) (Abstract available)

1.10 Goal and Objectives of this thesis

Accurate, robust, sensitive, specific, rapid, cheap and high-throughput assays for parasite detection are becoming essential for monitoring of many ongoing or planned parasite control or elimination programs. The overall goal of this PhD thesis is to contribute to the development and implementation of novel molecular based diagnostic approaches for monitoring highly neglected filariasis causing parasites.

Therefore, following objectives were addressed in this thesis:

Objective 1: To develop and evaluate a novel high-throughput procedure of NAs extraction from used mRDT followed by qPCR based amplification of *Plasmodium* spp. specific genes to allow for detection and quantification of malaria parasites and comparison with mRDT based malaria diagnosis.

Objective 2: To adapt the protocol developed for *Plasmodium* spp. detection to monitor rate and geographical distribution of *M. perstans* and *L. loa* infections and co-infections with malaria. Risk factor identification of filarial parasite infections on Bioko Island by inclusion of metadata collected during MIS 2018.

Objective 3: To investigate co-infections between Plasmodium *spp*., *M. perstans* and *L. loa* in pregnant women on Bioko Island to understand potential relationship of infection rates with bed net usage and resulting anemia levels.

Chapter II

Molecular malaria surveillance using a novel protocol for extraction and analysis of nucleic acids retained on used rapid diagnostic tests

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Molecular malaria surveillance using a novel protocol for extraction and analysis of nucleic acids retained on used rapid diagnostic tests

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The use of malaria rapid diagnostic tests (RDTs) as a source for nucleic acids that can be analyzed via nucleic acid amplification techniques has several advantages, including minimal amounts of blood, sample collection, simplified storage and shipping conditions at room temperature. We have systematically developed and extensively evaluated a procedure to extract total nucleic acids from used malaria RDTs. The co-extraction of DNA and RNA molecules from small volumes of dried blood retained on the RDTs allows detection and quantification of *P. falciparum* parasites from asymptomatic patients with parasite densities as low as 1 Pf/µL blood using reverse transcription quantitative PCR. Based on the extraction protocol we have developed the ENAR (Extraction of Nucleic Acids from RDTs) approach; a complete workflow for large-scale molecular malaria surveillance. Using RDTs collected during a malaria indicator survey we demonstrated that ENAR provides a powerful tool to analyze nucleic acids from thousands of RDTs in a standardized and high-throughput manner. We found several, known and new, non-synonymous single nucleotide polymorphisms in the propeller region of the kelch 13 gene among isolates circulating on Bioko Island, Equatorial Guinea.

Abbreviations

Pf	P. falciparum
pfk13	*Pf* Kelch 13
RDT	Rapid diagnostic test
DBS	Dried blood spot
ENAR	Extraction of nucleic acids from RDT
CHMI	Controlled human malaria infection
NA	Nucleic acid
NAT	Nucleic acid amplification technique

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PfIS	WHO International standard for *P. falciparum* NAT
LOD	Limit of detection
RT	Room temperature
qPCR	Quantitative polymerase chain reaction
PlasQ	Multiplex qPCR assay for quantification of *P. falciparum* and *Plasmodium* spp. Parasites
SNP	Single nucleotide polymorphism
Pf/µL	*Pf* Parasites per µL blood

Malaria remains a global public health issue with an estimated 228 million cases resulting in an estimated 405,000 deaths in 2018[1]. *P. falciparum* (*Pf*) is the most pathogenic malaria species accounting for the vast majority of malaria cases and deaths. Malaria surveillance, the continuous and systematic collection, analysis and interpretation of epidemiological data, is the core monitoring and evaluation tool for malaria control programs, and provides the framework for effective allocation of resources[2]. A critical surveillance measure, which closely reflects malaria transmission intensity, is the parasite rate; the proportion of the population found to carry parasites in their peripheral blood[3,4]. Malaria rapid diagnostic tests (RDTs) are the most widely used technique to measure parasite rates in endemic countries. In sub-Saharan Africa, RDTs have almost completely replaced light microscopy for malaria diagnosis, with an estimated 75% of malaria tests conducted using RDTs in 2017[1]. RDTs are relatively low cost, provide fast result turnaround time, are widely available and easy to use. However, there are also disadvantages including low sensitivity, resulting in poor performance among asymptomatic individuals [5]and the widespread emergence of *pfhrp2* deletions in certain regions[6]whereby RDTs fail to detect malaria infection.

Nucleic amplification techniques (NATs), such as polymerase chain reaction (PCR), not only show higher sensitivities than RDTs[5,7] but also allow further characterization of *Pf* isolates using molecular markers. Surveillance of drug-resistant *Pf* strains, based on analysis of resistance-associated molecular markers, is a widely used and valuable epidemiological tool[8]. In sub-Saharan Africa, malaria treatment relies heavily on artemisinin-based combination therapy (ACT). The implementation of surveillance programs for early detection of emerging artemisinin-resistant *Pf* strains will be the key to prevent the spread across the continent[9]. Artemisinin-resistant *Pf* strains were first reported in Cambodia[10,11] and remain a public health concern in South East Asia but have not yet been found to be widespread in Africa, South America or Oceania[12]. Non-synonymous mutations in the propeller region of the *Pf* kelch 13 gene (pfk13) were discovered as molecular markers for artemisinin resistance[13].

Residual blood from RDTs are an ideal source for nucleic acids (NAs) to be used for NAT-based resistance markers screening and present several advantages, including simplicity and cost-effectiveness of sample collection, as well as simplified storage and shipping conditions at room temperature (RT). Over the past decade, several reports have been published describing the use of DNA extracted from used RDTs for molecular analysis of malaria parasites (studies summarized in Supplementary Table S1)[14–24]. However, most studies that tried to address the question of using RDTs as source of DNA were conducted with small sample sizes and focused on demonstrating the feasibility of extracting DNA rather than fitting this approach for molecular surveillance of malaria at larger scale. We identified three key areas that are critical to develop a surveillance tool based on molecular analysis of used RDTs: (i) accessing a representative collection of RDTs and creating an effective selection and sorting strategies for RDTs of interest. (ii) high-throughput extraction and analysis of NAs from RDTs with minimal hands-on time and focus on reproducibility and quality control throughout the entire extraction process. (iii) increasing recovery of *Pf* NAs during the extraction process in order to include asymptomatic individuals with low parasite density infections.

This manuscript outlines an overall strategy and the protocols for collecting, sorting and processing RDTs to extract the retained NA at large-scale in order to screen for single nucleotide polymorphisms (SNPs) in an artemisinin-resistance molecular marker in a dataset of thousands of healthy, malaria asymptomatic individuals. We systematically developed and extensively evaluated a procedure to extract NA from RDT. The "Extraction of Nucleic Acids from RDTs" (referred to as ENAR) approach is supported by custom-made software solutions that allow the analysis of thousands of RDTs in a standardized, reproducible and high-throughput manner.

We developed the ENAR approach in Tanzania and implemented the ENAR approach within Bioko Island Malaria Elimination Project's (BIMEP) 2018 malaria indicator survey (MIS) conducted on Bioko Island, Equatorial Guinea. BIMEP is an island-wide intervention resulting in a substantial reduction in malaria, achieving a reduction in parasitemia of around 75% over the past 15 years[25]. Despite these achievements, malaria transmission remains stable on Bioko for an number of reasons, and recently a *Pf* isolate of African origin with artemisinin-resistance, including a novel non-synonymous mutation in pfk13, was identified in a 43-year-old man returning to China from Equatorial Guinea[26]. This reality underlies the importance of incorporating molecular techniques as monitoring and evaluation tools in malaria control programming.

Results

Blood stored on RDTs is a source of *Pf* DNA. First, we conducted a literature search of reports describing the use of NA extracted from RDTs as input templates for NAT-based detection of malaria parasites (Supplementary Table S1). A total of 11 studies were published between 2006 and 2019. All studies were limited to the extraction of DNA and used a variety of different extraction methods. Most extraction protocols were based either on the Chelex method (n = 7) or silica column-based DNA extraction kits (n = 6). One study extracted DNA from the entire RDT strip, all other studies used only predefined fragments of the RDT strip. These previous studies demonstrated that *Pf* DNA can be recovered from RDTs and amplified by NATs. Several studies genotyped drug resistance associated markers using sanger or next generation sequencing.

As the majority of these studies extracted DNA from RDTs of febrile clinical malaria cases, indicating high parasite densities, we first conducted a study to test feasibility of detecting Pf DNA from RDTs of asymptomatic

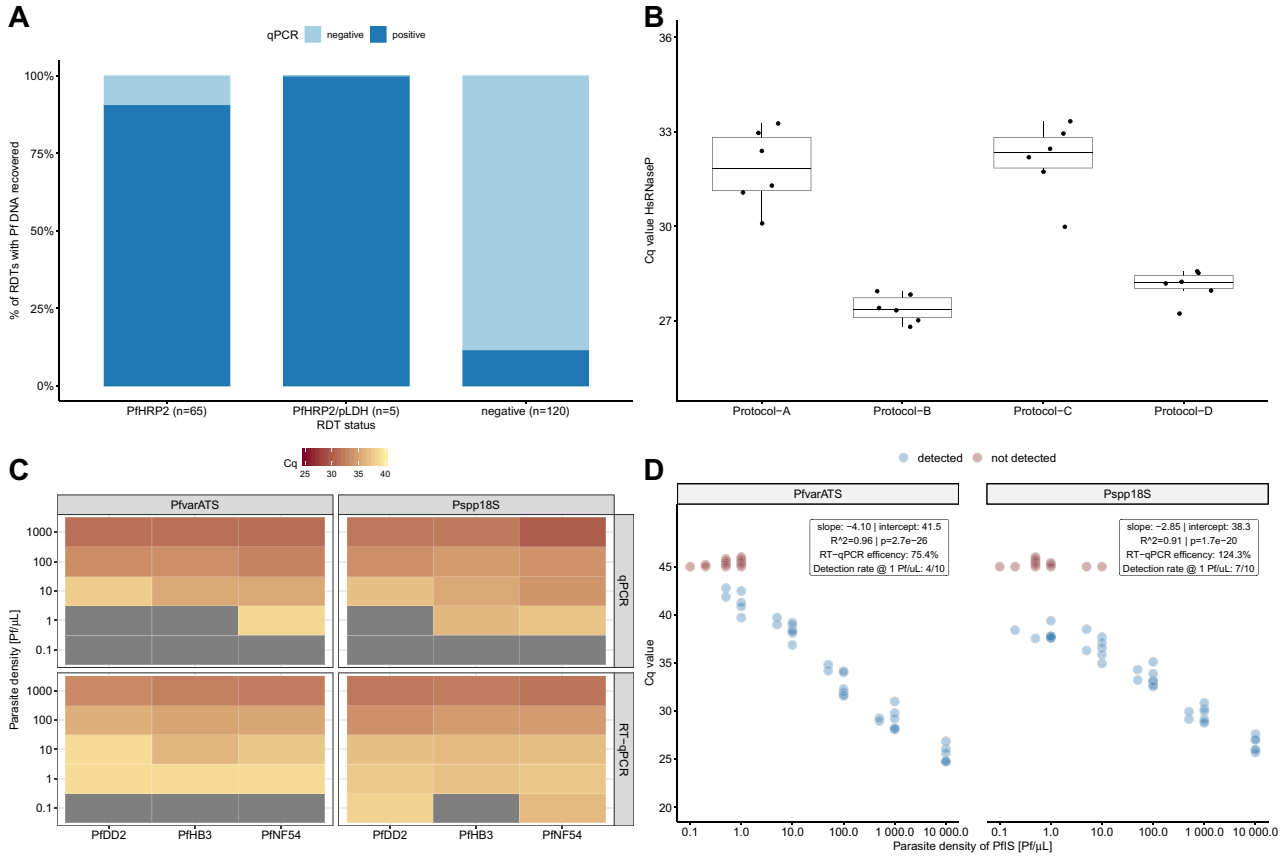

Figure 1. Extraction and detection of *Pf* NAs from used RDTs. (**A**) Recovery rates of *Pf* DNA from RDTs collected in asymptomatic Tanzanian school children. (**B**) Comparison of extraction performance of four protocols based on Cq values of the human *rnasep* gene. (**C**) Association of parasite densities and Cq values of freshly prepared *Pf* strains (PfDD2, PfHB3 and PfNF54). Gray colour indicates failed detection. (**D**) Correlation between parasite densities of serially diluted PfIS and Cq values for PlasQ targets. Red coloured dots represent samples where amplification failed.

individuals. We employed RDTs collected in a malaria survey conducted among asymptomatic children from three primary schools in the Mkuranga district of Coastal Tanzania. DNA was extracted from 190 RDTs and *Pf* DNA was recovered from 90.8% (59/65) of PfHRP2-positive RDTs, from 100% (5/5) of PfHRP2/pLDH-positive RDTs and from 11.7% (14/120) negative RDTs (Fig. 1A).

Encouraged by the outcome of the school-based survey, we aimed to improve the extraction method from RDTs. As a proxy for the amount of extracted NAs, the Cq value of the human *rnasep* gene (HsRNaseP target), which is the internal control of the previously published PlasQ assay, was used to assess the overall performance of four different extraction procedures (Fig. 1B). Side-by-side comparison of the four extraction procedures, named Protocol A through D, confirmed the superior performance of protocols B and D. Considering the costs and the fact that protocol D co-extracts RNA, we developed protocol D, which we renamed ENAR (Extraction of Nucleic Acids from RDTs). In order to identify the part of the RDT strip where most *Pf* NAs accumulate, we analyzed the sample pad (proximal part), the detection area (middle part), and the absorption pad (distal part) using ENAR. In RDTs probed with fresh blood, *Pf* NAs are found in all three parts, with more than 87% of the total extracted DNA concentrated in the middle part. RDTs spiked with frozen blood that is associated with red blood cell lysis resulted in an equal distribution of NA along the entire RDT strip (Supplementary Figure S1).

Detection and quantification of *Pf* parasites based on ENAR protocol. We evaluated the ENAR protocol with cultured *Pf* strains from different geographical locations including the strains PfDD2 (South East Asia), PfHB3 (Central America) and PfNF54 (Africa) by preparing ten-fold serial dilutions in whole blood with parasite densities corresponding to 0.1–1,000 Pf/µL. RDTs were spiked with 5 µL of diluted cultures, the NAs extracted by ENAR, and analyzed by qPCR and RT-qPCR (Fig. 1C). Only the RT-qPCR assay resulted in detection of all three strains with the 1 Pf/µL parasite density. Furthermore, the Pspp18S-based RT-qPCR assay even detected two (PfDD2 and PfNF54) out of the three *Pf* strains at a concentration of 0.1 Pf/µL. This result demonstrates that the ENAR clearly co-extracts DNA and RNA. The *Pf* 18S ribosomal RNA, detected by the Pspp18S RT-qPCR assay, is constantly and highly expressed during the life cycle of the parasite[27,28], while the acidic terminal sequence of the var genes (PfEMP1), detected by the PfvarATS assay, is associated with lower RNA levels[29]. The ability of the ENAR protocol to co-extract DNA and RNA was also demonstrated with the following

CHMI-1 (2016) > 18 months storage	CHMI-2 (2018) < 1 month storage
BinaxNOW Malaria RDT	CareStart Malaria (Pf/PAN) Combo
71	50
20 µL	5 µL
605 (596–616)	18 (10–48)
RT	RT
14.0 (10.5–18.0)	12.7 (9.0–18.0)
38.0% (27/71)	62.0% (31/50)
4.7 (0.05–840.0)	0.3 (0.01–1,041.0)

Table 1. Overview of blood samples collected during two CHMIs and stored on RDTs.

experiment: Five µL of an in vitro-generated stage V gametocyte culture was applied onto the RDTs and stored at RT for three weeks before NAs were extracted by ENAR. The gametocyte-specific transcript PF3D7_0630000 was reverse transcribed and amplified using a published assay which does not require DNase treatment for specific detection of gametocytes[30]. Extracted NAs from 5 µL undiluted and 1:100 diluted stage V gametocytes specifically amplified the gametocyte marker, while the control without a reverse transcription step did not result in amplification (Supplementary Figure S2).

The PfIS, an international standard with known parasite density, was used to explore the feasibility of quantifying *Pf* parasites extracted by ENAR. In total, 51 individual RDTs containing 5 µL PfIS with different parasite densities, ranging from 0.1 to 10,000 Pf/µL of the PfIS, were prepared. A high reproducibility and reverse correlation between parasite densities and Cq values were observed for both targets, the *Pf* specific PfvarATS and the pan-*Plasmodium* target Pspp18S (Fig. 1D). Based on the slope, RT-qPCR efficiencies of 75.4% and 124.3% were calculated for PfvarATS and Pspp18S, respectively. RDTs negative for PlasQ assay amplification (Cq > 45, colored in red) carried mostly dilutions representing parasite densities ≤ 1 parasite/µL. Two exceptions were observed where the Pspp18S assay failed to amplify two RDTs probed with higher parasitemia levels (5 and 10 Pf/µL, respectively). RDTs probed with 1 parasite/µL were detected in 4 (PfvarATS) and 7 (Pspp18S) out of 10 RDTs tested.

In summary, based on experiments conducted with standardized *Pf* reference samples we conclude that ENAR is able to recover both DNA and RNA, which results in an increased sensitivity of the RT-qPCR compared to the qPCR-based detection methods. The lower limit of detection (LOD) for RT-qPCR-based amplification of NAs from RDTs is around 1 Pf/µL, although 10× lower parasitemia levels can be detected as demonstrated with freshly cultured *Pf* parasites. RDTs are a reliable source of NAs and extraction by ENAR followed by analysis using RT-qPCR assays allows quantification of *Pf* parasites.

Evaluation of ENAR protocol using controlled human malaria infection studies as a platform. Blood collected from volunteers undergoing Controlled Human Malaria Infection (CHMI) studies represent well-characterized samples as the parasite strain, the timing and dosing of infection is known. Therefore, blood samples collected from volunteers undergoing CHMI are well suited for developing and validating novel malaria diagnostic tools[31].

The ENAR protocol was evaluated with venous blood samples collected during CHMIs assessing the efficacy of Sanaria's PfSPZ Vaccine in clinical trials in Bagamoyo, Tanzania in malaria pre-exposed volunteers. RDTs were spiked with blood and stored as part of two CHMIs, the first of which was conducted in 2016 (CHMI-1) and the second in 2018 (CHMI-2). As part of the standard diagnostic procedures during the CHMIs, whole blood was collected in EDTA tubes and DNA extracted from a total of 180 µL whole blood. A DNA-based qPCR assay was run and parasitemia quantified (defined as WB-qPCR). Parasite densities as low as 0.05 Pf/µL are detected with the WB-qPCR protocol. During both CHMIs, fresh blood from asymptomatic subjects collected 9 to 18 days post-CHMI was tested with RDTs (Table 1). CHMI-1 and CHMI-2 used two different types of RDTs, which required 20 µL and 5 µL of whole blood, respectively. RDTs collected during CHMI-1 were stored for an average of 605 days (categorized as > 18 months), while RDTs collected during CHMI-2 were stored for an average of 18 days (< 1 month) before processing following the ENAR protocol. For the entire storage period, RDTs were kept at RT in a closed box and protected from light. NAs were extracted from the RDTs using the ENAR protocol and parasites were detected and quantified by RT-qPCR using the PlasQ assay.

Impact of long-term storage on detection rate of parasite NA extracted by ENAR. First, we analyzed the impact of RDT storage time on parasite detection rates. Long-term storage (> 18 months) negatively affects the *Pf* detection rate in samples with a parasite density between 1 and 10 Pf/µL but has no negative impact on samples with initial parasite density greater than 10 Pf/µL (Fig. 2A). Long-term storage negatively affects the detection rate based on the Pspp18S target (33% vs. 100%, Fisher's exact test $p = 0.06$) more than the PfvarATS target (66% vs. 100%, Fisher's exact test $p = 0.46$). Interestingly, the parasite densities estimated from RDTs with shorter storage time (< 1 month) are closer to the reference parasite densities assessed by WB-qPCR using 180 µL freshly prepared blood than the estimates from RDTs with longer storage time (> 18 months) (Fig. 2B). This is an additional indicator that NAs conserved on RDTs might undergo degradation over time.

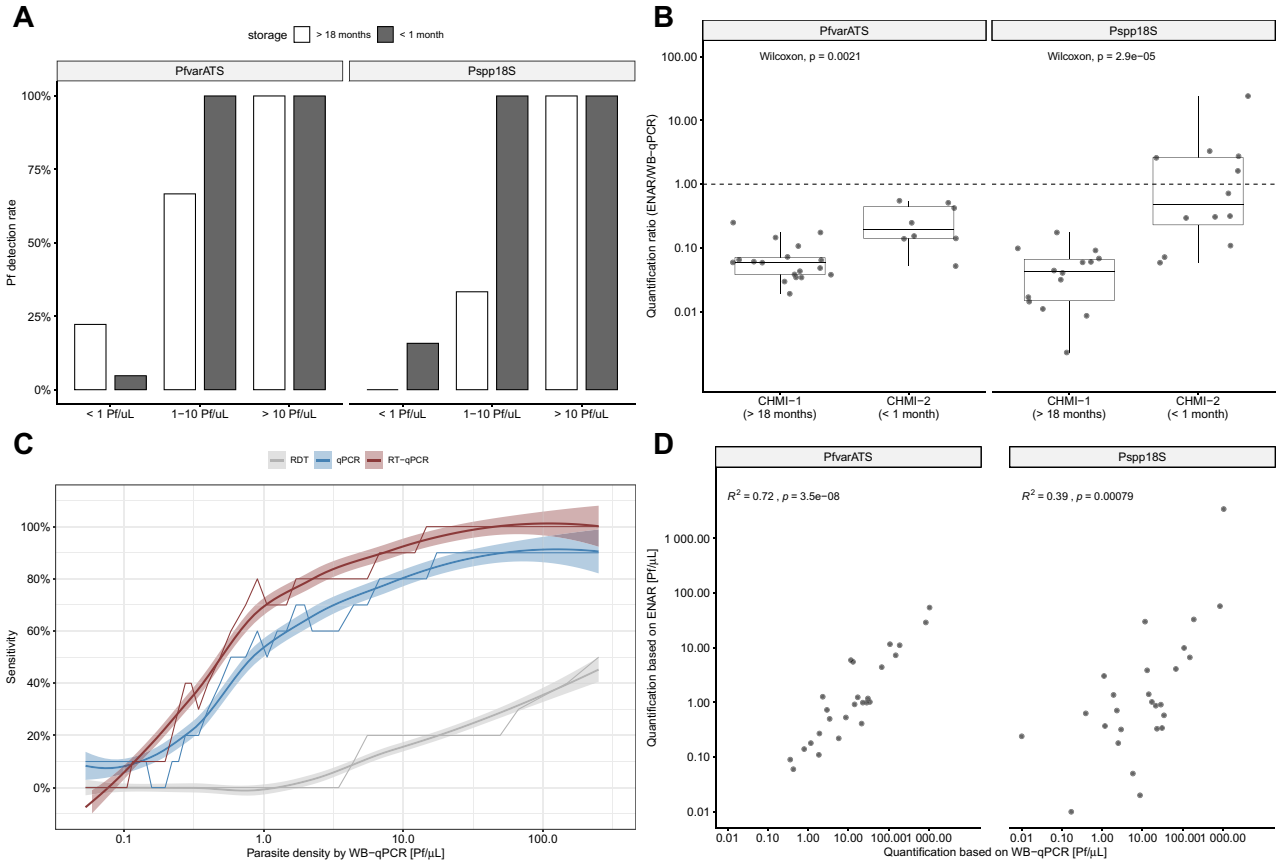

Figure 2. Evaluation of ENAR protocol using samples collected during CHMI studies. (**A**) *Pf* detection rates grouped by parasite density and storage time. (**B**) Quantification ratio between densities derived from ENAR and densities derived from whole blood qPCR (WB-qPCR). (**C**) Diagnostic sensitivity of rapid diagnostic test (RDT), ENAR followed by qPCR detection (qPCR) and ENAR followed by RT-qPCR detection (RT-qPCR) in relation to parasite density. Rolling means of 10 observations, using WB-qPCR as a gold standard, are shown with 95% CIs (shaded areas). (**D**) Correlation of parasite densities obtained from DNA extracted from fresh whole blood and NAs extracted by ENAR.

Clinical sensitivity and parasite quantification based on ENAR approach. If the data of both CHMIs are combined, the overall detection rate was 54% for the ENAR-based RT-qPCR when compared to WB-qPCR, which was significantly higher than detection by microscopy (9%) or PfHRP2 antigen capture by RDT (12%) using the same samples.

In order to understand the contribution of RNA on the detection rates in this clinical sample set, we compared RT-qPCR with qPCR. Detection rates of RT-qPCR in relation to parasite density reveals an improved diagnostic performance over the whole range of *Pf* densities compared to qPCR (Fig. 2C). RT-qPCR is significantly more sensitive than qPCR for the Pspp18S assay (27% vs 47%, McNemar test $p = 0.0026$), but not for the PfvarATS assay (47% vs. 47%, McNemar test $p = 1.0$). Interestingly, among the long-term stored RDTs collected in 2016, the detection rate of the Pspp18S assay was also significantly higher for RT-qPCR compared to qPCR (52% vs 22%, McNemar test $p = 0.01$). Even after long-term storage a significant proportion of (fragmented) RNA can be still extracted and used for RT-qPCR amplification.

Parasite densities determined by WB-qPCR versus densities obtained with the ENAR-based RT-qPCR method showed significant positive correlation supporting the quantitative character of our approach (Fig. 2D). The correlation was stronger with the PfvarATS assay ($r^2 = 0.72$) than with the Pspp18S assay ($r^2 = 0.39$).

Implementation of ENAR protocol within malaria indicator survey. We implemented the ENAR approach within a malaria indicator survey in which we aimed to screen for SNPs in the pfk13 propeller region to study the prevalence and type of mutations potentially associated with artemisinin resistance. We tested ENAR using samples and data derived from the 2018 BIMEP MIS which included more than 13,000 individuals (Fig. 3A). Instead of disposing the RDTs after use, the tests were labeled with a barcode to connect each RDT with other survey data collected in questionnaires (Fig. 3B). For each of these barcode-labeled RDTs, an extra informed consent for molecular analysis was obtained from the participants or their legal guardians. For the sorting and selection of distinct RDTs for analysis, we developed the *RDTselect* app (https://github.com/Sparclex/barcode-value-finder), a browser-based mobile phone application which identifies barcode-labeled RDTs based on an input list containing all barcodes of a certain selection (Fig. 3C).

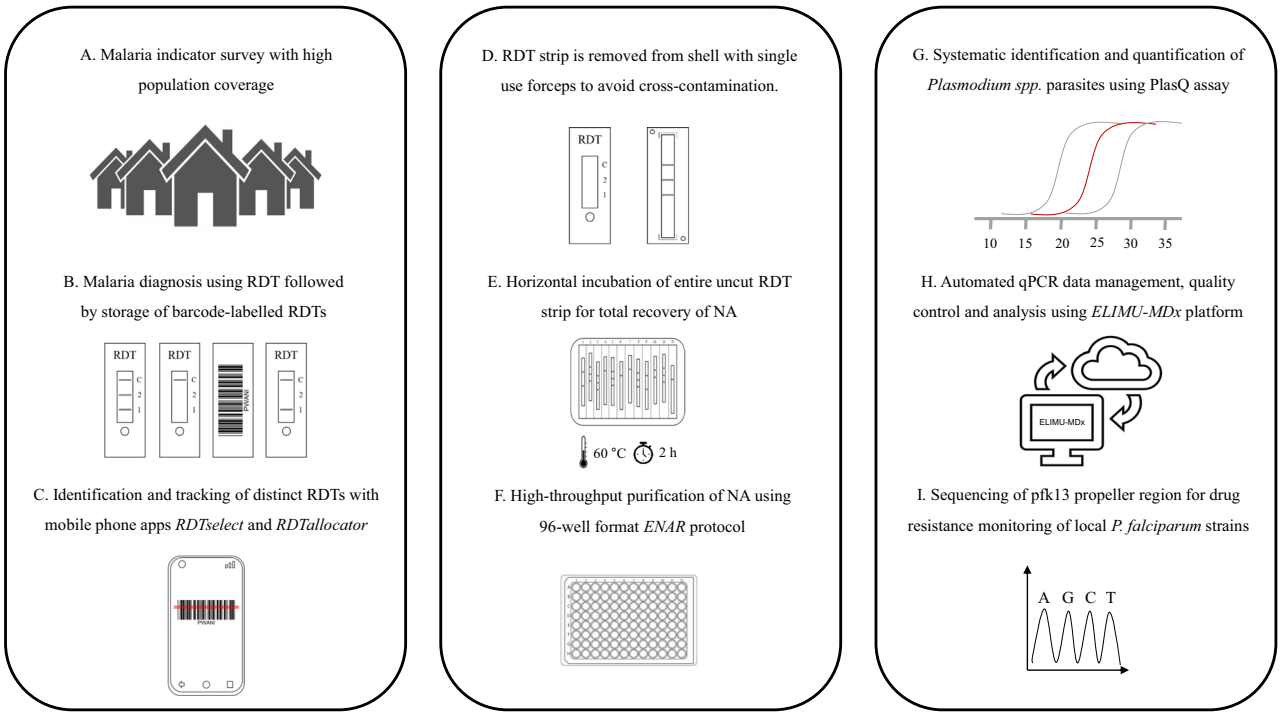

Figure 3. Adaptation of ENAR protocol for analyzing large numbers of barcoded RDTs. (**A**) Malaria indicator survey conducted including a detailed questionnaire. (**B**) Malaria prevalence is determined by RDT followed by storage of barcode-labelled RDTs. (**C**) Sorting and tracking of RDTs using smartphone apps. (**D**–**F**) High throughput protocol for extraction of NAs from RDTs using the ENAR approach (Image courtesy of LJNovaScotia/Pixabay). (**G**) Detection and quantification of *Pf* and non-*Pf* malaria parasite. (**H**) Automated analysis of qPCR data using ELIMU-MDx. (**I**) Genotyping of pfk13 propeller region for drug resistance monitoring.

To enable tracking of an individual RDT throughout the ENAR extraction process the *RDTallocator* app (https://github.com/Sparclex/position-allocator) was programmed. The barcodes are scanned with a mobile phone camera and the *RDTallocator* app allocates the associated RDT strip to the next available position in a 96-well plate (Fig. 3C). After opening the RDT shell the entire uncut RDT strip is removed with sterile, single-use forceps (Fig. 3D), incubated with lysis buffer in a 12-well long-format plate (Fig. 3E), and NAs are extracted in a high-throughput 96-well format of the ENAR protocol (Fig. 3F). All extracted samples undergo initial screening for presence of *Plasmodium* spp. parasites and quality control using the PlasQ RT-qPCR assay (Fig. 3G). All RT-qPCR data generated were managed and analyzed by a custom-designed laboratory management and information system. ELIMU-MDx is designed for automated quality control, management and analysis of qPCR data[32] (Fig. 3H). Samples positive for *Pf* were subjected to amplification and sequencing of pfk13 for identification of SNPs associated with drug resistance (Fig. 3I).

A total of 2,690 out of 13,270 (20.3%) RDTs were extracted by ENAR and analyzed for *Plasmodium* spp. parasites by RT-qPCR. The demographic information of the entire MIS population and the selected volunteers for the molecular analysis are given in Table 2. Only volunteers with body temperature < 37.5 °C were included. Volunteers with a positive RDT and pregnant women are intentionally over-represented in our sample set.

Malaria infections among asymptomatic MIS participants are characterized by *Pf* infections with mainly low parasite densities. Applying the approach described in Fig. 3, 30.8% (828/2,690) of the analyzed RDTs tested positive for *Plasmodium* spp. NAs (Table 3). A qPCR-based species identification revealed that 92.9% were *Pf*, 4.0% *P. malariae* and 1.0% *P. ovale* spp. No *P. vivax* or *P. knowlesi* NAs were found. In this asymptomatic population, *Pf* infections had on average parasite density of 29.2 Pf/µL, with densities being the highest among children below the age of five years (Fig. 4a). The rather low parasitemia levels of asymptomatic individuals in combination with the small amount of blood available have implications for pfk13 genotyping. Samples with parasitemia levels below 50 Pf/µL are rarely amplified successfully for pfk13 sequencing (Fig. 4b). In order to increase the efficiency of pfk13 genotyping process from RDTs, pre-selection based on RDT result is advised. For example, 84.5% of RDTs positive for both, PfHRP2 and pLDH carried parasite densities high enough to result in successful amplification of the pfk13 propeller region.

Low prevalence of SNPs in the pfk13 propeller region among *Pf* parasite isolates on Bioko. Sequence analysis of the pfk13 propeller region revealed a low prevalence of SNPs (Table 4). 97.6% (283/290) of Bioko's *Pf* isolates carried the wildtype allele. Two isolates had the A578S and one the V589I non-synonymous SNP, which have been described in sub-Saharan Africa before[12,33] and are not associated with arte-

	All individuals (n = 13,270)	Selected individuals (n = 2,690)
Gender		
Female	7,155 (53.9%)	1569 (58.3%)
Male	6,115 (46.1%)	1,121 (41.7%)
Age (years)		
Mean (SD)	21.2 (± 17.7)	27.1 (± 21.0)
Age group		
< 5	2,308 (17.4%)	276 (10.3%)
5–14	3,719 (28.0%)	660 (24.5%)
15–45	5,758 (43.4%)	1,208 (44.9%)
> 45	1,485 (11.2%)	546 (20.3%)
District		
Baney	1519 (11.4%)	400 (14.9%)
Luba	1,093 (8.2%)	268 (10.0%)
Malabo	10,121 (76.3%)	1814 (67.4%)
Riaba	537 (4.0%)	208 (7.7%)
RDT result		
Negative	11,842 (89.2%)	1623 (60.3%)
pLDH	43 (0.3%)	39 (1.4%)
PfHRP2	871 (6.6%)	653 (24.3%)
pLDH + PfHRP2	462 (3.5%)	367 (13.6%)
Pregnancy status		
Currently pregnant	237 (1.8%)	225 (8.4%)
Gave birth to live baby	918 (6.9%)	128 (4.8%)
Hemoglobin (g/dL)		
Mean (SD)	12.4 (± 1.79)	12.1 (± 1.88)
Anemia status		
No	8,874 (65.7%)	1593 (57.6%)
Mild	2,711 (20.1%)	634 (22.9%)
Moderate	1777 (13.2%)	502 (18.1%)
Severe	110 (0.8%)	34 (1.2%)

Table 2. Demographic information of MIS participants.

	Number of samples (%)
RDTs analysed by PlasQ	2,690
Positive for PlasQ RT-qPCR	828 (30.8%)
Plasmodium spp. Identification	
Positive for *P. falciparum*	769 (92.9%)
P. falciparum with > 100 Pf/uL	227 (29.5%)
Positive for *P. malariae*	33 (4.0%)
Positive for *P. ovale* spp.	8 (1.0%)
Positive for *P. knowlesi*	0 (0.0%)
Positive for *P. vivax*	0 (0.0%)
Pf/Pm co-infections	16 (1.9%)

Table 3. ENAR-based identification of malaria parasites using PlasQ RT-qPCR assay.

misinin resistance[34]. The P553L SNP was first described in Cambodia[13]. This SNP has previously been found at low prevalence in East Africa, in Kenya and Malawi[35] as well was recently found in an isolate from a Chinese national returned from Angola[36]. To our knowledge, the V517I SNP has never been described before. Compared to the other three known SNPs, the V517I SNP had the lowest PROVEAN[37] score, indicating no or neutral effects on the biological function of the kelch 13 protein. Two synonymous SNPs, namely, V510V and C469C, were also found.

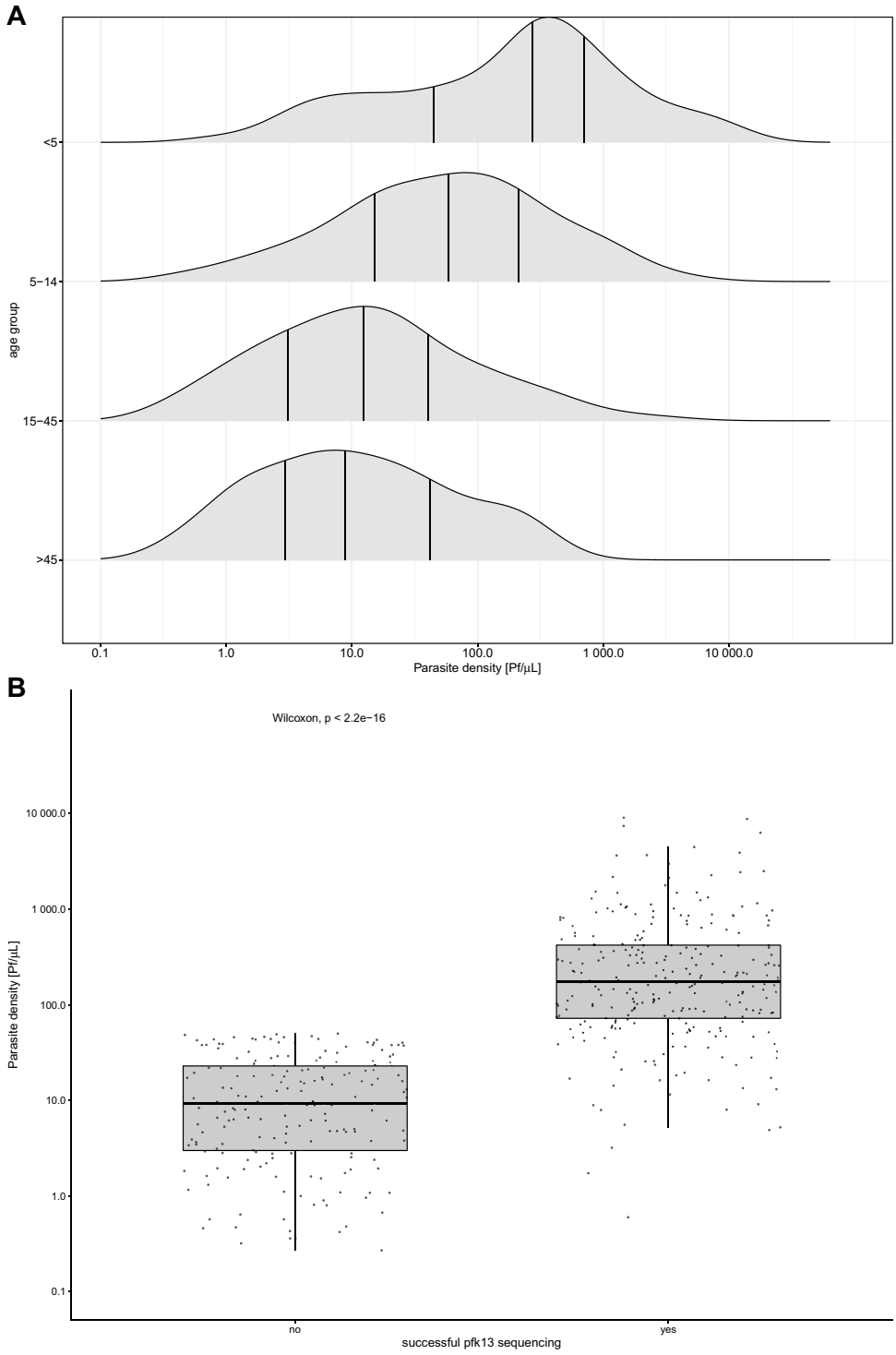

Figure 4. Parasite densities among asymptomatic individuals and implication for sequence analysis. (**A**) Age group dependent parasite densities. (**B**) Association between parasite density and successful amplification of pfk13 for sequence analysis.

Discussion

This report presents the development of an high-throughput approach for large-scale molecular surveillance of malaria parasites based on extraction of NAs from RDTs. During the development of ENAR, special attention was given to the evaluation of its reproducibility and the impact of long-term storage on the detectability of the NAs. Using samples from CHMI studies as a standardized platform allowed us to conclude that NAs can be reliably recovered and amplified from RDTs, even after long-term storage at RT. The small amount of blood in combination with low parasite density is a challenge when it comes to detecting *Pf* in asymptomatic patients.

		PROVEAN score
Kelch13 propeller genotyping		
P. falciparum strains sequenced	290	
PfNF54 allele	283 (97.6%)	
Non-synonymous SNPs		
A578S (G1732T)	2 (0.69%)	−1.962
V589I (G1765A)	1 (0.35%)	−0.663
V517I (G1549A)	1 (0.35%)	−0.562
P553L (C1659T)	1 (0.35%)	−1.721
Synonymous SNPs		
V510V (G1530A)	1 (0.35%)	
C469C (C1407T)	1 (0.35%)	

Table 4. *Pf*k13 propeller polymorphisms observed in MIS population on Bioko Island.

Therefore, we aimed to maximize the amount of NA recovered from RDTs by expanding the pool of possible target molecules to RNA by using RT-qPCR. Even after a storage period of over 18 months at RT, the detection rate of the RT-qPCR assay was still significantly higher compared to qPCR only, indicating long-term preservation of DNA and RNA.

We aimed to transform the ENAR approach into an flexible tool for larger scale surveillance studies by increasing extraction and analysis throughput. The ENAR approach was successfully integrated into the 2018 BIMEP MIS on Bioko Island. More than 13,000 individuals gave extra consent for storage and molecular analysis of their RDT. This high acceptance rate was also described by others[21] and can be attributed to the convenience of blood collection by finger prick and the small blood volume, usually 5 to 10 µL, needed for RDTs. With a total of 2,750 RDTs, we analyzed blood from more than 20% of the MIS participants. This was made possible by the development of custom-made software solutions for sorting and identification of RDTs and by a significant reduction in processing time by using the entire RDT strip instead of cutting it into pieces.

Robust (quantitative) data, as generated by ENAR, in combination with a large-scale MIS adds substantial value to our understanding of malaria endemicity on Bioko Island without conducting additional expensive and time consuming epidemiological studies. In addition this process allows for researches to detect various species of malaria parasites. For instance, we found *P. malariae* and *P. ovale* spp., but did not find *P. vivax*, as in previous studies when surveys carried out in 1996 and 1998 found two[38] and one[39] case of *P. vivax* infection on Bioko Island.

In addition, we screened for SNPs in the propeller region of the pfk13 gene among asymptomatic individuals to obtain data of possible artemisinin-resistant *Pf* strains circulating on the island. We found that 1.7% (5/290) of the analyzed *Pf* isolates had non-synonymous SNPs in the pfk13 propeller region, which is comparable to the prevalence found in other African countries[33]. Among the five isolates with non-synonymous SNPs, two isolates had the A578S, one the V589I, one the P553L and one the V517I SNP. The A578S and V589I allele had been reported in the region already[40,41], and we found one new previously unreported non-synonymous SNP, V517I. Interestingly, the P553L SNP is the only mutation we found which was previously associated with delayed parasite clearance[12]. Although the prevalence of pfk13 SNPs seems to be low in the moment, the spread of *Pf* parasites with pfk13 SNPs needs to be closely monitored. A molecular surveillance approach as presented may offer a unique opportunity to support policy makers regarding choice and change of drugs for malaria treatment[42].

Based on the presented results, we propose that ENAR provides a powerful tool for molecular malaria surveillance and could be reliably used for retrospective quantitative and in-depth molecular studies of malaria.

Material and methods

***Pf* reference samples.** *Pf* reference samples were used to test the performance of the ENAR procedure. Experiments with *Pf* reference samples were conducted using Carestart HRP2/pLDH Combo RDTs (Access Bio, Inc., Somerset, NJ, USA). Serial dilutions of the WHO International Standard for *Pf* DNA Nucleic Acid Amplification Techniques (NIBSC code: 04/176, herein referred to as PfIS)[43] were used to quantify *Pf* parasitemia by (RT)-qPCR. Whole blood was spiked with different parasite densities, ranging from 10,000 to 0.1 Pf/µL and 5 µL of this suspension applied onto RDT.

Additionally, ten-fold serial dilutions, ranging from 10,000 to 0.1 Pf/µL, of freshly cultured *Pf* strains PfNF54, PfDD2 and PfHB3 were prepared and 5 µL were applied onto RDTs. 5 µL of stage V gametocytes were obtained from in vitro parasite culture as described previously[44]. RDTs probed with these stage V gametocytes were extracted using the ENAR protocol after a three-week storage period at RT.

School-based survey in Mkuranga district. Carestart HRP2/pLDH Combo RDTs were used to determine the parasite rate among asymptomatic children from three primary schools in the Mkuranga district of Coastal Tanzania. Extraction protocol A, which is based on the Quick-DNA Miniprep Kit (Zymo Research Corporation, Irvine CA, USA), was used to extract DNA from a total of 190 RDTs collected during this school-based survey. *Pf* was detected by amplifying the acidic terminal sequence of the var genes (PfvarATS)[45].

Sample collection, analysis and storage during CHMI studies. RDTs were collected during two CHMI studies conducted to evaluate Sanaria's PfSPZ Vaccine in Bagamoyo, Tanzania (Clinical Trials.gov registration numbers NCT02613520 and NCT03420053, respectively). The first CHMI was conducted in 2016 (referred to CHMI-1) and the second CHMI was conducted in 2018 (referred to CHMI-2). Fresh venous whole blood collected in EDTA tubes was analyzed by RDTs within 45 min after blood collection. During CHMI-1, 20 µL was applied to BinaxNOW Malaria RDT (Alere, Cologne, Germany) and during CHMI-2, 5 µL was applied to Carestart HRP2/pLDH Combo RDT. The RDTs were read according to the manufacturers guidelines and then stored in a box at RT until extraction of NA.

The same samples were used to monitor parasitemia during CHMI by thick blood smear microscopy and qPCR as described elsewhere[46,47]. All samples were processed and analyzed at the laboratory of the Bagamoyo branch of the Ifakara Health Institute in Tanzania.

Malaria indicator survey on Bioko Island, Equatorial Guinea. The 2018 BIMEP Malaria Indicator Survey (MIS) was carried out between August and October 2018 on a representative sample of 13,505 individuals from 4,774 households selected from all communities across Bioko Island. All consenting permanent residents and short-term visitors were tested for malaria using the CareStart Malaria HRP2/pLDH Combo RDT. Used RDTs were stored at RT in plastic bags containing desiccants and transported to the Swiss Tropical and Public Health Institute for further molecular analysis.

Nucleic acid extraction methods from RDTs. The RDT cassettes were opened, the entire RDT strip removed and cut into four small pieces in order to fit into a 1.5 mL micro-centrifuge tube. A set of cleaned forceps and scissors were used with special attention given to prevent cross-contamination between samples. After processing a sample, the scissors and forceps were cleaned in 10% sodium hypochlorite, wiped with ethanol-sprayed tissues and dried before processing the next sample. The four nucleic extraction protocols tested, named A through D, were all based on silica columns.

Protocol A: ZR Quick-DNA Miniprep Kit. The protocol is based on the Quick-DNA Miniprep Kit (Zymo Research Corporation, Irvine CA, USA). Briefly, 1 mL of Genomic Lysis Buffer was added to the pre-cut RDT strip in a 1.5 mL micro-centrifuge tube and incubated at 95 °C for 20 min. The mixture was then transferred onto the extraction column and the manufacturers guide was followed for extraction. DNA was eluted in 50 µL of DNA Elution Buffer.

Protocol B: ZR Quick-DNA Miniprep Plus Kit. The protocol is based on the Quick-DNA Miniprep Plus Kit (Zymo Research Corporation, Irvine CA, USA). We added 600 µL of Solid Tissue Buffer (Blue) and 40 µL of Proteinase K to the pre-cut RDT strip in a 1.5 mL micro-centrifuge tube and incubated at 55 °C for 60 min. The supernatant was transferred to a clean 1.5 mL micro-centrifuge tube and 640 µL of Genomic Lysis Buffer was added and thoroughly mixed. The mixture was transferred onto the extraction column and extracted per manufacturers guidelines. DNA was eluted in 50 µL of DNA Elution Buffer.

Protocol C: NukEx Pure RNA/DNA Kit. The protocol is based on NukEx Pure RNA/DNA Kit (Gerbion GmbH, Kornwestheim, Germany), which co-extracts DNA and RNA. We created a working solution of 500 µL of Binding Buffer, 4 µL of Poly A and 50 µL of Proteinase K. The working soution was added to the pre-cut RDT strip in a 1.5 mL micro-centrifuge tube following incubation at 60 °C for 10 min. The supernatant was transferred onto the NukEx Spin Column and textraction was carried out per manufacturer's guidelines. Total NAs were eluted in 50 µL of Elution Buffer.

Protocol D: Zainabadi et al. extraction method for DBS. The protocol is based on a recently published extraction protocol for total NAs from dried blood spots[48]. Identical buffer compositions were used, and the protocol was adapted to extraction of NAs from RDT strips. We incubated the pre-cut RDT strip in 900 µL lysis buffer at 60 °C for 2 h. The supernatant was transferred onto Omega HiBind RNA Mini Columns (Omega Bio-Tek, Norcross, USA) and NAs extracted as described. NAs were eluted in 50 µL of Elution Buffer (Quick-DNA Miniprep Kit, Zymo Research Corporation, Irvine CA, USA).

High-throughput extraction protocol of NAs from RDTs (ENAR protocol). We adapted protocol D to extract NAs from used RDTs in a high-throughput manner. The main modification included a horizontal incubation of the entire uncut RDT strip by using sterile, RNase-/DNase-free 12-channel reservoirs (Axygen, Corning Inc, USA) and switching to a 96-well format for extraction. By removing the cutting step, the hands-on time during the extraction process is significantly reduced, as well the risk of cross-contamination by carryover during the cutting process is minimized. Up to eight 12-channel reservoirs, with a total of 96 samples, were processed in one batch. Lysis was conducted by adding 900 µL lysis buffer to each RDT strip placed in the 12-channel reservoir followed by incubation at 60 °C for 2 h with gentle shaking. All supernatants were then transferred to Omega E-Z 96 wells DNA plates (Omega Bio-Tek, Norcross, USA), washed with Wash Buffer 1 and 2 and lastly eluted into a 96 well plate (DNA LoBind Plates, Eppendorf) with 50 µL pre-warmed (60 °C) Elution Buffer (Zymo Research Corporation, Irvine CA, USA). With these adaptations to the protocol, NA from 96 RDTs can be extracted in about three hours, with minimal hands-on time needed. One positive control (RDT spiked with 5 µL blood containing 200 Pf/µL) and one negative control (Lysis Buffer only) were included with each extraction

plate to control for plate-to-plate consistency and cross-contamination. A standard operating procedure (SOP) for ENAR can be found in Supplementary Protocol S1.

Detection and quantification of *Plasmodium* spp. parasites. We used the PlasQ assay, a multiplex qPCR assay for *Plasmodium* spp. and *Pf* detection and quantification to analyze the NAs extracted from RDTs[47]. The PlasQ assay targets the Pan-*Plasmodium* 18S DNA and RNA (Pspp18S)[49,50] and the *Pf*-specific acidic terminal sequence of the var genes (PfvarATS) [45]. The human *rnasep* gene (HsRNaseP) [49] served as an internal control to assess the quality of NA extraction and qPCR amplification. To run the PlasQ as a RT-qPCR assay, targeting both DNA and RNA templates, we added 1 × Luna WarmStart RT Enzyme Mix (New England Biolabs, Ipswich, USA) and started the RT-qPCR program with a reverse transcription step at 55 °C for 15 min. All qPCR and RT-qPCR assays were run on a Bio-Rad CFX96 Real-Time PCR System (Bio-Rad Laboratories, California, USA). Samples were analyzed in duplicate with positive (PfNF54 DNA), negative (malaria negative individual) and non-template (molecular biology grade H_2O) controls added to each qPCR run.

Multiplex pre-amplification of *Plasmodium* spp. DNA. The *Plasmodium* spp. 18S rDNA and pfk13 genes of all PlasQ-positive samples were amplified in a multiplex reaction by conventional PCR. We amplified 3 µL of extracted NAs in a total volume of 20 µL using 1 × HOT FIREPol MultiPlex Mix (Solis Biodyne, Tartu, Estonia). Using 0.25 µM of the published primers, AGT GGA AGA CAT CAT GTA ACC AG and CCA AGC TGC CAT TCA TTT GT, 986 bp of the pfk13 propeller region were amplified[26]. Simultaneously, 1,407–1,469 bp of the pan-*Plasmodium* 18S rDNA were amplified using 0.5 µM of GRA ACT SSS AAC GGC TCA TT[51] and AGC AGG TTA AGA TCT CGT TCG[49]. The conditions of the multiplex PCR were the following: 95 °C for 12 min; 25 cycles of 95 °C for 20 s, 57 °C for 40 s and 72 °C for 1 min 45 s; and 72 °C for 10 min.

Detection of gametocytes and *Plasmodium* spp. species identification. *Gametocyte-specific RT-qPCR assay.* A previously published RT-qPCR assay for identification of *Pf* gametocytes based the PF3D7_0630000 transcript was used[30]. Briefly, 2 µL of extracted NAs were added to 8 µL reaction mix consisting of 0.6 µM of primers, 0.3 µM probe and Luna Universal Probe One-Step RT-qPCR Kit (New England Biolabs, Ipswich, USA). The qPCR program included a reverse transcription step for 15 min at 53 °C, followed by polymerase activation for 1 min at 95 °C, and 45 cycles with 15 s at 95 °C and 45 s at 53 °C.

Plasmodium spp. species identification. Non-*falciparum Plasmodium* species identification based on the 18S rDNA gene was performed. 2 µL of the product from the *Plasmodium* spp. multiplex pre-amplification were added to the master mix containing 1 × Luna Universal Probe qPCR Master Mix, 0.8 µM forward (GTT AAG GGA GTG AAG ACG ATC AGA) and 0.8 µM reverse primers (AAC CCA AAG ACT TTG ATT TCT CAT AA) to amplify a 157- to 165-bp segment of the *Plasmodium* spp. 18S rDNA gene[52]. Species-specific probes were selected to differentiate between the species. *P. malariae* was detected using a Yakima Yellow-labelled MGB probe (CTA TCT AAA AGA AAC ACT CAT)[53], *P. ovale* spp., inclduing *P. ovale curtisi* and *P. ovale wallikeri*, using a novel designed Texas Red-labelled and LNA-modified probe (GGA [LNA-A]AT [LNA-T]TC TTA GAT TGC TTC CT[LNA-T] CAG), *P. vivax a* Cy5-labelled probe (GAA TTT TCT CTT CGG AGT TTA)[54] and *P. knowlesi* a Cy5-labelled probe (CTC TCC GGA GAT TAG AAC TCT TAG ATT GCT)[55]. The conditions for the qPCR were: 95 °C for 3 min and 45 cycles of 95 °C for 15 s and 57 °C for 45 s.

Genotyping of pfk13 propeller region. In a second PCR reaction with a 15 µL total volume, 1.5 µL of the product from the *Plasmodium* spp. multiplex pre-amplification was amplified using 1 × HOT FIREPol MultiPlex Mix (Solis Biodyne, Tartu, Estonia) and 0.33 µM forward (TGA AGC CTT GTT GAA AGA AGC A) and reverse (TCG CCA TTT TCT CCT CCT GT) primers. Except for an annealing temperature of 58 °C, the PCR conditions were similar to the first reaction. The 798 bp product of the second PCR was evaluated using agarose gel electrophoresis and samples which failed amplification were repeated. Amplicons were sequenced by Microsynth (Microsynth AG, Balgach, Switzerland).

Data analysis and statistics. All (RT)-qPCR assays were run in duplicates and initial data analysis of the (RT)-qPCR data was conducted using CFX Maestro Software (Bio-Rad Laboratories, California, USA). In the case where one replicate interpreted as positive and the other negative, then the assay was repeated and the result was considered positive if two positive replicates were obtained out of the total four replicates. All (RT)-qPCR data generated were managed and analyzed by a custom-designed laboratory management and information system named ELIMU-MDx[32]. The ELIMU-MDx platform supports automated quality control, management and analysis of qPCR data. Oligo design and sequence analysis was performed using Geneious Prime 2019.1.1 (https://www.geneious.com). Statistical analysis and visualization of data was conducted using R version 3.5.1 based on packages *dplyr*, *ggpubr*, *ggplot2*, *gridextra*, *reshape2* and *scales*.

Ethics approval and consent to participate. For the school-based survey in Mkuranga district, sample collection was approved by the Senate Research and Publication Committee (SRPC) of the Muhimbili University of Health and Allied Sciences (Ref. No. 2012-04-04180) and the respective authorities at Mkuranga district. Both clinical trials were performed in accordance with Good Clinical Practices (GCP). CHMI-1 (Clinical Trials.gov: NCT02613520) protocol was approved by IRBs of the Ifakara Health Institute (IHI) (Ref. No. IHI/IRB/ No: 32-2015), the National Institute for Medical Research Tanzania (NIMR) (NIMR/HQ/R.8a/Vol.IX/2049), and the Ethikkommission Nordwest- und Zentralschweiz (EKNZ) Switzerland (reference number 15/104). The pro-

tocol was also approved by the Tanzania Food and Drug Authority (TFDA) (Auth. No. TZ15CT013). CHMI-2 (Clinical Trials.gov: NCT03420053) protocol was approved by IHI's IRB (Ref. No. IHI/IRB/ No: 32-2015), NIMR (NIMR/HQ/R.8a/Vol.IX/2049), EKNZ (reference number 15/104) and TFDA (Auth. No. TZ15CT013). The 2018 malaria indicator survey was approved by the Ministry of Health and Social Welfare of Equatorial Guinea and the Ethics Committee of the London School of Hygiene & Tropical Medicine (Ref. No. LSHTM: 5556). Written informed consent was obtained from all adults and from parents or guardians of children who agreed to participate. Only samples for which an additional consent for molecular analysis was obtained were included in this study. We confirm that all experiments were performed in accordance with relevant guidelines and regulations.

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Author contributions

Conceptualization: EAG, TS, CD, Data curation and validation: EAG, TS, OTD, Formal analysis and visualization: EAG, TS, Funding acquisition: CD, MT, CM, BMN, Investigation: JS, NS, HM, Methodology: EAG, SH, GC, AD, LG, MM, CAY, Resources: SA, NS, JS, SLH, GM, CCF, WPP, GAG., Software: SK, Project administration and supervision: CD, TS, Writing—original draft: EAG, TS, CD, All authors reviewed the manuscript.

Competing interests

SL Hoffman is salaried and full-time employee of Sanaria Inc, the developer and sponsor of PfSPZ Vaccine. He was not responsible for the collection, recording or entry of the parasitological data used in this study. The other authors have no conflicts of interest.

Additional information

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Chapter III

Analysis of nucleic acids extracted from rapid diagnostic tests reveals a significant proportion of false positive test results associated with recent malaria treatment

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Analysis of nucleic acids extracted from rapid diagnostic tests reveals a significant proportion of false positive test results associated with recent malaria treatment

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Abstract

Background: Surveillance programmes often use malaria rapid diagnostic tests (RDTs) to determine the proportion of the population carrying parasites in their peripheral blood to assess the malaria transmission intensity. Despite an increasing number of reports on false-negative and false-positive RDT results, there is a lack of systematic quality control activities for RDTs deployed in malaria surveillance programmes.

Methods: The diagnostic performance of field-deployed RDTs used for malaria surveys was assessed by retrospective molecular analysis of the blood retained on the tests.

Results: Of the 2865 RDTs that were collected in 2018 on Bioko Island and analysed in this study, 4.7% had a false-negative result. These false-negative RDTs were associated with low parasite density infections. In 16.6% of analysed samples, masked *pfhrp2* and *pfhrp3* gene deletions were identified, in which at least one *Plasmodium falciparum* strain carried a gene deletion. Among all positive RDTs analysed, 28.4% were tested negative by qPCR and therefore considered to be false-positive. Analysing the questionnaire data collected from the participants, this high proportion of false-positive RDTs could be explained by *P. falciparum* histidine rich protein 2 (PfHRP2) antigen persistence after recent malaria treatment.

Conclusion: Malaria surveillance depending solely on RDTs needs well-integrated quality control procedures to assess the extent and impact of reduced sensitivity and specificity of RDTs on malaria control programmes.

Keywords: Molecular malaria surveillance, False-positive malaria rapid diagnostic test, PfHRP2 persistence, *pfhrp2* gene deletion

Background

According to the World Health Organization (WHO), more than 409,000 malaria-related deaths were reported in 2019, most of them in children below the age of 5 years [1]. The majority of malaria infections (94%) and malaria-related deaths (95%) occurred in sub-Saharan Africa (SSA) [2], where *Plasmodium falciparum* is the dominant

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malaria parasite [1]. The test-treat-track strategy advised by WHO is one of the backbones of current malaria control and elimination programmes [3]. This strategy entails every suspected malaria case be tested, every confirmed case be treated, and the disease be tracked through surveillance systems [4]. Testing relies heavily on rapid diagnostic tests (RDTs), exemplified by the more than 348 million RDTs distributed globally in 2019 [1]. In SSA, RDTs have almost completely replaced light microscopy for malaria diagnosis, accounting for an estimated 75% of all malaria tests conducted in 2017 [5]. RDTs are point-of-care tests that detect circulating antigens, such as the *P. falciparum*-specific histidine rich protein 2 (PfHRP2) or histidine rich protein 3 (PfHRP3), as well as the pan-*Plasmodium* spp. enzymes, lactate dehydrogenase (pLDH) or aldolase [6]. More than 90% of RDTs currently in use target the PfHRP2 antigen because of its higher sensitivity compared to non-PfHRP2 antigens [7]. PfHRP2-based RDTs used for the diagnosis of febrile patients that suffer from malaria infection are highly sensitive and specific [8]. RDTs are often used by national malaria surveillance programmes. However, when individuals are asymptomatic with low parasite densities, RDTs often fail to detect the parasites due to low antigen concentrations [9, 10].

A recent study showed that false-negative RDTs (FN-RDT) are more common in lower malaria transmission settings, younger subjects and in urban areas in SSA [11]. Reduced diagnostic performance of RDTs has also been attributed to genetic diversity of the *pfhrp2* gene [12], differences in expression levels of PfHRP2 antigen in parasite field strains [13], or deletion of *pfhrp2* and *pfhrp3* genes in isolates [14]. *Pfhrp2* gene deletions appear to be common and therefore are relevant as they might be a threat to malaria control programmes based on monitoring of malaria prevalence through RDTs [15, 16].

Less attention has been given to the specificity of malaria RDTs used in malaria surveys that potentially result in false-positive results. False-positive RDTs (FP-RDT) have been associated with high levels of circulating rheumatoid factor [17–19] or acute typhoid fever [20]. There is evidence of FP-RDTs in patients infected with *Schistosoma mekongi* [21] or human African trypanosomiasis [22]. FP-RDTs are also caused by persisting antigen circulation in peripheral blood after successful *P. falciparum* drug treatment. A meta-analysis revealed that around half of the PfHRP2-detecting RDTs remain positive 15 days (95% CI 5–32) post *P. falciparum* treatment, which is 13 days longer than RDTs based on the pLDH antigen [23]. The latter study also reported a higher persistent RDT positivity among individuals treated with artemisinin combination therapy (ACT) than those treated with other anti-malarial drugs. Since RDTs are instrumental to malaria surveillance programmes, their diagnostic performance should be systematically monitored over time using sensitive and highly specific methods detecting *Plasmodium* spp. molecular markers. Described here is an approach for quality control of field-deployed RDTs by retrospective molecular analysis of the parasite DNA retained on them using RT-qPCR.

Methods

The 2018 malaria indicator survey conducted on Bioko Island as a biobank of RDTs for molecular malaria surveillance

A malaria indicator survey (MIS) has been conducted annually since 2004 on the island of Bioko, Equatorial Guinea, to evaluate the impact of malaria control interventions [24]. The survey uses a standard questionnaire developed by the Roll Back Malaria initiative to gather information on selected households and their occupants. The 2018 Bioko Island MIS covered 4774 households with 20,012 permanent residents, among whom 13,505 persons consented to storage and molecular analysis of their RDT. Briefly, consenting individuals living in surveyed households are tested for malaria and malaria-related anaemia. Malaria testing was done with the CareStart™ Malaria HRP2/pLDH (Pf/PAN) combo test (ACCESS BIO, NJ, USA). PfHRP2-positive RDTs were recorded as *P. falciparum*, pLDH-positive RDTs as *Plasmodium* spp. and RDT-positive for both antigens as mixed. The haemoglobin level in peripheral blood was measured during the MIS using a battery-operated portable HemoCue system (HemoCue AB, Ängelholm, Sweden). The anaemia status (mild, moderate, severe) was categorized based on definitions published by WHO [25] stratified by age, gender and pregnancy status. Households were assigned scores based on the type of assets and amenities they own to derive a surrogate of their socio-economic status (SES), using principal component analysis (PCA). After ranking all households based on their score, they were divided into five equal categories (quintiles), each with approximately 20% of the households. The first quintile corresponded to the lowest wealth index and the fifth to the highest wealth index. The household wealth index categories were also assigned to permanent household members.

Detection and quantification of *Plasmodium* spp. nucleic acids extracted from RDTs

A previously published dataset generated with the Extraction of Nucleic Acids from RDTs (ENAR) protocol developed by the authors was extended for this study [26]. Briefly, RDTs were barcoded, stored at room temperature and shipped to Basel, Switzerland, for nucleic acid (NA) extraction and detection. This approach simplifies small

volume blood collection, transport and storage logistics, and allows linking outcomes of molecular-based detection of parasite-derived NA with the demographic and socio-economic information collected from each corresponding MIS participant at high throughput.

All 2865 samples were initially screened with the PlasQ RT-qPCR assay [27]. In this RT-qPCR assay, the high copy number *P. falciparum*-specific varATS region [28] and the pan-*Plasmodium* 18S rDNA gene were targeted [29, 30]. Samples with cycle of quantification (Cq) value < 45 in two replicates of either of the two targets, varATS or 18S rDNA, were considered positive for active blood-stage malaria infection. *Plasmodium falciparum* parasites were quantified based on their Cq value for varATS [26]. In addition, only samples with Cq value < 35 for amplification of the internal control gene, the human *rnasep* gene were included, to demonstrate that the NA extracted from the RDTs is sufficient for reliable molecular analysis of malaria parasites. Non-*falciparum* malaria species identification of samples positive for the pan-*Plasmodium* target 18S rDNA was performed with a multiplex RT-qPCR assay based on species-specific 18S rDNA sequences as described previously [26].

Quality control and categorization of RDT outcomes

A RDT was considered positive if a healthcare worker recorded a positive signal for the PfHRP2, pLDH or both targets during the MIS. Among these positive RDTs, a true-positive RDT (TP-RDT) result was defined as a RDT with detectable *Plasmodium* spp. NA (two replicates with varATS and/or 18S rDNA Cq < 45 and human *rnasep* Cq < 35). A FP-RDT result was defined as positively read and recorded RDT in the field but with a negative outcome for *Plasmodium* spp. NA based on PlasQ RT-qPCR in the presence of human *rnasep* Cq < 35. Negative RDTs were classified as being read as negative by a healthcare worker during the MIS and recorded in the database. A true-negative RDT (TN-RDT) result was defined as a RDT whose negative result collected in the field was confirmed by the PlasQ RT-qPCR. A FN-RDT result was defined as negatively read by a healthcare worker in the field with a positive PlasQ RT-qPCR result based on two replicate amplifications with varATS and/or 18S rDNA Cq < 45 and the human *rnasep* Cq < 35.

qHRP2/3-del assay for detection of *pfhrp2* and *pfhrp3* deletions

The previously published qHRP2/3-del assay that simultaneously amplifies the *pfhrp2* and *pfhrp3* genes together with the internal control gene *pfrnr2e2* was adapted to accommodate for the lower input of NA [31]. Briefly, the probe for the internal control gene *pfrnr2e2* was labelled with fluorescein (FAM) instead of Cy5 to improve its detectability. Additionally, the final concentration of all primers was increased from 0.3 µM to 0.45 µM. Concentrations of 0.15 µM were used for the *pfrnr2e2* probe, and 0.225 µM for the *pfhrp2* and *pfhrp3* probes each. All samples were run in triplicates and the number of amplification cycles was increased from 45 to 50. Every 96-well qPCR plate contained control DNA extracted from a known *pfhrp2*-deleted *P. falciparum* strain (Dd2), a *pfhrp3*-deleted *P. falciparum* strain (HB3), and a *P. falciparum* strain without *pfhrp2* and *pfhrp3* gene deletions (NF54) as well as a non-template control (NTC). Successful amplification was defined as a mean Cq < 40 for *pfrnr2e2* calculated from at least two replicates for each sample. The qHRP2/3-del assay only was run with NA extracted from RDTs that had displayed a Cq < 35 for the *varATS* target in the PlasQ RT-qPCR.

Pfrnr2e2, *pfhrp2* and *pfhrp3* are all single-copy genes and they show comparable performances in the multiplex qPCR assay [31]. One approach to detect *P. falciparum* strains with *pfhrp2* and/or *pfhrp3* gene deletions in mixed *P. falciparum* strain infections (herein defined as masked gene deletions), is to calculate the difference in Cq values obtained between *pfhrp2* or *pfhrp3* and *pfrnr2e2* amplifications (ΔCq values). This is done by subtracting the Cq value obtained during the amplification of *pfrnr2e2* from the Cq value of *pfhrp2* or *pfhrp3*, respectively. Combining all runs that were conducted, the mean ΔCq for *pfhrp2* in controls (NF54 and HB3) was 0.00 (SD ± 0.52) and for *pfhrp3* the mean ΔCq in controls (NF54 and Dd2) was 1.19 (SD ± 0.83). For *pfhrp2* the ΔCq cut-off value of 2.0 determined by Schindler et al. [31] was used to identify masked gene deletions. For *pfhrp3* a ΔCq cut-off value of 4.0 was chosen to identify masked gene deletions due to the shift in mean ΔCq in the controls.

Genotyping of *Plasmodium falciparum pfmsp1* and *pfmsp2 genes*

Genotyping with *pfmsp1* and *pfmsp2* was performed following published procedures using nested PCR [32]. The first two PCR reactions amplify conserved sequences within the polymorphic regions of *pfmsp1* and *pfmsp2*, respectively. The second, nested PCR targets allele-specific sequences in five separate reactions. Samples were run in 20-µL total volume with 1 × Hot Firepol Master Mix (Solys BioDyne, Estonia), 0.25 µM of forward and reverse primers and 2-µL template DNA. The cycling conditions for the first PCR were 95 °C for 12 min, 25 cycles of 95 °C for 30 s, 58 °C for 1 min and 72 °C for 2 min and 72 °C for 10 min. For the second PCR, the cycling conditions for the three allele-specific *pfmsp1* primer pairs were 95 °C for 12 min, 35 cycles of 95 °C for 30 s, 56 °C for 40 s and 72 °C for 40 s and 72 °C for 10 min. For the two *pfmsp2* allele-specific reactions the conditions were: 95 °C for 12 min, 35 cycles of

95 °C for 30 s, 58 °C for 40 s and 72 °C for 40 s and 72 °C for 10 min. Presence and size of PCR products was determined and documented visually on a 1% agarose gel with a 100 bp DNA ladder.

Genotyping of *Plasmodium malariae* circumsporozoite protein (pmcsp)

The *pmcsp* gene was amplified by semi-nested PCR for all samples with a positive signal for *Plasmodium malariae* in the non-*falciparum* malaria species identification assay [26]. The first PCR was run with 3 µL of DNA template in a reaction volume of 20 µL. The reaction mix contained 1 × Hot Firepol Master Mix and 0.25 µM of each of the primers csp_OF [33] and csp-R [34]. The conditions for the first PCR were: 95 °C for 12 min; 35 cycles of 95 °C for 15 s, 53 °C for 30 s and 65 °C for 90 s and final elongation at 65 °C for 10 min. The second, semi-nested PCR used 1.5 µL of the product from the first reaction in a total volume of 15 µL. The reaction mix contained 1 × Hot Firepol Master Mix and 0.33 µM of the primers csp_IF [33] and csp-R. The conditions for the second PCR were: 95 °C for 12 min; 35 cycles of 95 °C for 15 s, 52 °C for 30 s and 62 °C for 90 s and final elongation at 62 °C for 10 min. The PCR product was sent to Microsynth (Microsynth AG, Switzerland) for bidirectional sanger sequencing. The 15 sequences of *P. malariae* circumsporozoite protein from Bioko Island have been deposited into GenBank under the Accession Numbers MW963324–MW963338.

Data analysis and statistics

The generated (RT)-qPCR data was initially analysed with the CFX Maestro Software (Bio-Rad Laboratories, CA, USA). Thresholds for each fluorescence channel were set manually and Cq values were then uploaded to the ELIMU-MDx platform for data storage and analysis [23]. Sequence analysis was performed using Geneious Prime 2019.1.1 (https://www.geneious.com). Statistical analysis and data visualization was performed using the R statistical language (version 4.0.3) based on packages data.table, dplyr, epiDisplay, epitools, ggplot2, ggpubr, ggridges, gridExtra, lme4, readxl, reshape2, scales, stringr, tidyr, tidyverse. Wilcoxon rank sum test was used for numeric values. Fisher's exact test (two-sided) was used for contingency tables. A generalized linear mixed-effects model with fixed and random effects was used for calculation of odds ratios and their confidence intervals.

Results

Integration of molecular diagnostic methods into the national malaria control programme to assess the performance of malaria RDTs

In total, 2865 RDTs (21.2%) collected during the 2018 MIS were included in this study. The median age of volunteers included in this sample collection was 22 years (interquartile range 9 to 38 years), female participants were over-represented (58.2%), and 97.8% of the participants were asymptomatic, non-febrile individuals. Of the 507 (17.7%) participants that reported to have been sick in the 2 weeks preceding the survey, 81.5% (413/507) had had fever. Other common symptoms were headache in 34.1% (173/507), followed by articular and bone pain in 21.3% (108/507), pallor and weakness in 13.0% (66/507), vomiting in 11.9% (60/507), and shaking chills in 7.5% (38/507). Fever was accompanied by other symptoms in 87.2% (360/413) of those who reported to have had fever. More than two-thirds of the RDTs were collected in the urban areas of the capital city Malabo on Bioko Island.

Following NA extraction, a PlasQ RT-qPCR result was generated for 1800 malaria negative and 1065 positive RDTs, as recorded in the MIS database. By comparison between PlasQ RT-qPCR results with the RDT results collected in the field, RDTs were grouped into four categories, namely true-positive (TP), true-negative (TN), false-positive (FP), and false-negative (FN), respectively (Fig. 1). The PlasQ RT-qPCR was used as a gold standard to evaluate the performance of the RDT, and this resulted in an overall sensitivity of 90.0% and specificity of 85.0% of field-deployed RDTs.

When stratified by type of antigen, RDTs classified as FP-RDTs were predominantly those that detected the PfHRP2 antigen (88.4%); whereas 8.9 and 2.6% of the FP-RDTs were those that detected both PfHRP2 plus pLDH antigens or the pLDH antigen only, respectively. Around half of RDTs classified as TP-RDTs were those that detected the PfHRP2 antigen only (50.1%), followed by those that detected PfHRP2 plus pLDH antigens (45.9%) and lastly, those that detected the pLDH antigen only (4.0%).

Low parasite density infections are likely to cause FP-RDT results in the field

The ENAR approach used in this study detects 10–100 times lower asexual blood stage parasite densities than the PfHRP2-based RDT [26]. The data confirms that a clear association exists between FN-RDT, TP-RDT and *P. falciparum* parasite densities assessed by the PlasQ RT-qPCR outcome. TP-RDT had higher geometric mean parasite densities (35.0 *P. falciparum*/µL, IQR: 7.2–166.0) compared to FN-RDTs (4.6 *P. falciparum*/µL, IQR: 1.1–20.0) (Fig. 2a, Wilcoxon rank sum test, $p < 0.001$). Although *P. falciparum* was the most common (93.8%) *Plasmodium* spp. species among RT-qPCR positive RDTs, *P. malariae* (4.0%) and *Plasmodium ovale* spp. (1.1%) were also identified. Co-infections between *Plasmodium* spp. species were included in these prevalence calculations. In 3.2% (27/847) of *Plasmodium*

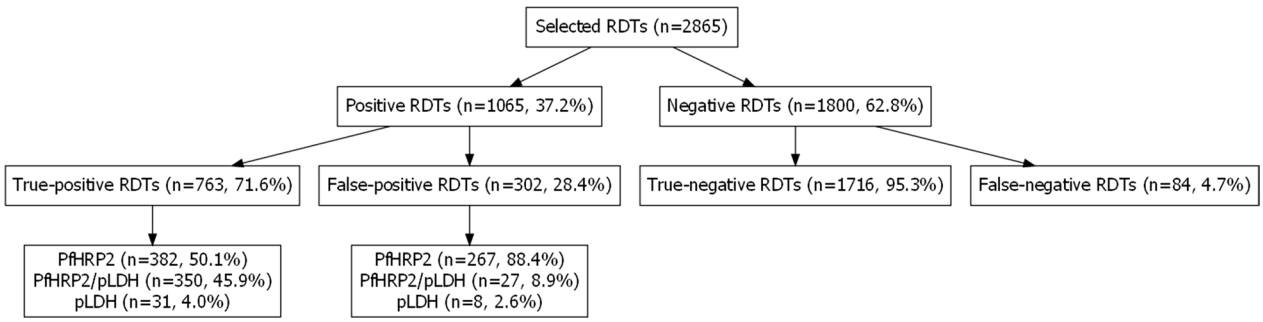

Fig. 1 Comparison of rapid diagnostic test outcomes with PlasQ RT-qPCR results obtained after nucleic acid extraction and amplification. Nucleic acids were extracted from 2865 RDTs collected during the 2018 MIS and subsequently amplified with the PlasQ RT-qPCR to detect *Plasmodium* specific nucleic acids

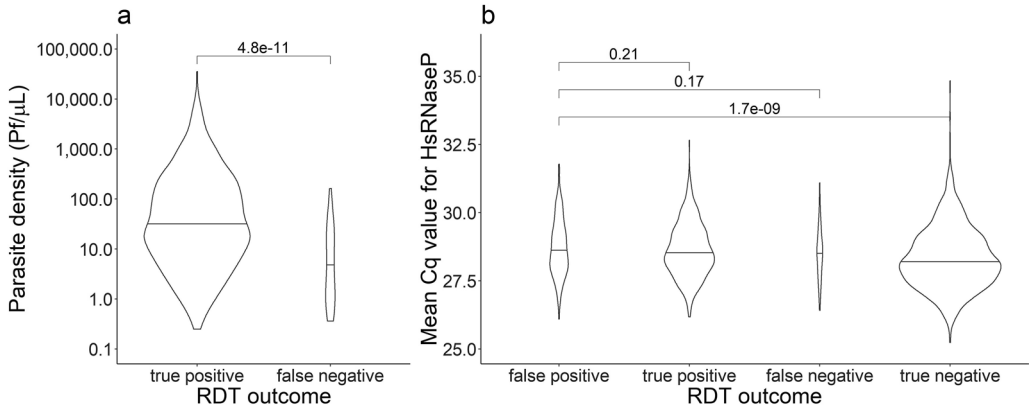

Fig. 2 Rapid diagnostic test outcome in relation to *Plasmodium falciparum* parasite densities and qPCR amplification of human *rnasep* endogenous control. **a** *Plasmodium falciparum* parasite densities compared between true positive and false negative RDT outcomes. Parasite densities for *P. falciparum* were estimated based on the varATS target of the PlasQ RT-qPCR assay. Wilcoxon rank sum test was used to compare the two groups. **b** Comparison of the Cq values obtained with the amplification of the human *rnasep* gene used as internal control of the PlasQ RT-qPCR assay, across all samples stratified by RDT outcome. The group of RDTs with a false-positive result was compared to the other RDT outcomes by Wilcoxon rank sum test

spp.-positive samples, no species could be assigned, possibly due to low parasite density and the generally lower sensitivity of the species-specific qPCR assays. No *Plasmodium vivax* and *Plasmodium knowlesi* parasite NAs were detected. The central repeat region of the *P. malariae* circumsporozoite protein (*pmcsp*) was amplified by PCR and Sanger sequenced to reconfirm the presence of *P. malariae* derived NA (Additional file 1: Fig. S1b). Nucleotide sequences were unique among all the 15 *P. malariae* PCR fragments sequenced and also the number of NAAG and NDAG repeats varied between these isolates indicating high diversity of the local *P. malariae* population. *Plasmodium malariae* was found among 6.6% of FN-RDTs compared to 3.8% among TP-RDTs. Similarly, *P. ovale* spp. was more prevalent in FN-RDTs (2.6%) than in TP-RDTs (0.9%).

To exclude the possibility that FP-RDTs are the consequence of failed amplification related to the degradation of NA retained on the RDTs, an additional analysis was carried out. During the PlasQ RT-qPCR, the human *rnasep* gene was used as an internal control to monitor the amount of NA extracted from each RDT. On average, the human *rnasep* was amplified with a Cq value of 28.5 (SD ± 1.0). There was no significant difference in the Cq values of the human *rnasep* gene amplification among RDTs, which were categorized as FP (28.6, SD ± 1.0), TP (28.5, SD ± 1.0), or FN (28.6, SD ± 1.0). TN-RDTs had a significantly lower median Cq value (28.2, SD ± 1.1) (Fig. 2b). These results indicate that the lack of detectable *P. falciparum* NA in the blood retained on FP-RDTs is not related to poor NA extraction performance or a failure in detecting NAs.

FN-RDT results are not associated with parasites carrying *pfhrp2* and *pfhrp3* gene deletions

Plasmodium falciparum strains were genotyped to identify strains with *pfhrp2* and/or *pfhrp3* gene deletions. The number of samples available was limited based on the combination of low parasite density infections and the limited amount of blood retained on RDTs as a source of NA. The single copy gene *pfrnr2e2*, serving as the internal control of the qHRP2/3-del assay, was amplified with Cq < 40 in 184/406 (45.3%) samples. To avoid false reporting of *pfhrp2* and/or *pfhrp3* gene deletions, the analysis was restricted to samples that had an additional successful amplification in either *pfmsp1* (32/47, 68.1%) or *pfmsp2* (31/47, 66.0%). No amplification in *pfmsp1* or *pfmsp2* was observed in 23.4% (11/47) of samples. Based on the available data from the 27 samples with successful *pfmsp1* and *pfmsp2* genotyping (Additional file 1: Fig. S1a), polyclonal infections consisting of two or more distinct *P. falciparum* clones were found in 63.0% (17/27) of samples. Association between parasite density and amplification of each of the three distinct reference genes (*pfrnr2e2, pfmsp1* or *pfmsp2*) is shown in Fig. 3a–c. At least two out of three reference genes were amplified in 36 samples, which were then included in the analysis of the *pfhrp2* and *pfhrp3* deletion status. No evidence for parasites carrying a *pfhrp2* gene deletion was found in these 36 samples, but 4 out of 36 samples (11.1%) were likely to carry *pfhrp3* gene deletions. All 4 samples with *pfhrp3* deletion were recorded as positive for PfHRP2 by RDT.

The qHRP2/3-del assay was used to identify *pfhrp2* and/or *pfhrp3* gene deletions in polyclonal *P. falciparum* infections by calculating the ΔCq values as the difference of Cq values between *pfhrp2 and pfhrp3* gene amplification and the *pfrnr2e2* internal control. Figure 3d shows the distribution of samples with their respective ΔCq values for *pfhrp2* and *pfhrp3.* Of the 36 samples included, 2 samples (5.6%) had increased ΔCq values for both genes, 2 samples (5.6%) only for the *pfhrp2* gene and 2 samples (5.6%) only for the *pfhrp3* gene, respectively. Importantly, all 36 samples, which were screened for *pfhrp2* and *pfhrp3* gene deletions, were positive for PfHRP2 by RDT. Three out of 6 samples with increased ΔCq values for *pfhrp2* and/or *pfhrp3* were successfully genotyped with *pfmsp1* and *pfmsp2*. Two genotypes were found in one sample with increased ΔCq value for *pfhrp2* and *pfhrp3* each and a single genotype in one sample with increased ΔCq value for *pfhrp3*.

FP-RDT results are associated with recent use of anti-malarial drugs

The rate of FP-RDTs differed across age, level of anaemia, geographical location of residence, and the SES (Additional file 1: Fig. S2). Interestingly, no study participant with a FP-RDT had a fever (> 37.5 °C) at the time of survey, while 1.6% (12/754) of those with TP-RDTs were recorded with fever. Eight variables collected during the MIS were used to identify risk factors associated with FP-RDTs through multivariate logistic regression analysis in which the outcome of the test was set as the outcome variable (Additional file 1: Table S1). FP-RDTs (n = 297) were compared to TP-RDTs (n = 754). Because sample collection was clustered within communities, community affiliation was introduced as a random effect to the model. The MIS included 299 communities, of which 201 (67.2%) were represented in the dataset. The median number of samples from a community was 3. Survey participants belonging to higher socio-economic classes (aOR 1.51 p = 0.01) had increased odds of having a FP-RDT. Participants who were reported to have been treated with an anti-malarial drug 2 weeks preceding the survey had more than four times the odds of a FP-RDT result than a TP-RDT (aOR 4.52, p < 0.001). Noteworthy, 46.6% (136/292) of the participants who had received an anti-malarial treatment in the 2 weeks preceding the survey did recall what drug they had been treated with. The majority of MIS participants (80.9%, 110/136), who reported to have received recent anti-malarial treatment, mentioned that they had received artemisinin derivates or ACT. Due to the small number of MIS participants treated with non-ACT anti-malarials, the variety of anti-malarials used within this group, and the fact that this information is self-reported, it was decided not to include any further analysis, including a breakdown into individual drugs. In contrast, moderate to severe anaemia reduced the odds of having a FP-RDT (aOR 0.60, p = 0.02). Those who reside in the rural Bioko Sur Province had also decreased odds of having a FP-RDT (aOR 0.44, p = 0.01). Age, gender, bed-net use, and reported sickness in the 2 weeks preceding the survey were not significantly associated with FP-RDTs (Fig. 4).

The impact of asymptomatic malaria infections on anaemia status might be underestimated by FP-RDT results

It was hypothesized that high rates of FP-RDTs are likely to result in underestimating the impact of asymptomatic malaria infections on the anaemia status. Among malaria-infected children aged < 5 years, the prevalence of anaemia was 67.7% if malaria status was assessed by RDT. Proportion of anaemic children with FP-RDT result (48.9%) is similar to children with TN-RDT result (41.4%) (p = 0.85, Fisher exact test), whereas children with a TP-RDT result are more likely to suffer from anaemia (78.3%) (p = 0.0005, Fisher exact test) (Fig. 5). This significant effect is even more pronounced among

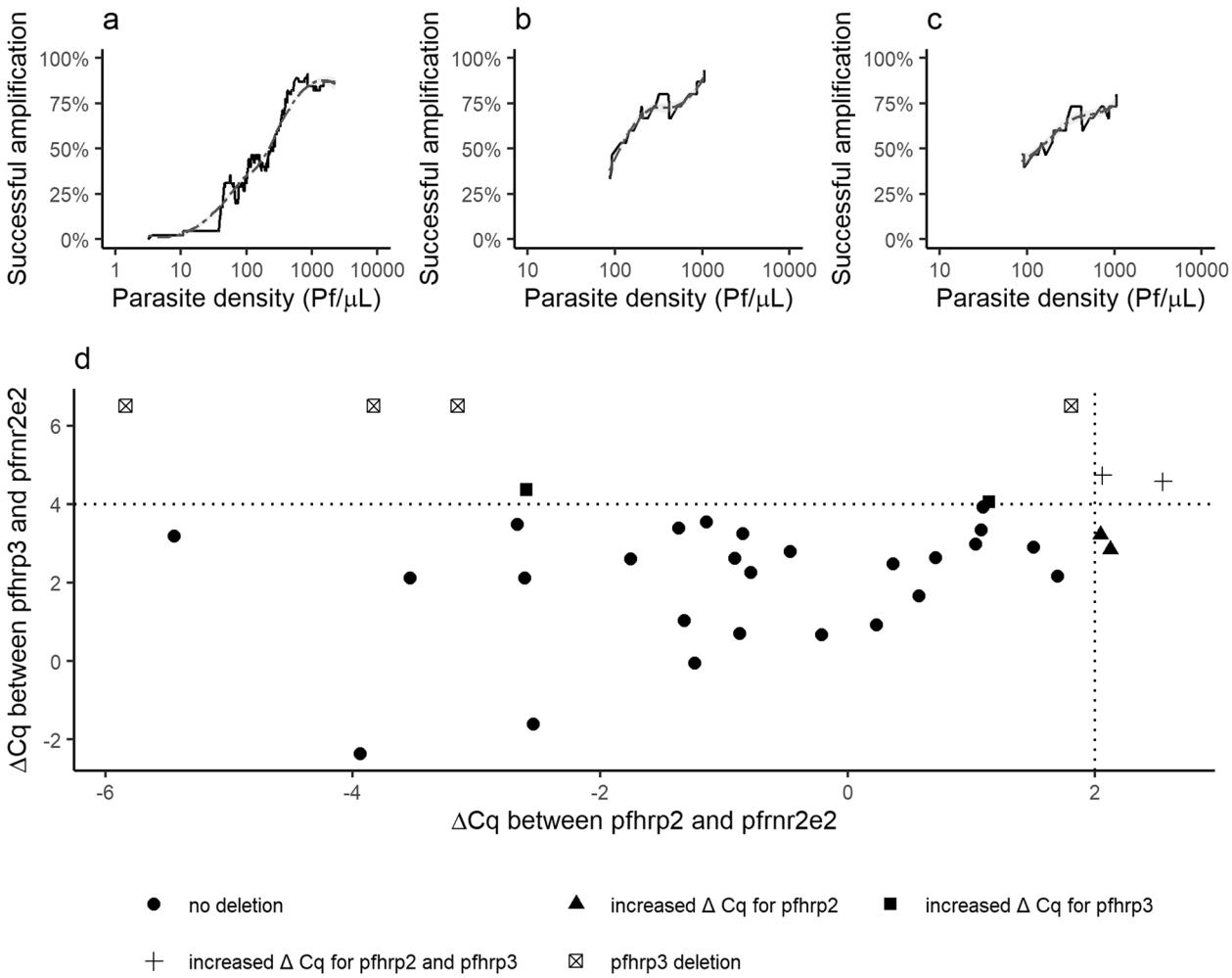

Fig. 3 Amplification rate for three single copy genes and ΔCq values of the qHRP2/3-del assay. Amplification rate (rolling mean) for the genotyping reference genes **a** *pfrnr2e2*, **b** *pfmsp1* and **c** *pfmsp2* as a function of the parasite density of the sample. Parasite densities for *P. falciparum* were estimated based on the *varATS* target of the PlasQ assay. **d** The distribution of ΔCq values between *pfhrp2* (x-axis) or *pfhrp3* (y-axis) and *pfrnr2e2*. ΔCq thresholds (dashed lines) were set at 2.0 for *pfhrp2* and 4.0 for *pfhrp3*. To show the ΔCq for *pfhrp2* for samples with a *pfhrp3* deletion, the ΔCq for *pfhrp3* was set arbitrarily at 6.5

children < 5 years with moderate and severe anaemia if compared to mild anaemia. Removing all FP-RDTs in this association between malaria infection status and anaemia levels in children < 5 years reveals that the association between asymptomatic malaria with moderate or severe anaemia might be even stronger. In older children and adults, the impact of FP-RDTs on assessing the anaemia status is negligible.

Discussion

Malaria control programmes rely on continuous and systematic collection of surveillance data for decision making and resource allocation [35]. A critical measure that closely reflects malaria transmission intensity is the parasite rate, which is the proportion of the population found to carry parasites in their blood [36]. RDTs, more specifically PfHRP2-based RDTs, are the most widely used test to measure parasite rates in endemic countries and are a cornerstone of malaria control. However, diagnostic performance issues of PfHRP2-based RDTs were identified to be particularly related to limited specificity. Therefore, malaria surveillance depending solely on RDTs might profit from well-integrated quality control procedures assessing the potential impact of reduced sensitivity and specificity of the RDT used. Presented in this report is an efficient approach to assess the performance of field-deployed RDTs used for

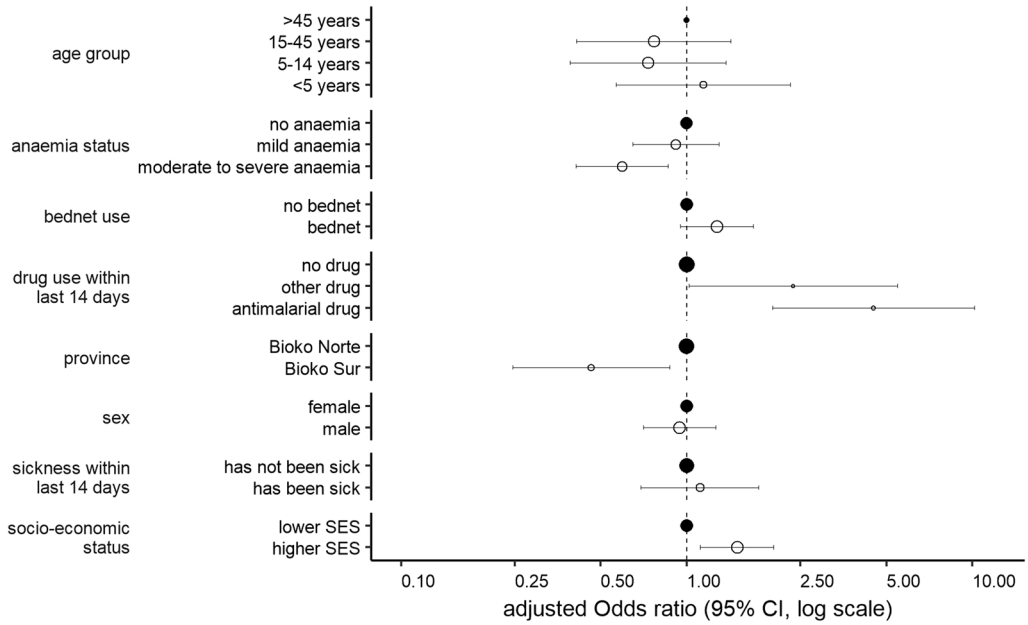

Fig. 4 Risk factors associated with false-positive rapid diagnostic test results by multivariate logistic regression analysis. The size of the circles corresponds with the number of responses for each variable outcome. The reference group is marked by filled circles and the other groups have open circles. Higher socio-economic status (SES) included people from the 4th and 5th wealth quintiles

malaria surveillance based on NA extraction from the RDTs followed by qPCR analyses.

Plasmodium spp. NA was found in 4.7% (84/1800) of the negative RDTs and were classified as FN-RDTs. The low proportion of FN-RDTs can be explained by the low parasite densities in these asymptomatic individuals (geometric mean of 5.4 *P. falciparum*/µL) and the low amount of blood (one drop corresponds to approximately 5 µL) used as starting material for the molecular analysis. This is a certainly one of the major limitations of the approach. In a previous study conducted among asymptomatic blood donors in Malabo, PfHRP2-based RDTs showed a sensitivity of only 23.1% and more than 75% of infections had densities below 100 *P. falciparum*/µL [27]. Therefore, the true proportion of FN-RDTs in a high prevalence setting such as Bioko Island is likely to be higher than reported here.

Plasmodium falciparum isolates were identified with potential *pfhrp3* deletions but not a single isolate with a confirmed *pfhrp2* deletion. Given the overall high frequency of polyclonal *P. falciparum* infections in this setting (63.0% by *pfmsp1/pfmsp2* genotyping), it was assumed that if *P. falciparum*-carrying *pfhrp2* deletions exist, then they would be most likely masked by co-infecting *P. falciparum* isolates without *pfhrp2* gene deletions. Of all the samples included for final analysis, 11.1% had an increased ΔCq value for *pfhrp2* and 11.1% for *pfhrp3* amplification, indicating for the first time that there are likely *P. falciparum* strains circulating on Bioko Island carrying deletions in their *pfhrp2* and/or *pfhrp3* genes. So far, one report described *P. falciparum* strains carrying *pfhrp2* and *pfhrp3* deletions in blood samples collected on the continental region of Equatorial Guinea [37]. Since travel activity between Bioko Island and the mainland of Equatorial Guinea is high, it can be assumed that parasite strains are exchanged frequently between these locations [38]. Most importantly, blood samples with *P. falciparum* clones indicative of masked *pfhrp2* and *pfhrp3* gene deletions were recorded as PfHRP2 positive by RDT. Likely, the co-circulating *P. falciparum* clones compensate for the lack of PfHRP2 expression resulting in RDT-positive testing. In 462 clinical samples from different African countries, 7.4% (34/462) samples carried a *pfhrp2* deletion and 10.6% (49/462) a *pfhrp3* deletion, while masked *pfhrp2* and *pfhrp3* deletions were found in 3.0 and 3.2% of samples, respectively [39].

The data support the notion that in settings where polyclonal *P. falciparum* infections are common assays with the ability to identify masked *pfhrp2* and/or *pfhrp3* gene deletions should be used [40]. Importantly, to avoid false reporting of *pfhrp2* and/or *pfhrp3* gene deletions, a robust and multi-layered approach was used by which only samples with a pre-defined parasite density, successful amplification of the assays' internal control, and additional, independent amplification of either *pfmsp1* or *pfmsp2* genes were included into the analysis.

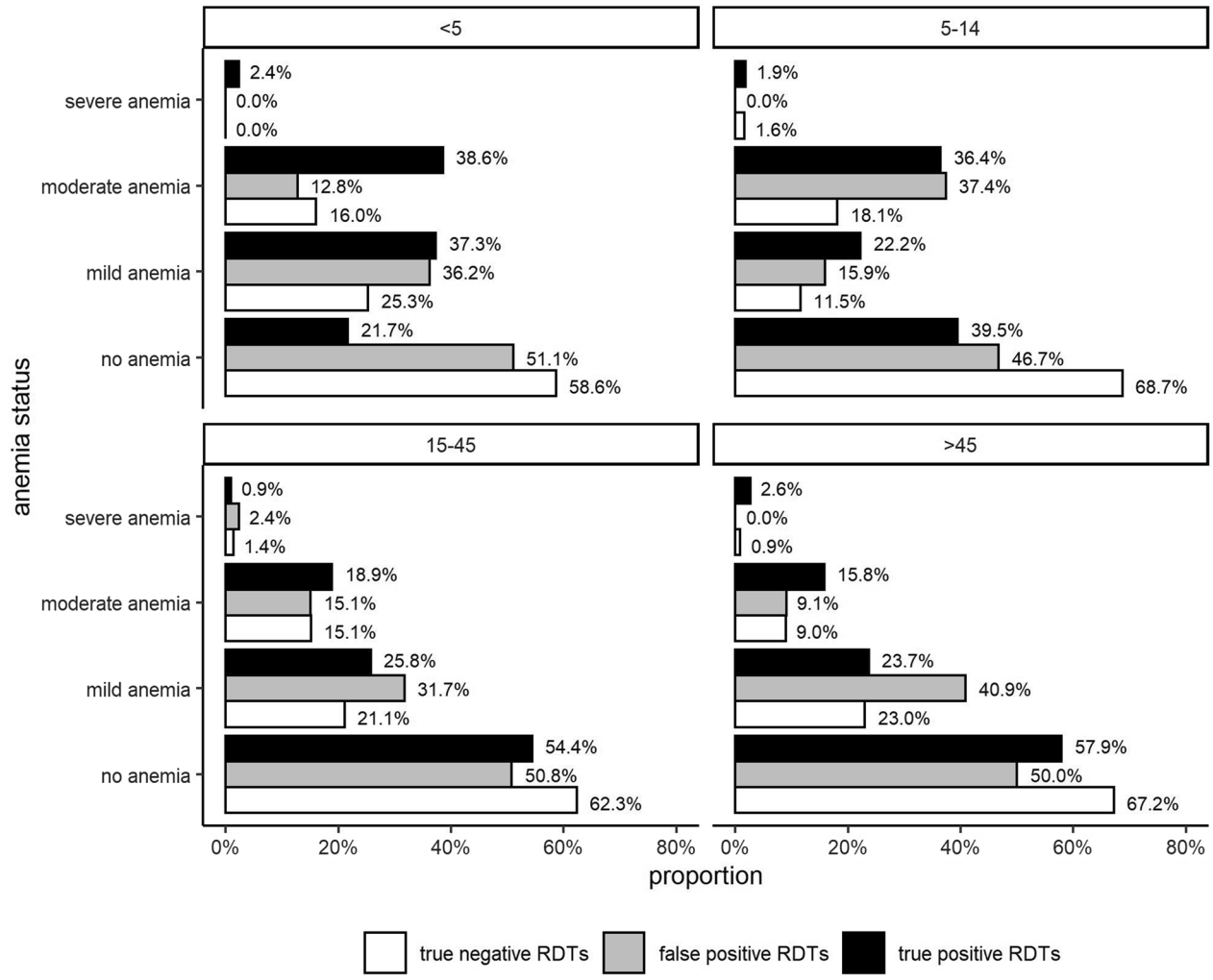

Fig. 5 Proportion of volunteers with different anaemia status stratified by true-positive, false-positive and true-negative rapid diagnostic test outcomes. The anaemia status was stratified by age group (< 5, 5–14, 15–45 and > 45 years). For TP-RDT, FN-RDT and TN-RDT test outcomes, the proportion of all participants of each age group with no, mild, moderate or severe anaemia was calculated

In this study, a significant proportion of FP-RDTs were discovered. The findings are not unique to Bioko Island. In a study conducted in Tanzania, 22% of malaria-positive RDTs were negative by molecular analysis for *P. falciparum* [41]. A study performed in Guinea-Bissau reported 26% FP-RDTs [42], and in Western Kenya, approximately one-third of positive RDTs were negative by molecular detection methods for *P. falciparum* [43]. With introduction of a novel RDTs labelled as 'ultra-sensitive', detecting lower concentrations of the PfHRP2 antigen, the problem of FP-RDT results is expected to become greater, as already shown in a recent study [44].

The wrong positivity of RDTs based on PfHRP2 detection could be associated with recent use of anti-malarial drugs confirming previous reports [23, 45–48]. It has been well established that anti-malarial treatment leads to FP-RDT results because the PfHRP2 antigen persists in the blood days to weeks after parasite clearance [23, 45–48].

In addition, an association was found between FP-RDTs and potential access to anti-malarial drugs based on higher SES and on living in urban parts of the Island.

The impact of FP-RDTs differs greatly depending on the setting in which RDTs are deployed. In clinical settings, FP-RDTs might be less common, but the consequences are serious since wrong prescription of anti-malarials might increase risk of overlooking other life-threatening diseases causing fever [49]. In cases where RDTs are used for epidemiological surveys, a high proportion of FP-RDTs due to PfHRP2 antigen persistence might lead to

an overestimation of malaria prevalence, particularly in populations with good access to anti-malarial treatment. Using RDT only as test for malaria infection status might underestimate the negative consequences of asymptomatic malaria infections on haemoglobin levels, particularly in children < 5 years of age [50].

The benefits and the challenges that come with large-scale deployment of molecular techniques for malaria surveillance in malaria-endemic regions have been discussed [51]. Alternative and non-molecular approaches such as automated malaria diagnosis using haematology analysers [52] should be further evaluated for malaria surveillance purposes. The ongoing COVID-19 pandemic has raised the awareness of the value of introducing novel methods as surveillance tools in the public health systems in Africa [53]. Building on this experience will potentially accelerate efforts to integrate sensitive and specific tools for continous, large-scale surveillance of malaria in control programmes.

Conclusion

Malaria surveillance programmes based on RDT assessments of malaria prevalence should be strengthened by the integration of molecular epidemiological data in the same setting. These data will serve as an early warning system for (i) spread of *P. falciparum* strains evading widely used diagnostic tests; (ii) understanding overuse of malaria drugs; (iii) help with identifying fever-causing diseases beyond malaria; and, (iv) help to clarify the burden of asymptomatic malaria as a cause of severe to moderate anaemia, particularly in children < 5 years.

Abbreviations

RDT: Rapid diagnostic test; PfHRP2/3: Histidine rich protein 2/3; ENAR: Extraction of nucleic acids from RDTs; MIS: Malaria indicator survey; RT-qPCR: Reverse transcription quantitative polymerase chain reaction; Cq: Quantification cycle; sSA: Sub-Saharan Africa; ACT: Artemisinin combination therapy; SES: Socio-economic status; NA: Nucleic acid; WHO: World Health Organization; PCA: Principal component analysis; FAM: Fluorescein; NTC: Non-template control.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12936-022-04043-7.

Additional file 1: Figure S1. Genetic diversity of *Plasmodium falciparum* and *Plasmodium malariae* length polymorphic genes. **Figure S2.** False-positive rapid diagnostic tests as a proportion of all positive rapid diagnostic tests. **Table S1.** Multivariable logistic regression analysis of risk factors associated with false-positive rapid diagnostic tests.

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Authors' contributions

Conceptualization: SH, CD, TS. Data curation and validation: SH, TS, OTD. Formal analysis and visualization: SH. Funding acquisition: CD, MT, GAG, WPP. Investigation: OTD, GAG, WPP, MOA, CAG. Methodology: SH, CAY, EAG, JPD, KB. Resources: MM, EN, OTD, GAG, WPP, CAG. Project administration and supervision: CD, TS. Writing original draft: SH, TS, CD. All authors read and approved the final manuscript.

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The funding sources had no role in the study design, the collection, analysis, and interpretation of data, as well as in writing this manuscript and in the decision to submit the paper for publication.

Availability of data and materials

All data needed to evaluate the conclusions in the paper are present in the manuscript or the Additional files. Further information will be made available to interested researchers.

Declarations

Ethics approval and consent to participate

The Ministry of Health and Social Welfare of Equatorial Guinea and the Ethics Committee of the London School of Hygiene and Tropical Medicine (Ref. No. LSHTM: 5556) approved the 2018 malaria indicator survey. Written informed consent was obtained from all adults and from parents or guardians of children who agreed to participate. Only samples for which an additional consent for molecular analysis was obtained were included in this study. We confirm that all experiments were performed in accordance with relevant national and international guidelines and regulations.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Publisher's Note

Chapter IV

Characterising co-infections with *Plasmodium* spp., *Mansonella perstans* or *Loa loa* in asymptomatic children, adults and elderly people living on Bioko Island using nucleic acids extracted from malaria rapid diagnostic tests

PLOS NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Characterising co-infections with *Plasmodium spp*., *Mansonella perstans* or *Loa loa* in asymptomatic children, adults and elderly people living on Bioko Island using nucleic acids extracted from malaria rapid diagnostic tests

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Abstract

Background

Regular and comprehensive epidemiological surveys of the filarial nematodes *Mansonella perstans* and *Loa loa* in children, adolescents and adults living across Bioko Island, Equatorial Guinea are lacking. We aimed to demonstrate that blood retained on malaria rapid diagnostic tests, commonly deployed for malaria surveys, could be used as a source of nucleic acids for molecular based detection of *M. perstans* and *L. loa*. We wanted to determine the positivity rate and distribution of filarial nematodes across different age groups and geographical areas as well as to understand level of co-infections with malaria in an asymptomatic population.

Methodology

M. perstans, *L. loa* and *Plasmodium* spp. parasites were monitored by qPCR in a cross-sectional study using DNA extracted from a subset malaria rapid diagnostic tests (mRDTs) collected during the annual malaria indicator survey conducted on Bioko Island in 2018.

Principal findings

We identified DNA specific for the two filarial nematodes investigated among 8.2% (263) of the 3214 RDTs screened. Positivity rates of *M. perstans* and *L. loa* were 6.6% and 1.5%,

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respectively. *M. perstans* infection were more prominent in male (10.5%) compared to female (3.9%) survey participants. *M. perstans* parasite density and positivity rate was higher among older people and the population living in rural areas. The socio-economic status of participants strongly influenced the infection rate with people belonging to the lowest socio-economic quintile more than 3 and 5 times more likely to be *L. loa* and *M. perstans* infected, respectively. No increased risk of being co-infected with *Plasmodium* spp. parasites was observed among the different age groups.

Conclusions/Significance

We found otherwise asymptomatic individuals were infected with *M. perstans* and *L. loa*. Our study demonstrates that employing mRDTs probed with blood for malaria testing represents a promising, future tool to preserve and ship NAs at room temperature to laboratories for molecular, high-throughput diagnosis and genotyping of blood-dwelling nematode filarial infections. Using this approach, asymptomatic populations can be reached and surveyed for infectious diseases beyond malaria.

Author summary

Mansonella perstans and *Loa loa* are filarial nematodes that infect millions of people living in less developed areas, predominantly in sub-Saharan Africa. Both parasites are neglected among other filarial nematodes because both are regarded as causing mainly asymptomatic infections. The aim of this study was to explore the feasibility of using malaria rapid diagnostic tests (mRDTs) deployed during malaria surveys as a convenient sampling strategy for molecular surveillance of blood-dwelling filarial nematode infections. Our findings demonstrate the potential of mRDTs as a source of parasite DNA beyond malaria, providing an opportunity to expand current knowledge on the distribution and populations mostly affected by *M. perstans* and *L. loa* infections to Equatorial Guinea, located in Central-West Africa.

Introduction

Human filariases are vector-borne infectious diseases that encompass Mansonellosis and loiasis [1]. Mansonellosis is caused by three main nematode species, *M. perstans*, *M. streptocerca* and *M. ozzardi* [2]. Recently, an additional species has been described in Gabon named *Mansonella sp* "DEUX" [3]. Mansonellosis is one of the most neglected tropical diseases despite the fact that in large parts of sub-Saharan Africa, as well as in Latin America an estimated 100 million people are infected [2,4]. The life cycles of *Mansonella* spp. generally alternate between an insect vector and humans who are the final hosts. The insect vectors transmitting *M. perstans* belong to the genus *Culicoides* [2]. When feeding on an infected human, female vectors pick-up microfilariae (mf) circulating in the blood. The mf penetrate the insect´s gut and undergo developmental stages in the thoracic flight muscle, migrate to the head and proboscis from where *M. perstans* is transferred to humans during the next feeding round [2]. The third-stage infective larvae (L3) actively penetrate the human skin before migrating and maturing into adult worms that can be found in serous body cavities, mainly the peritoneal cavity [2]. Adult male and female worms mate and female worms begin to produce unsheathed mf circulating

in peripheral blood. Unsheathed mf of *M. perstans* are detected by microscopic examination in thick or thin blood films stained with Giemsa in blood samples taken at any time of the day [5,6]. Little is known about the clinical outcome of chronic *M. perstans* infections in endemic populations, and as for other filarial infections most infections seem to be clinically silent [2,5]. Clinical symptoms attributed to *M. perstans* infections include eosinophilia, angioedema, arthralgia, fever, headache, pruritus, skin eruption, serositis, neurologic manifestations, ocular or palpebral pruritus, visual impairment and chest pain [1].

At least 10 million people are infected with *Loa loa* in endemic countries of Central, and Western Sub-Saharan Africa [7]. *L. loa* larvae are transmitted to humans during blood feeding of an infected vector fly belonging to the genus *Chrysops* spp. [1]. The sheathed adult worms live freely in the subcutaneous tissues where they produce thousands of sheathed mf daily, usually with a peak between 10 AM and 4 PM [8]. Diagnosis of *L. loa* can be challenging since adult worms can be present without detectable mf in blood [8].

In areas where different filarial nematodes are co-endemic, misclassification of mf by microscopy can be problematic [8,9]. Therefore, molecular methods have been developed to improve filarial nematode detection [10] and qPCR-based molecular techniques have shown higher sensitivity to detect low parasite density infections, and to discriminate between different filarial nematode species [8]. Large-scale implementation of molecular diagnostic methods for neglected tropical diseases has been regarded as a challenge in the public health systems of low and middle-income countries based on cost, human resource requirement and complexity of supply chain management [11].

The aim of this work was to demonstrate that blood retained on malaria rapid diagnostic tests (mRDTs) is a source of nucleic acids for molecular based detection of *M. perstans* and *L. loa* in otherwise asymptomatic individuals. In doing so, we described the association of filarial infections with demographic and geographic factors and assessed the level of multi-parasitism of these nematodes with the highly endemic malaria parasites on Bioko Island. We also used Cq values as a proxy of filarial parasite density and compared this measurement against demographic and geographic characteristics of the investigated population as well as the time of the day of RDT sample collection.

Material and methods

Ethics statement

The MIS in 2018 was approved by the Ministry of Health and Social Welfare of Equatorial Guinea and the Ethics Committee of the London School of Hygiene & Tropical Medicine (Ref. No. LSHTM: 5556). Consent was sought from eligible respondents before the administration of the questionnaire. A signed authorization was requested from the parents or legal guardians of children, and adult participants to use their samples for further analyses. Laboratory experiments were performed in accordance with relevant guidelines and regulations.

Study area

Bioko Island is located on the West African continent shelf, precisely in the Gulf of Guinea and separated from Cameroon by no more than 32 kilometres of shallow ocean. With its total land surface of approximately 2000 km^2, Bioko forms part of the nation of Equatorial Guinea and is administratively divided into four districts: Malabo, the capital of Equatorial Guinea, and Baney both located in the northern part of the Island; and Luba and Riaba both located in the Southern part. The Island has an estimated population size of 270´000 people, with the majority (85%) living in Malabo [12]. Bioko has a typical equatorial climate, with high temperatures, high humidity, and heavy rainfall [13].

Malaria has historically been hyper-endemic in Bioko Island with a prevalence of 45% among 2–14 year old children before the launch of the Malaria control program [14,15]. The Bioko Island Malaria Control Program—implemented since 2003—has successfully reduced malaria prevalence and malaria related morbidity and mortality [14,16]. Malaria indicator surveys (MIS) have been conducted annually since 2004 within sentinel sites across the island to enable monitoring and evaluation of this programme [14,17].

Study design

The 2018, the MIS on Bioko Island was conducted on a representative sample using the Malaria Pf/PAN (HRP2/pLDH) Ag Combo rapid diagnostic test (ACCESS BIO, NJ, USA). Consent to store used mRDTs for further molecular analyses was obtained from 13,505 survey participants and unique barcode labels were affixed to the used mRDTs that were shipped to the Swiss Tropical and Public Health Institute for further analyses. Each mRDT barcode was linked to a household unique identification code [18]. This allowed a detailed geographic allocation of the mRDT results. The age distribution of MIS 2018 participants differed slightly between the four districts. Malabo district with median age of 17 years, (IQR 7–30) and is characterized by a slightly younger MIS 2018 population than Riaba (17 years, IQR 7–40), Luba (19 years, IQR 8–45) or Baney (16 years, IQR 7–32). To derive parasite positivity rate estimates, all individuals found positive and all tested in the sample were aggregated at a 1x1 km^2 grid. Coinfections were estimated at the same spatial resolution, for comparison.

A subset of the mRDT were selected for molecular analysis of the blood retained on the mRDT based on the mRDT test outcome for malaria. Out of the 1376 malaria positive mRDTs identified during the 2018 MIS, we analysed the nucleic acids from 1065 mRDTs (77.4%). In addition, other malaria negative mRDT were selected for the molecular analysis.

Extraction of nucleic acids from used mRDTs

We reused the extracted nucleic acids (NAs) from a study which was published recently by our group [19]. Briefly, the entire and uncut nitrocellulose strip in the used mRDT was carefully removed and incubated in a lysing buffer at 60˚C for two hours. After several washing steps, NAs were eluted in a final volume of 75 μL and stored at -20˚C. The extracted NAs were amplified and detected by reverse transcription quantitative real time PCR (RT-qPCR) to identify and quantify *Plasmodium* spp. parasites [19]. For the study presented here, we extracted and analysed NAs from additional mRDTs collected during the same MIS and extend the approach by using the extracted NAs to detect the filarial nematodes, *M. perstans* and *L. loa*, by qPCR using well described marker genes [20,21]. We have calculated the median number of days between blood collection onto the mRDT during the MIS 2018 and extraction of NA in our laboratory in Basel to be 253 days (IQR 138–352 days).

Loa loa and *Mansonella perstans* detection by a multiplex qPCR assay

A multiplex qPCR assay, herein referred to as llmp-qPCR, was developed and performed to detect *L. loa* and *M. perstans* DNA. In a multiplex qPCR reaction, the *L. loa* specific qPCR assay amplifies a 62 base pair (bp) fragment from the hypothetical protein LLMF72 [20] and the *M. perstans* specific qPCR assay is based on amplification of a 187 bp fragment of the 18S ribosomal RNA gene [21]. The human rnasep gene (RNaseP) served as an amplification internal control to monitor the successful extraction and amplification of DNA [22]. All reactions were run in duplicates on 96-well plates. Molecular biology grade H_2O was run as a non-template control, and a mix of *M. perstans* and *L. loa* DNA served as a positive control for each run. Each reaction contained 2 μL of template DNA and 8 μL master mix consisting of 1 x

Luna Universal Probe qPCR Master Mix (New England Biolabs, Ipswich), 0.4 μM of *M. perstans* forward primer, 0.4 μM of *M. perstans* reverse primer, 0.25 μM of the Yakima Yellow-labelled *M. perstans* probe, 0.8 μM of *L. loa* forward primer, 0.8 μM of *L. loa* reverse primer, 0.4 μM of FAM-labelled *L. loa*-specific minor groove binder (MGB) probe, 0.4 μM HsRNaseP forward primer, 0.4 μM of HsRNaseP-reverse primer and 0.4 μM of Cy5-labelled HsRNaseP probe. Using the Bio-Rad CFX96 Real-Time PCR System (Bio-Rad Laboratories, California, USA), amplification program was 1min at 95˚C, followed by 50 cycles of 15s at 95˚C and 45s at 55˚C. Samples were considered positive if the quantification cycle (Cq) value was <50.

Co-infection of *Mansonella perstans* and *Loa loa* with *Plasmodium* spp.

The PlasQ is a multiplex RT-qPCR assay for *Plasmodium* spp. and *P. falciparum* detection and quantification that has been developed by our group and described previously [19,22]. This qPCR assay consisted of amplification of two targets combined in a multiplex assay, namely the Pan-*Plasmodium* 18S rDNA sequence (Pspp18S) and the *P. falciparum*-specific acidic terminal sequence of the var genes (PfvarATS). The human RNaseP (HsRNaseP) gene served as an internal control to assess the quality of DNA extraction and qPCR amplification. The PlasQ was performed on NA extracted from mRDT and samples with Cq value <45 of either of the two targets, PfvarATS or Pspp18S, were considered positive for *Plasmodium* spp. Then, results obtained were linked to llmp-qPCR results obtained from the same aliquot of NA extracted from identical mRDT to assess co-infection status between Plasmodium spp., *L. loa* and *M. perstans*. Coinfections were estimated at the same 1x1 km^2 grid, for comparison.

Sanger sequencing analysis of *Mansonella perstans* and *Loa loa*

The ribosomal internal transcribed spacer 1 region was amplified with a set of primers that bind universally to all filarial species and are designed to highlight interspecific differences [23]. The PCR products were 484 base pairs (bp) for *M. perstans* and 457 bp for *L. loa*. PCR products of 10 and 23 samples tested positive by llmp-qPCR for *L. loa* and *M. perstans*, respectively, were sequenced from both ends by Sanger Sequencing (Microsynth AG, Balgach, Switzerland) to confirm specificity of the qPCR assays. Samples covering a large and representative range of different Cq values were selected for sanger sequencing of the ribosomal internal transcribed spacer 1 region (S4 Fig). Sequence analysis was realized using Geneious Prime 2019.1.1 (https://www.geneious.com). A consensus sequence of all 23 *M. perstans* and 10 *L. loa* sequences of 417 bp and 390 bp length, respectively, served as a query sequence to identify all GenBank entries with >90% identity and >95% coverage using BLAST. Additionally, representative sequences for *M. streptocerca* (KR868771) and *M. ozzardi* (EU272180) deposited to GenBank were included. Geneious Prime software (version 2021.0.3) was used for the multiple sequence alignment and to generate the phylogenetic analysis using its in-build neighbour-joining (NJ) clustering method [24]. Branch lengths were estimated with the Tamura-Nei model [25] with *Onchocerca volvulus* (EU272179) as an outgroup. The resulting newick file was imported into R for final phylogeny and visualization using the ggplot2, ggtree, and treeio packages.

Data analysis

Households were assigned scores based on the type of assets and amenities they own (radio, television, sofa, fan, air-conditioner, car, etc) to derive a surrogate of Socio-Economic Status (SES), using Principal Component Analysis (PCA). Households were ranked based on their score and the distribution was further divided into five equal categories (quintiles), each with approximately 20% of the households. The first quintile corresponded to the lowest wealth

index (WI) and the fifth to the highest WI. The household WI categories were also assigned to permanent (de jure) household members. Predicted co-infection rates for *Plasmodium* spp. and *M. perstans* or *L. loa* were expressed as the product and 95% confidence interval (95% CI) of *Plasmodium* spp., *M. perstans* and *L. loa* the prevalence stratified by age group. The ELIM-U-MDx platform was used for quality control, management and analysis of qPCR data [26]. Statistical analysis and visualization of data were conducted using R version 3.5.1. Univariate analysis (Fisher's exact test and Wilcoxon-Mann-Whitney-Test, as appropriate) was used for comparison between groups. P-value < 0.05 was considered statistically significant.

Results

Sample selection and study population characteristics

A total of 4774 households, including 20'012 individuals, from across Bioko Island participated in the MIS 2018. 13'505 participants provided an additional consent for molecular analysis of the mRDT collected. To increase the probability to identify individuals with filarial nematodes and *Plasmodium* spp. co-infections, we over-sampled mRDTs from two specific sub-populations. Firstly, malaria positive mRDTs were preferentially selected and processed and secondly, for filarial nematodes infections, mRDTs from adults living in rural districts were enriched for selection and analysis. A graphical representation of the over-sampling is shown in S1 Fig. Among the mRDTs selected for NAs extraction, 1065 mRDTs were malaria positive, accounting for 75.8% of all positive mRDTs identified during the 2018 MIS. Significantly higher proportions of adults and people living outside of urban Malabo were included. The subset of mRDTs which were selected to investigate the positivity rates of *M. perstans* and *L. loa* infections stratified by geographical location, age and socio-economic status is shown in Table 1. Noteworthy, from each district or age group at least 10% of the collected mRDTs were included into the analysis. In summary, the majority of the samples included were collected in Malabo (64%). The proportion of mRDTs collected from women was higher compared to men. The mean age was 22 years (SD = 19.7) and participants aged <20 belonged to the most common age group (45.4%). Socio-economic status was higher in participants living in Malabo and Baney compared to the two southern districts (Luba and Riaba). The mean haemoglobin value of all participants was 12.02 g/dl (SD = 1.9) and 99.4% of people did not have fever at time of the sample collection.

Positivity rates of *L. loa* and *M. perstans* among participants of the annual malaria indicator survey

Using the llmp-qPCR assay, detecting simultaneously *M. perstans* and *L. loa* in a single qPCR reaction (S2 Fig), of the 3214 mRDTs that were tested, 8.2% (263) were positive for *M. perstans* and/or *L. loa*. The proportion of mRDTs positive for *M. perstans* was 6.6% (213) compared to 1.5% (50) for *L. loa*. Fig 1 details the positivity rates of *M. perstans* and *L. loa* stratified by age (A), socio-economic status (B) and gender (C). People living in rural districts have significantly higher positivity rates for *M. perstans* than people living in the urban areas. Positivity rates in rural districts ranged from 17.1% (Luba) to 13.2% (Baney) compared to 2.1% in the urban district of Malabo. On the contrary, no significant differences in *L. loa* positivity rates were observed between rural and urban districts. *L. loa* was more prevalent in the two Southern districts, Riaba (3.9%) and Luba (2.7%), compared to the Northern districts of Malabo (1.5%) and Baney (0.7%). *M. perstans* infection rates in high-endemic rural districts increased significantly with age and the highest positivity rate was observed in participants older than 60 years ($p < 0.00001$). *L. loa* was found at higher rates in participants older than 20 years of age

Table 1. Distribution of population included by age, gender, sociodemographic status and district of residence.

Characteristics	*Malabo (n* = 2064)	Baney (n = 690)	Luba (n = 257)	Riaba (n = 203)	Total (n = 3214)
Gender					
Women (%)	1261 (61.1)	384 (55.7)	139 (54.1)	100 (49.3)	2086 (55.0)
Men (%)	803 (38.9)	306 (44.3)	118 (45.9)	103 (50.7)	1704 (45.0)
Age (years)					
0–19 (%)	1261 (61.1)	110 (15.9)	42 (16.4)	47 (23.2)	1460 (45.4)
20–39 (%)	576 (27.9)	379 (54.9)	58 (22.7)	46 (22.7)	1059 (33)
40–59 (%)	111 (5.4)	141 (20.4)	81 (31.6)	58 (28.6)	391 (12.2)
≥ 60 (%)	116 (5.6)	60 (8.7)	75 (29.3)	52 (25.6)	303 (9.4)
Socio-economic status (quintile)					
1 lowest	185 (9.0)	153 (22.5)	93 (36.3)	94 (46.3)	525 (16.4)
2 second lowest	348 (16.8)	132 (19.4)	66 (25.8)	53 (26.1)	599 (18.7)
3 middle	473 (22.9)	130 (19.1)	29 (11.3)	32 (15.8)	664 (20.7)
4 second highest	487 (23.6)	119 (17.5)	49 (19.1)	22 (10.8)	677 (21.1)
5 highest	571 (27.7)	145 (21.4)	19 (7.4)	2 (1.0)	737 (23.0)

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in urban as well as in rural areas ($p = 0.0001$) (Fig 1A). Among children below the age of five, a positivity rate of 1.4% (4/296) for *M. perstans* and not a single infection for *L. loa* was observed. In older children and adolescents, positivity rates of 2.4% and 0.7% were estimated for *M. perstans* and *L. loa*, respectively. Infection rates were strongly influenced by the socio-economic status of the individuals (Fig 1B). People from rural district assigned to the lowest SES were three times more likely to harbour an *M. perstans* infection than people belonging to the highest SES. The same was observed for *L. loa* where the positivity rate was also higher among lowest SES compared to highest SES.

While the proportion of *L. loa* infections was comparable between male and female individuals combined for rural and urban districts ($p = 0.4$), *M. perstans* was significantly higher among male (21.1%) compared to the female (9.0%) inhabitants of rural districts ($p < 0.00001$) (Fig 1C).

Filarial nematode species identification by ribosomal internal transcribed spacer 1 region sequence analysis

Specificity of the qPCR-based species identification was confirmed by sequence analysis of the conserved ribosomal internal transcribed spacer 1 region that encompassed 390 to 450 bp depending on the filarial species (Fig 2). To our knowledge, this is the first time that a molecular marker, regularly used for filarial nematode species identification, was amplified and sequenced from DNA extracted from blood retained on mRDTs. Twenty-three samples positive for *M. perstans* and ten samples positive for *L. loa* identified by the llmp-qPCR assay were all confirmed by sequence analysis. The *M. perstans* sequences clustered closely with each other and other *M. perstans* sequences, but are distinct from *Mansonella* spp. DEUX sequences. The *L. loa* sequences generated in this study are closely related to sequences from Central- and West-Africa deposited in GenBank.

Geographical distribution of *M. perstans*, *L. loa* and *Plasmodium* spp. show distinct patterns

On Bioko Island the environmental living conditions for the population differ starkly between the urban centre in Malabo and the rural districts in Baney, Riaba and Luba. Therefore, we

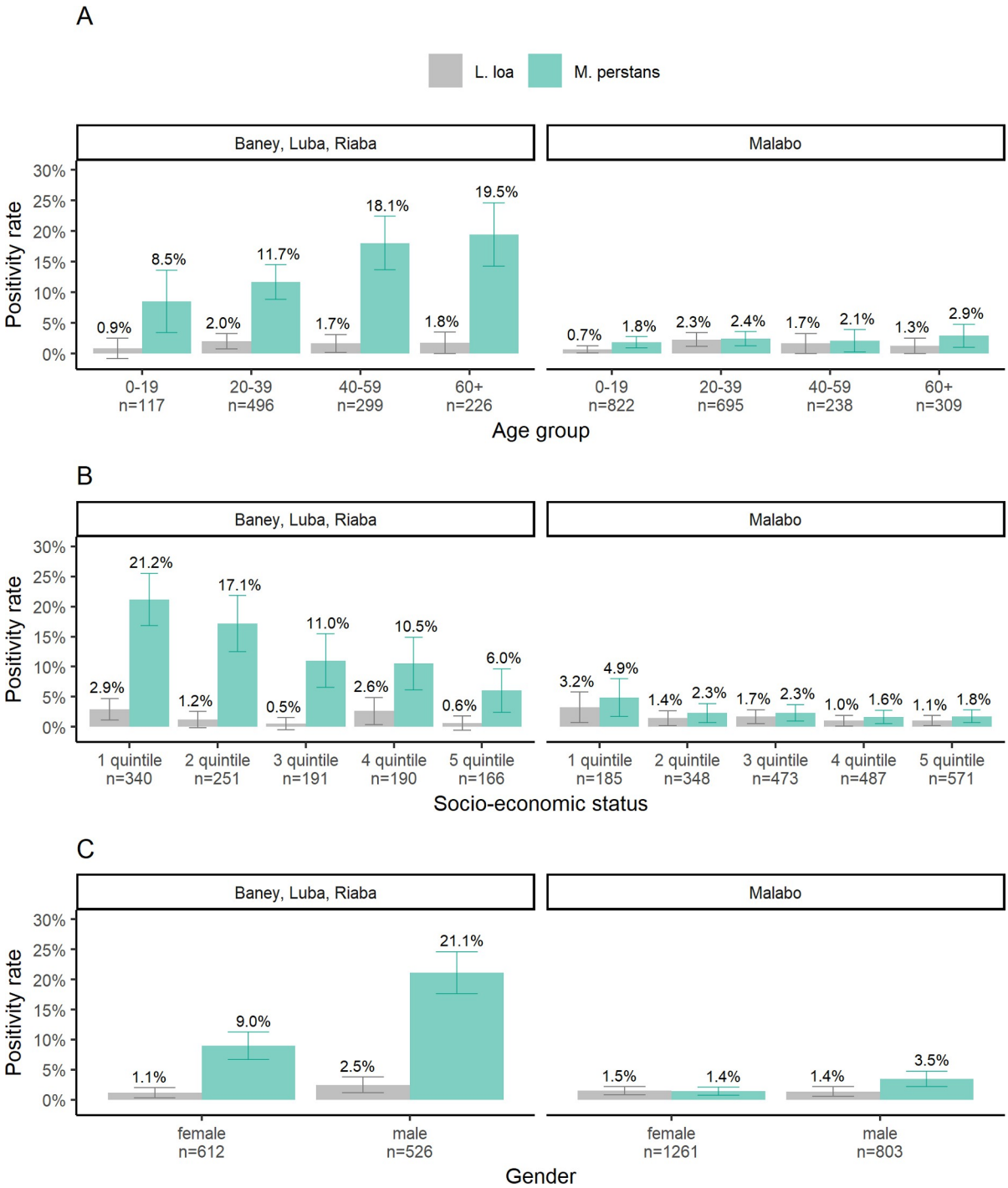

Fig 1. Positivity rates of *M. perstans* and *L. loa* by rural and urban districts. (A) across all age groups investigated, (B) grouped according to socio-economic status and (C) stratified by gender. Positivity rates and 95% confidence intervals were calculated as the proportion of llmp-qPCR positive mRDTs in all tests carried out in each group and are given on top of each bar. Data supporting the Fig 1 are detailed in S2 Table.

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mapped the prevalence and geographical distribution of *P. falciparum* for all samples collected during the MIS based on mRDT positivity in Fig 3A. To investigate the level of co-infections between malaria and filarial parasites in different populations, malaria positive RDTs were

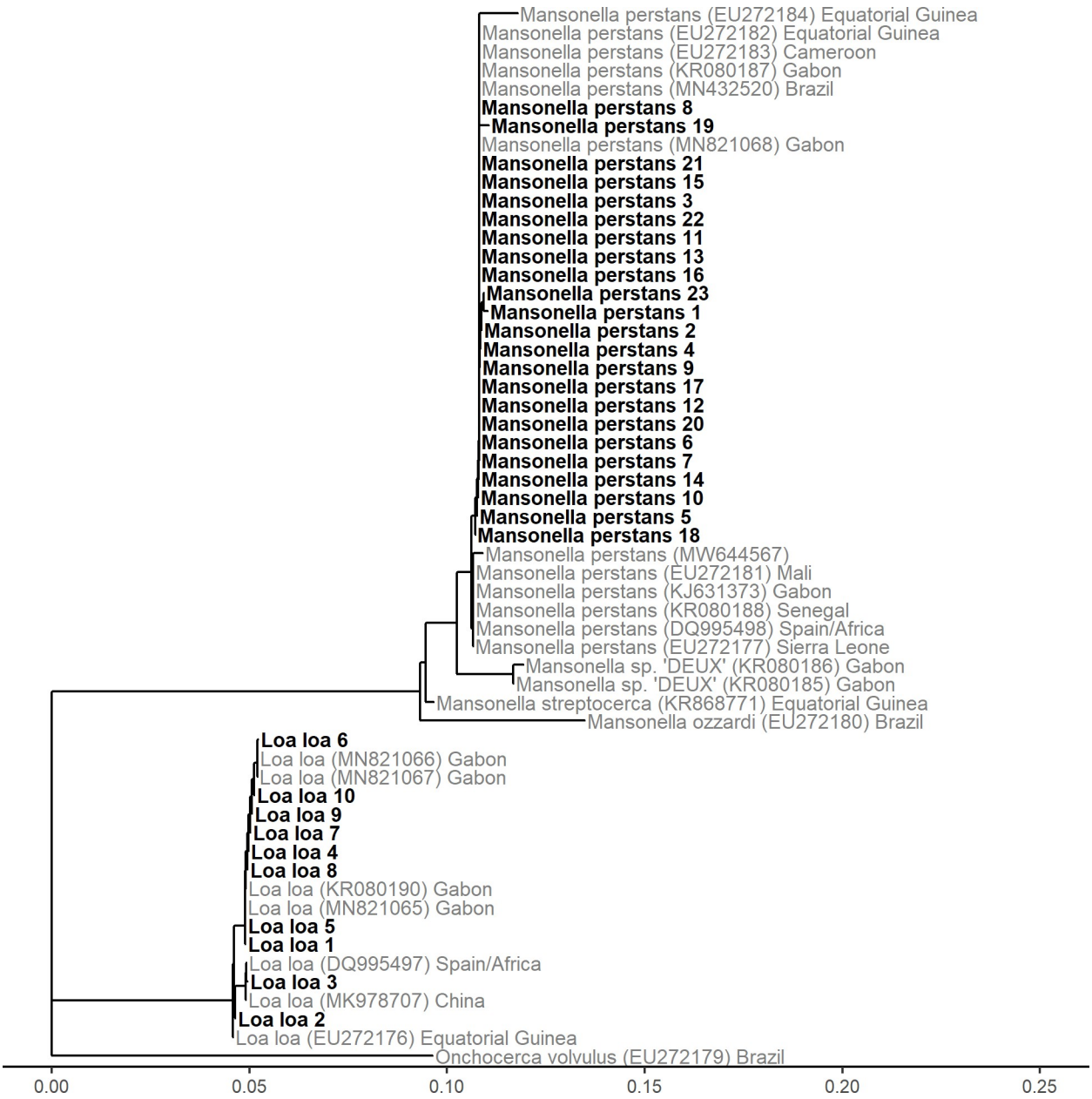

Fig 2. Phylogenetic tree of the ribosomal internal transcribed spacer 1 region of *M. perstans* and *L. loa*. PCR products were amplified from DNA extracted from mRDTs and sequenced. The scale on the x-axis corresponds to the number of substitutions per site (number of changes/sequence length).

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given priority when selected for molecular analysis described here. This intentional enrichment resulted in higher positivity rates of *Plasmodium* spp. when analysed by RT-qPCR (Fig 3B). High positivity rates of *M. perstans* were found in areas along the East coast as well as in the Southern districts; while in urban areas around Malabo, *M. perstans* was found at low rates or was even absent (Fig 3C). *L. loa* positivity rates are generally low and no distinct patterns are seen. Interestingly, no *L. loa* were found in the district of Baney, where positivity rates of *M. perstans* are the highest (Fig 3D).

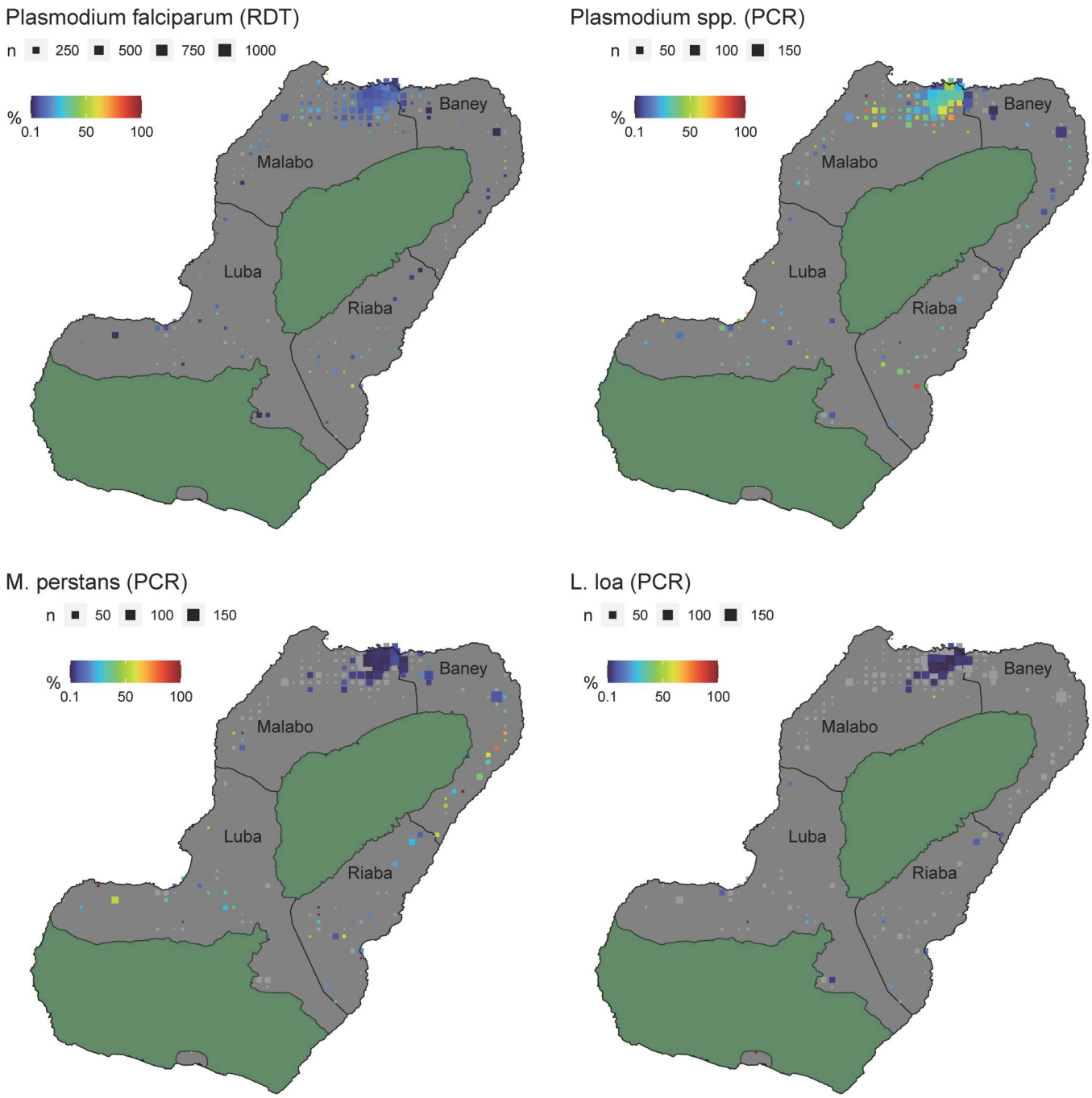

Fig 3. Positivity rate of *Plasmodium* spp, *M. perstans* and *L. loa* across Bioko Island. (A) Prevalence of PfHRP2-positive mRDTs for the entire MIS 2018 population. (B) *Plasmodium* spp. RT-qPCR positivity rate of mRDTs selected for molecular analysis. (C) *M. perstans* qPCR based positivity rate. (C) *L. loa* qPCR based positivity rate. The size of the squares represents the number of people analysed living in the corresponding 1x1 km^2 grid. The areas marked in green are nature reserves. Greyed out spots on the maps represent settlements from which mRDTs were collected with null positivity rate for Plasmodium *spp*, *M. perstans* and *L. loa*.

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Co-infection of *M. perstans* or *L. loa* with *Plasmodium* spp. parasites

Next, we wanted to estimate the proportion of malaria positive individuals co-infected with filarial nematodes at the molecular level. From the total 3214 mRDTs extracted, we analysed

2775 mRDTs for *Plasmodium* spp., by using the PlasQ assay because of limited availability of NA. Ten out of 2775 mRDTs were positive for both, *Plasmodium* spp., and *L. loa*, 32 were positive for both, *Plasmodium* spp., and *M. perstans*, and six were positive for both *M. perstans* and *L. loa*. Triple infection of *M. perstans*, *L. loa*, and *Plasmodium* spp., was found in three samples (Fig 4A). Then, we analyzed the likelihood that predicted co-infection rates between *Plasmodium* spp. and either *M. perstans* or *L. loa* differed from observed proportions indicative of biological interaction between these infectious diseases as described previously [27]. We did not find evidence for unexpected higher or lower proportions of co-infections in any of the investigated age groups (Fig 4B). Coinfections were mapped with areas positive for either *M. perstans* (Fig 4C) or *L. loa* (Fig 4D). Infections were highlighted according to the presence of people infected with more than one of the investigated parasites. Most coinfections were observed in Malabo, the area with the highest population density.

Age is associated with variation of microfilaria levels in peripheral blood

The llmp-qPCR Cq values for both, *M. perstans* and *L. loa*, were used as approximations of parasite density and compared between infected individuals older and younger than 40 years of age. The median age of all *M. perstans* positive individuals was 40 years. Persons older than 40 years had a significantly lower *M. perstans* Cq values compared to individuals below the age of 40 years (geom. Mean of 34.0 versus 35.1, $p = 0.02$) (Fig 5A). The difference in *M. perstans* infection parasite densities associated with age is not the result of variation in blood volumes analysed or amount of DNA amplified since the Cq values of the human RNaseP gene is similar between the two groups. The same outcome can be observed in Fig 5B. For instance, at a Cq value of 33, the cumulative frequency of individuals older than 40 years of age is 33.6% compared to only 15.5% for younger individuals. Analysing the distribution of the *M. perstans* Cq values reveals a clear shift towards lower Cq values in infected individuals above the age of 40 years (Fig 5C). Combining all these findings directs towards the conclusion that older individuals have a tendency to higher *M. perstans* parasite densities. No significant differences were observed among the individuals infected with *L. loa*. Apart from age (S3A Fig), no significant difference was observed between female and male gender (S3B Fig), while the parasite density of *M. perstans* infections was higher in rural areas comprising districts of Baney, Luba and Riaba compared to the more urban district of Malabo ($p = 0.018$) (S3C Fig). Interestingly, no difference of llmp-qPCR Cq values were seen in samples collected during the morning versus afternoon (S3D Fig).

Discussion

We conducted a larger scale, cross-sectional study of samples including paediatric, adolescent, adult and elderly populations residing in urban and rural regions of Bioko Island. To the best of our knowledge, this report represents the first molecular epidemiological study of *M. perstans* and *L. loa* in Equatorial Guinea that also includes the evaluation of geographical distribution and association with socio-economic status.

Between 1978 and 2020, a total of 20 publications described Mansonellosis, Loasis, Onchocerciasis, and lymphatic filariasis in the context of Equatorial Guinea (S1 Table). Five of these publications described *L. loa* and *M. perstans* infections among Equato-Guineans living in Spain [28–31] or Singapore [32], while three were case reports of foreigners returning from Equatorial Guinea [33–35]. One cross-sectional study investigated the prevalence of *L. loa* and *M. perstans* on Bioko Island using microscopy and qPCR-based detection [36].

We have extended our mRDT-based molecular surveillance tool, originally developed for malaria, to the filarial nematodes *M. perstans* and *L. loa*. The widespread availability and use of

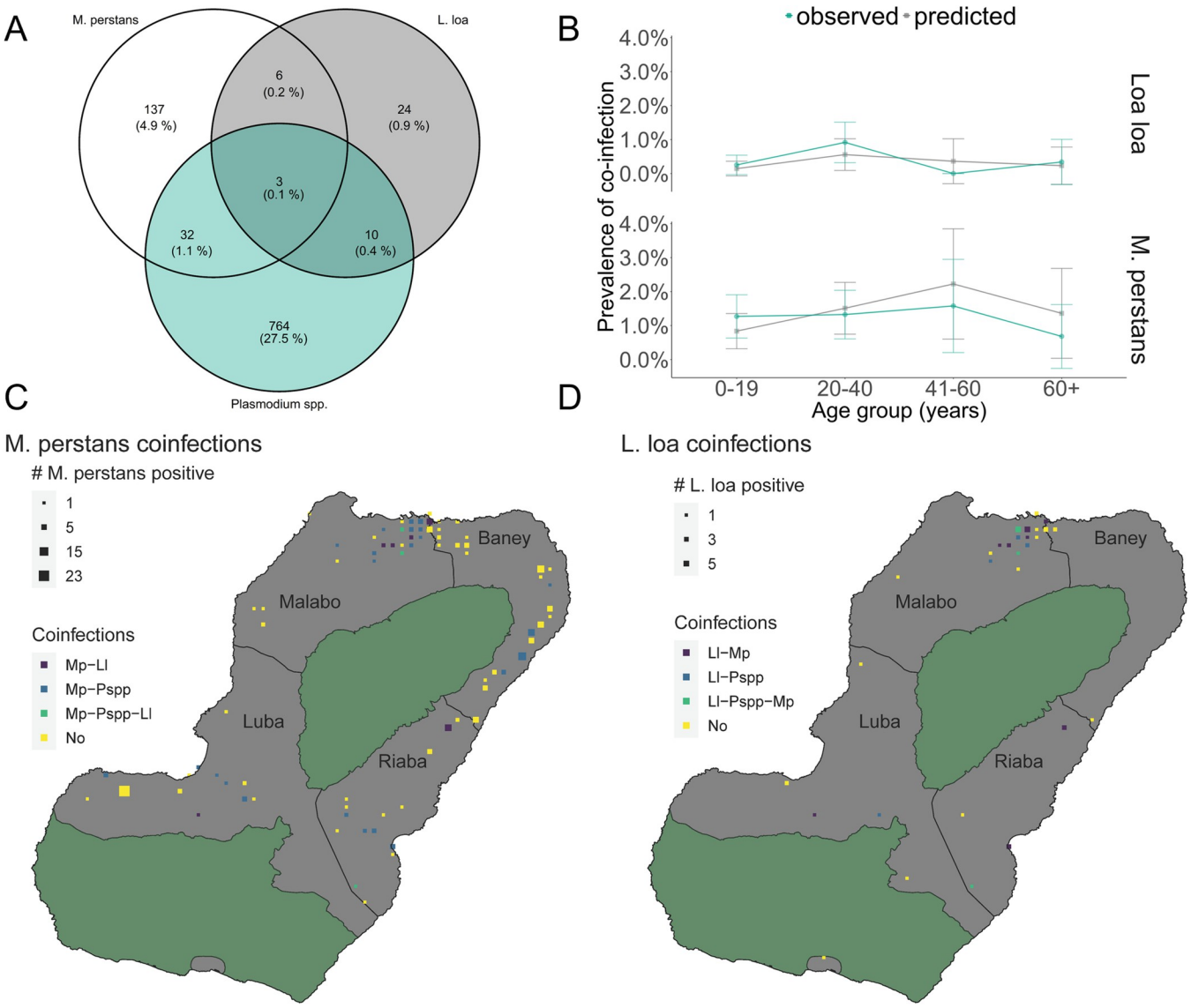

Fig 4. *Plasmodium* spp., *M. perstans* and *L. loa* multi-parasitism infections on Bioko Island. (A) Number of *M. perstans*, *L. loa* and *Plasmodium* spp. co-infections amongst 2775 individuals tested. (B) Positivity rates of *Plasmodium* spp., *M. perstans* and *L. loa* co-infections, stratified by age group. The blue lines and orange lines depict the observed and predicted co-infection rates, respectively. (C) Geographical distribution of *M. perstans* co-infections with malaria and *L. loa*. (D) Geographical distribution of *L. loa* co-infections with malaria and *M. perstans*. In (C) and (D) only 1x1 km^2 grids with at least one case of *M. perstans* or *L. loa* infection are presented. The areas marked in green are nature reserves.

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mRDTs in malaria endemic regions that are also endemic to *M. perstans* and *L. loa*, the simplicity of mRDT collection and storage, would make this approach convenient for large-scale molecular epidemiological studies covering *Plasmodium* spp., *M. perstans* and *L. loa*. Using our extraction protocol based on mRDTs, high quality and sufficient quantities of *M. perstans* and *L. loa* specific DNA fragments were obtained as demonstrated by successful Sanger sequencing of the ribosomal internal transcribed spacer 1 region. Therefore, apart from amplifying short DNA fragments usually used for qPCR, our NA extraction method also allows to amplify larger fragments suitable for genotyping of the pathogens of interest. In future, switching to more polymorphic markers for genotyping in combination with next generation sequencing technologies might improve tracking of infections and importantly help to

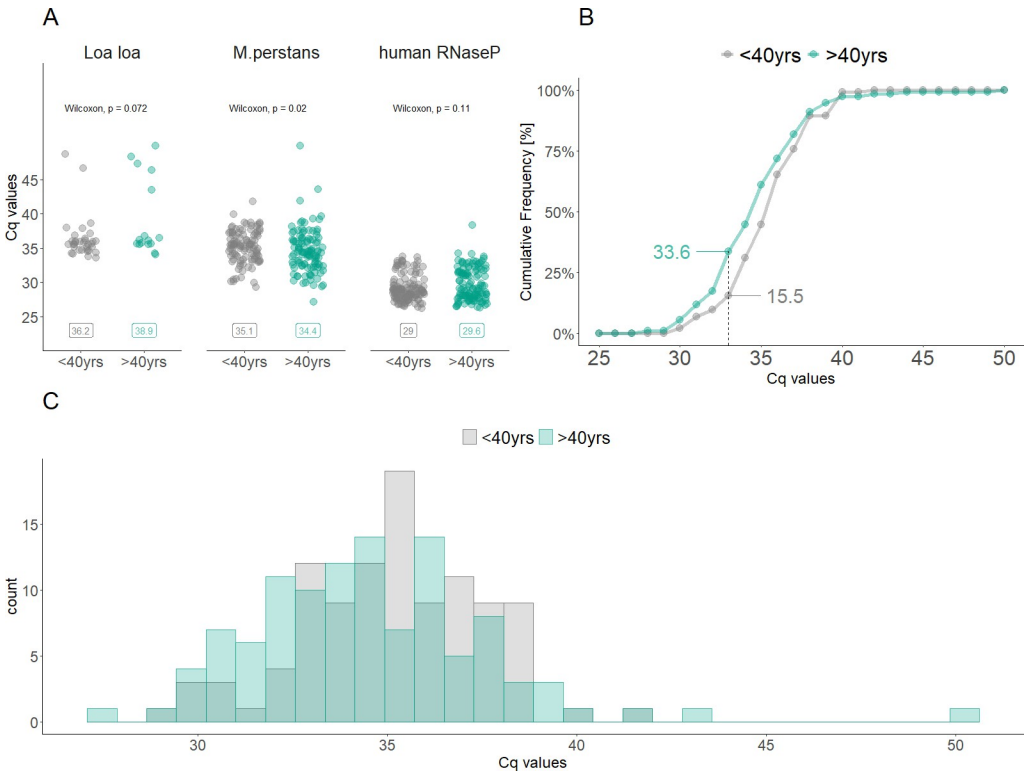

Fig 5. Comparison of Cq values obtained from *M. perstans* and *L. loa* infected individuals aged above and below 40 years. (A) Scatter plots of Cq values for *L. loa*, *M. perstans* qPCR and the corresponding Cq values for the human RNaseP gene qPCR. The geometric mean values for each group are shown. (B) Cumulative frequency of Cq values for *M. perstans* infected individuals. At a Cq value of 33 (dashed line), the cumulative frequency among individuals aged above 40 years is 33.6% compared to 15.5% among individuals aged below 40 years. (C) Histogram of the distribution of Cq values for *M. perstans* infected individuals stratified according to age.

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understand if there are multiple strain infections that possibly accumulate over time in the elderly population showing the highest parasite density of *M. perstans* infections by qPCR.

The cost of our mRDT-based *M. perstans* and *L. loa* test system was estimated to be $4 per sample, from which $3 were spent for NA extraction and $1 on the llmp-qPCR assay. Noteworthy, the same aliquot of extracted NA was used for genomic characterization and quantification of malaria parasites as described [19] making this approach highly cost efficient. The low cost and high scalability of our approach enables systematic monitoring of impact of public health interventions against blood borne pathogens through large scale surveillance.

Here, we found that infections with *M. perstans* (6.6%) are more prevalent than *L. loa* (1.5%) and that *M. perstans* infections can be mostly found in the older, male, population living in rural parts of Bioko Island. This finding reconfirms previous reports [37–39]. We found similar prevalence data for *M. perstans* and *L. loa* compared to a qPCR-based cross-sectional study conducted on Bioko Island in 2018. Ta and colleagues found that 8.8% and 0.7% of persons tested were infected with *M. perstans* and *L. loa*, respectively [36]. The similar proportions found in these two independent studies indicate that detection rates for both filarial nematode species are comparable in spite of different sampling methods (dried blood recovered from mRDT versus freshly collected whole blood) and blood volumes (5 μL versus 200 μL) used. The prevalence of *L. loa* found in both studies could be underestimated and partially explained by the fact that 70% of infected individuals do not show mf circulation in peripheral blood, with occult infection or occasional presence of adult worms under conjunctive tissue [40].

The higher parasite density, as expressed by the qPCR's Cq values, of *M. perstans* infection found in rural areas on the East coast of Bioko Island might reflect the distribution or abundance of the vector and its active transmission in those areas. The vector *Culicoides* presence is more likely associated to aquatic environments, banana and plantain stems [9] that might describe the environmental characteristics of rural areas in Bioko Island. Entomological monitoring for the presence of these vectors would be justified to improve the understanding of the geographic patterns observed and inform control interventions. Elderly people above the age of 60 were proportionally the most affected age group. The increased infection rates in combination with higher *M. perstans* parasite densities compared to younger people might be due to the cumulative effect of reinfection during their lifespan [39].

It has been shown that filarial worms including *M. perstans* and *L. loa* cause chronic infections that are associated with strong immune modulation in the human host [41,42]. These long- standing and strong immune modulatory effects particularly of *M. perstans* might negatively impact on mf clearance [37] as well as on co-infections like malaria or tuberculosis outcomes in the same host [43]. In addition, albeit not clinically overt, *M. perstans* infections might impair vaccine induced immune responses and protection by exerting strong immune modulatory effects as described for other helminth infections [44,45]. Therefore, molecular epidemiological studies using the methodology outlined here may prove critical in identifying cofounders of the protective efficacy of experimental malaria vaccine studies currently ongoing in Equatorial Guinea [46,47].

Quantitative measurements of filarial nematodes might become important in the context of development of novel drug interventions against Mansonellosis, loiasis, lymphatic filariasis and onchocerciasis in areas with high co-infections between these parasites [48]. The lack of an international standard with predefined numbers of mf of each of the filariasis causing parasites that could be used for quantitative assessment of microfilaremia based on Cq values measured is one of the tools limiting our approach.

Our study presented had some limitations. We restricted our analysis to *L. loa* and *M. perstans*, both blood-borne pathogens. *Onchocercha volvulus* and *M. stretocerca* were described on Bioko Island [36] but their mf are located in the skin and therefore are detected using primarily skin biopsies for microscopy for molecular analyses. Although Lymphatic filariasis has not been reported on Bioko Island, using mRDTs collected during daylight as a source of the blood sample would not allow exploring the presence of *Wuchereria bancrofti*. Also, we have used primers/probe combinations in our Llmp-qPCR assay that could most likely not amplify the newly identified Mansonella sp "DEUX, thereby potentially omitting this novel *Mansonella* species described recently [4]. We conducted a feasibility study to demonstrate that it is possible and sensible to use the mRDTs and metadata collected during an annual MIS to assess at very low additional costs the positivity rate of highly neglected nematode filarial infections for different demographic and socio-economic groups. However, a full analytical and clinical performance evaluation to determine the sensitivity and specificity of our approach based on a direct comparison with microscopy would be needed to fully understand the limitations of our molecular testing for routine surveillance of filarial nematodes in endemic regions.

Conclusion

In summary, our approach of repurposing used mRDT as source of NA provides a promising, future tool that enables a cost-effective approach to monitor the prevalence, genotypes, parasite densities and co-infections of filarial nematodes and potentially other blood borne infectious diseases. Also, PCR amplification and sequencing of DNA fragments allowing for genotyping extends the range of possible applications of using NA stored on mRDTs. This

method might be of particular interest in settings with limited access to cool chains, laboratory infrastructure and in populations not necessarily served by clinics and health posts in rural areas.

Supporting information

S1 Table. Literature review on studies carried out on filarial nematodes in Equatorial Guinea or Equato-Guineans living abroad.
(DOCX)

S2 Table. Positivity rates of *M. perstans* and *L. loa* stratified by gender, age, district and socio-economic status.
(DOCX)

S1 Fig. Selection of mRDTs used for NA extraction and molecular analysis stratified by mRDT result (A), age groups (B) and district (C).
(TIFF)

S2 Fig. Representative amplification plots for the llmp-qPCR assay. (A) Multiplex qPCR amplification of the human RNase P gene, *M. perstans* and *L. loa*. (B) Curves in purple show amplification of the RNaseP gene used as an internal extraction and qPCR amplification control. (C) Curves in yellow show the amplification and detection of *M. perstans*-specific 18S target by qPCR. (D) Curves in blue show the amplification and detection of the *L. loa*-specific LLMF72 target by qPCR.
(TIF)

S3 Fig. Variation of Llmp-qPCR Cq values for *L. loa* (white) and *M. perstans* (grey). Grouped by age (A), gender (B), urban or rural residence (C) and day time of blood collection (D).
(TIFF)

S4 Fig. Graphical representation of samples selected for the Sanger Sequencing experiment of the ribosomal internal transcribed spacer 1 region. All *M. perstans* or *L. loa* positive samples, sorted by their Cq values are shown. Samples selected for Sanger Sequencing are highlighted in red.
(TIFF)

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Chapter V

Understanding determinants of *Mansonella perstans* infections in Bioko Island's at-risk population using machine learning classifiers

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Abstract

Background: The risk factors for *Mansonella perstans* infections, a filarial disease transmitted by midges of the genus *Culicoides* spp., on Bioko Island are not well described. This study aimed to investigate, geographical, environmental and socio-economic determinants of *M. perstans* infections among the at-risk population to guide future targeted research of this highly neglected filarial disease.

Methods: We used data from different sources including the dataset from the Bioko's Malaria Indicator Survey (MIS) conducted in 2018 and a *M. perstans* qPCR data from a previous report to establish a so-called at-risk population (adult male living in rural regions of Bioko). Then, we identified determinants for *M. perstans* infection among this population. For that, six Machine Learning (ML) classifiers were trained with the data from the MIS conducted in 2018 and then applied in the MIS conducted one year later (2019) for prediction of the infection probability. The performance of the ML classifiers was evaluated and using the measure of importance attributed to each feature of the dataset, we aimed to identify risk factors.

Results: The at-risk population had a *M. perstans* positivity rate of 24.2%. Among this population, agriculture or fisheries represented the sector of occupation the most at risk of *M. perstans* infection. Evaluating the six ML classifiers with the test dataset from the same MIS as the training dataset derived, revealed that the accuracy was above 70% for three ML classifiers. Using a real-world dataset including individuals from the MIS conducted one year later showed a lower performance of the ML classifiers. Accuracies of the six ML classifiers ranged from 70.5% to 37.2%. Importantly, all six ML classifier had specificities of above 65%, compared to only 17% based on the assumption that all adult men living in rural areas of Biko Island are positive for *M. perstans.* Each ML classifier ranked the predictors based on the importance for infection status classification. Predictors from the category environmental/geographical contained the highest fraction of important predictors (69.2%), followed by individual/health (4.0%) and housing/infrastructure (3.4%).

Conclusion: Environmental and geographical factors delineate the occurrence of *M. perstans* within the at-risk population living on Bioko Island. Here, the combination of different datasets enabled to provide meaningful information used to make prediction using ML classifiers. In-depth analysis of the environmental/geographical predictors point to isolated, rural areas with moist tropical forest located 100 meters and more above sea level as the regions on Bioko Island where *M. perstans* infection risk is the highest. This approach might support the control of highly neglected tropical diseases by providing better targeted approaches to the population most affected.

Introduction

Mansonella perstans is one of the most prevalent human filarial nematode infections in Sub-Saharan Africa with an estimated 114 million people infected throughout the continent.[1] Despite its wide distribution, understanding of risk factors associated with infection is still limited. This knowledge gap impacts the development and implementation of treatment, control, and elimination approaches. *M. perstans* is a vector-transmitted disease with its transmitting vector, biting midges of the genus *Culicoides* spp., found in or near dense forest in Central Africa or along the forest fringes between the rain forest and open land in West Africa.[2–5] Environmental factors affecting *Culicoides* spp. abundance include sufficient rainfall and warm climate as well as breeding sites with fallen leaves, decaying plantains and banana stems.[1] Previous investigations on risk factors associated with *M. perstans* infection focussed on risk factors for the general population (e.g. age, gender, geographical location) and less on the level of at-risk-people or individuals (e.g. detailed socio-economic status). In endemic areas, elderly men living in rural areas are often described to be the most affected by *M. perstans* infections.[4–7] Furthermore, high *M. perstans* infection rates have been reported from farmers as well as from nomadic populations.[8]

Machine learning (ML) is a powerful approach that intersects artificial intelligence and statistical learning in the process of discovering relationships or patterns. ML algorithms have shown superior predictive ability in addressing classification problem when compared with classical statistical models.[9] Various ML algorithms have been successfully applied for risk factor identification associated with communicable[10,11] as well as with non-communicable[12,13] diseases.

On Bioko, an Equato-Guinean Island located in the Gulf of Guinea, an annual Malaria Indicator Survey (MIS) is conducted to monitor the impact and progress of the Bioko Island Malaria Control Program (BIMCP). In 2018, more than 3200 volunteers participating in the MIS were additionally tested for *M. perstans* infection.[6] The positivity rate was the highest among adult men living in rural

areas of the island. In the current study we explored the usefulness of ML classifiers to identify predictors and risk factors of *M. perstans* positivity among adult male living in rural regions of Bioko, the at-risk population we identified previously.

Methods

Data sources and study variables

This study used data from different sources. Dataset from Bioko's MIS conducted in 2018 and 2019 were included. During both surveys, participants were asked to provide capillary blood by finger prick for malaria testing using malaria rapid diagnostic test (mRDT) and for hemoglobin measurement. In addition, the participants filled an extensive questionnaire describing the socio-economic and occupational situation and the geographical location of their houses was recorded. For this study only the at-risk population (adult male living in rural regions of Bioko) and for those with *M. perstans* qPCR data available were included.

The BIMCP divides Bioko into 2,091 uniquely coded, 1 × 1 km map-areas and each MIS participant is linked to its map-area. For each of these map-areas the population density, distance to the closest health facility and distance to the closest military camp are known. We used satellite remote sensing data from 2018 obtained by the Malaria Atlas Program (MAP)[14] to further characterize the environment and geographical features of these map-areas. Vegetation indices were included to characterize potential vector habitats and as a proxy for precipitation. We used the enhanced vegetation index (EVI), the tasseled cap greenness (TCG), and the normalized difference vegetation index (NDVI) to characterize and quantify vegetation. Tasseled cap brightness (TCB) was also included as an additional measure for vegetation, as this metric will be high in areas with bare soils. To quantify the surface moisture and to identify potential vector breeding sites, metrics designed to capture the moisture present in pixels, such as tasseled cap wetness (TCW) and distance to and type of waterbodies were included. Additional geographical and environmental features included were temperature, elevation, and slope.

All datasets were combined and variables with less than 50% information filled-in, and less than 20% variability in the answers collected were removed. After filtering a total of 65 variables were selected derived from all datasets that were included in the further analysis process (referred to predictors throughout this article). A list of all variables included here is provided in the supplementary data (Table S1).

M. perstans infection status

The outcome of interest in this study was the *M. perstans* infection status, which was determined using our recently developed versatile and cost-efficient molecular approach based on extraction of nucleic acids from used malaria RDTs followed by qPCR detection and quantification of *M. perstans*.[6]

ML classifier selection and analytical strategy

The R programming language (version 4.1.2) and the R package *caret* were used to perform the ML data processing and analysis based on six ML classifiers. The different ML classifier algorithms selected for this study were "random forest" (*rf*), "extreme gradient boosting decision trees" (*xgbTree*), "neural networks" (*nnet*), "support vector machine with radial basis kernel" (*svmRadial*), "naïve bayes" (*naiveBayes*) and "generalized linear model by likelihood-based boosting" (*glmBoost*). The MIS-2018 dataset was split into two sets (training and testing) to learn from the data, train the ML classifiers and identify patterns predicting *M. perstans* positivity (herein referred to "ML predictors"). All six ML classifiers were applied to a random subset of 80 % of the individuals (MIS-2018 training dataset, n=358) and validated with the remaining 20 % of the individuals (MIS-2018 test dataset, n=88). All ML classifiers were trained based on 10-fold cross-validation. As a real-world

evaluation of this ML approach, the MIS-2018 trained ML classifiers were applied to the MIS-2019 real-world dataset and performance was assessed independently. MIS-2019 real-world dataset consisted of data from randomly selected 258 at-risk individuals.

Analytical evaluation of ML classifiers

The selected ML classifiers' performance in terms of correct classification of the *M. perstans* infection status was assessed using the MIS-2018 test dataset and the MIS-2019 real-world dataset. The R package *epiR* was used to calculate all performance measures such as accuracy, sensitivity, specificity, and Cohen's kappa coefficient of agreement (Kappa). The 95% Confidence Intervals (95%-CI) were calculated for each performance measure.

Identification of ML predictors for *M. perstans* positivity

Based on the importance assigned by the ML classifiers, the top 5% of predictors were identified and if shared among at least two different ML classifiers, considered as predictors of high importance. Predictors identified as part of the ML classifier training using the MIS-2018 dataset were used for further analysis and important predictors found in less than 5 individuals were removed.

Ethics approval and consent to participate

The Ministry of Health and Social Welfare of Equatorial Guinea and the Ethics Committee of the London School of Hygiene and Tropical Medicine (Ref. No. LSHTM: 5556) approved the malaria indicator surveys in 2018 and 2019. Written informed consent was obtained from all adults and from parents or guardians of children who agreed to participate. Only participants for which an additional

consent for molecular diagnostics of pathogens in their blood retained on the mRDT was obtained were included in this study.

Results

Defining and descriptive analysis of the at-risk population

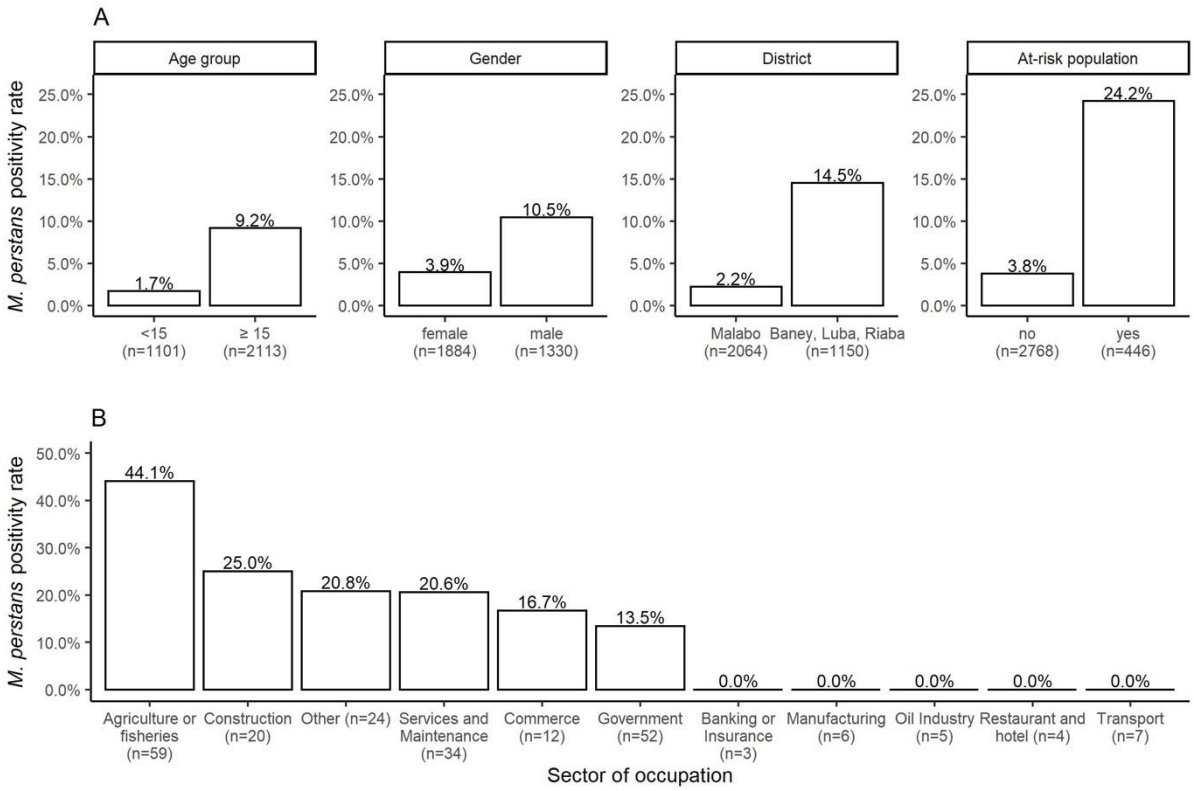

Figure 1: (A) Age group, gender and district are all factors with differences in *M. perstans* positivity rates between the at-risk population and the general population. (B) *M. perstans* positivity rates for the at-risk population differ between sectors of occupation.

In this study we focused on identifying predictors among the at-risk population for *M. perstans* infection. Using the demographic information available from the MIS-2018, we find that the

population aged 15 years and above has a more than five times higher *M. perstans* positivity rate compared to the younger population. In general, male participants are 2.6 times more likely to be positive for *M. perstans* than female participants and the positivity rate for *M. perstans* among participants living in the urban capital city Malabo is much lower than in the rural districts Baney, Luba and Riaba (Figure 1A, panel 1-3). Using these three demographic characteristics, we defined male participants aged 15 and above and living outside of Malabo as the at-risk-population. In the at-risk population almost a quarter of participants (24.2%) are positive for *M. perstans* by qPCR, compared to 3.8% in the remainder of the MIS-2018 participants (Figure 1A, panel 4). Among the at-risk population, the sector of occupation plays a role for *M. perstans* positivity. *M. perstans* positivity is highest among participants working in agriculture or fisheries (44.1%, 26/59), followed by construction (25.0%, 5/20), other sectors (20.8%, 5/24) and services and maintenance (20.6%, 7/34) (Figure 1B).

Data pre-processing and analytical strategy

The flow chart in Figure 2 outlines the data processing steps and analytical strategy of this study. We extracted the at-risk population from a previously published dataset[6] based on age, gender, and district. Three datasets were available for the at-risk population: the MIS questionnaire that consisted of 48 selected variables, the laboratory dataset with 4 variables and finally 13 variables from the satellite remote sensing data. The MIS questionnaire dataset describes mainly the socio-economic, occupational, and housing situation of the participants. The laboratory dataset consisted of hemoglobin measurements and qPCR results for *M. perstans*, *L. loa* and *Plasmodium* spp. The satellite remote sensing dataset, provided by the Malaria Atlas Program (MAP)[14], characterizes the environment and geographical features of the area where each of the participants live. Vegetation indices as well as other geographical and environmental features were included to identify potential

vector breeding sites. Eighty percent of the MIS-2018 at-risk population dataset was used for training the six different ML classifiers, namely *rf*, *xgbTree*, *nnet*, *svmRadial*, *naiveBayes* and *glmBoost*. The ML classifiers were tested with the remaining 20% of the MIS-2018 dataset (test dataset) and their respective performances were evaluated. The ML classifiers were further evaluated on an independent real-world dataset of samples collected from the at-risk population during the 2019 MIS. The most important ML predictors shared between two or more ML classifiers were investigated in more detail.

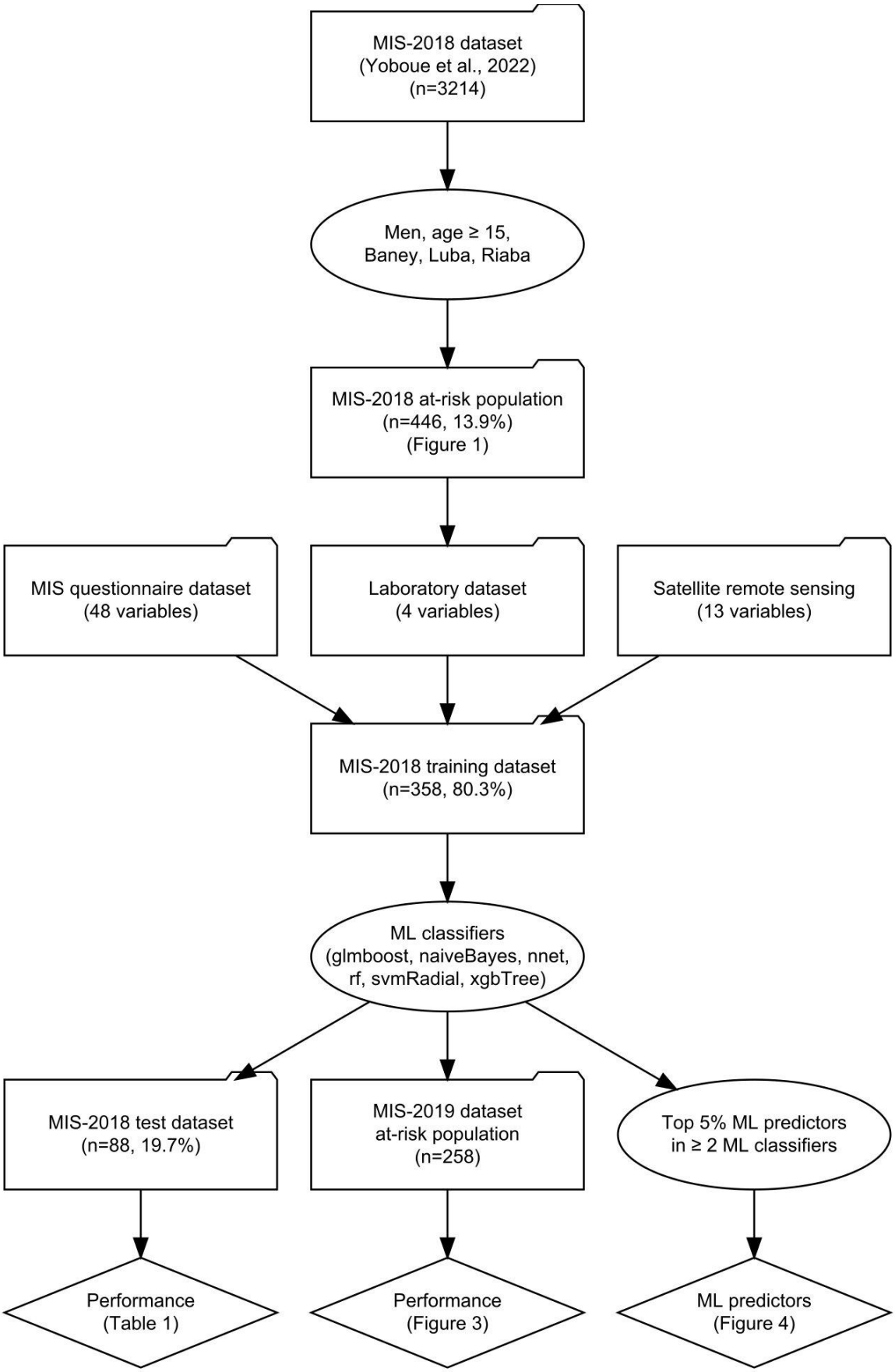

Figure 2: Flow chart outlining the data pre-processing and analytical strategy

ML classifier performance using MIS-2018 test and MIS-2019 real-world dataset

Using the MIS-2018 test dataset the performance of the six ML classifiers was assessed (Table 1). In terms of accuracy and Cohens Kappa agreement, *glmboost*, *nnet* and *svmRadial* outperformed the three other ML classifiers. The highest sensitivity was observed for the *nnet* classifier, while *rf* and *svmRadial* showed the highest specificity.

ML classifier	Accuracy	Kappa	Sensitivity	Specificity
glmboost	73.9% (63.4%-82.7%)	35.5% (12.8%-58.1%)	77.6% (65.8%-86.9%)	61.9% (38.4%-81.9%)
naiveBayes	68.2% (57.4%-77.7%)	20.3% (-4.1%-44.6%)	74.6% (62.5%-84.5%)	47.6% (25.7%-70.2%)
nnet	77.3% (67.1%-85.5%)	41.3% (18.7%-63.9%)	82.1% (70.8%-90.4%)	61.9% (38.4%-81.9%)
rf	53.4% (42.5%-64.1%)	14.9% (-4.1%-33.9%)	46.3% (34.0%-58.9%)	76.2% (52.8%-91.8%)
svmRadial	71.6% (61.0%-80.7%)	37.2% (16.4%-58.0%)	70.1% (57.7%-80.7%)	76.2% (52.8%-91.8%)
xgbTree	56.8% (45.8%-67.3%)	14.7% (-5.8%-35.1%)	53.7% (41.1%-66.0%)	66.7% (43.0%-85.4%)

Table 1: Performance of six different ML classifiers with the MIS-2018 at-risk population test dataset. Accuracy, Kappa agreement, sensitivity and specificity were calculated with their respective 95% confidence intervals (indicated in brackets).

With a second and real-world dataset which derived from the next MIS conducted one year later (MIS-2019), we further evaluated the performance of the ML classifiers to correctly identify the *M. perstans* infection status in this at-risk population (Figure 3A). Compared to the evaluation with the MIS-2018 test dataset, the performance of all six ML classifiers was significantly lower. The *nnet* classifier showed the best performance in terms of accuracy (70.5%) and Cohens Kappa agreement (25.7%), while *rf* had issues to correctly classify the infection status in this dataset as indicated by an accuracy of 37.2%. Interestingly, all six ML classifier had specificities of above 65%, reducing the chance of false positive results significantly. The *rf* and *svmRadial* classifiers had the highest

specificities with 95.3% and 83.7%, respectively. As a comparison, the assumption that all men aged 15 and above and not living in Malabo are positive for *M. perstans* results in a specificity of only 17%. The highest sensitivity was achieved by *nnet* (71.6%), while the lowest sensitive classifier was *rf* (25.6%).

All six ML classifier calculate the probability of a sample being positive for *M. perstans*. In Figure 3 B, samples positive for *M. perstans* by qPCR are colored dark blue, samples negative for *M. perstans* are colored light blue. *M. perstans* qPCR positive samples have significantly higher probabilities assigned than negative samples, particularly in classifiers *glmboos*t, *rf* and *svmRadial* (Figure 3B).

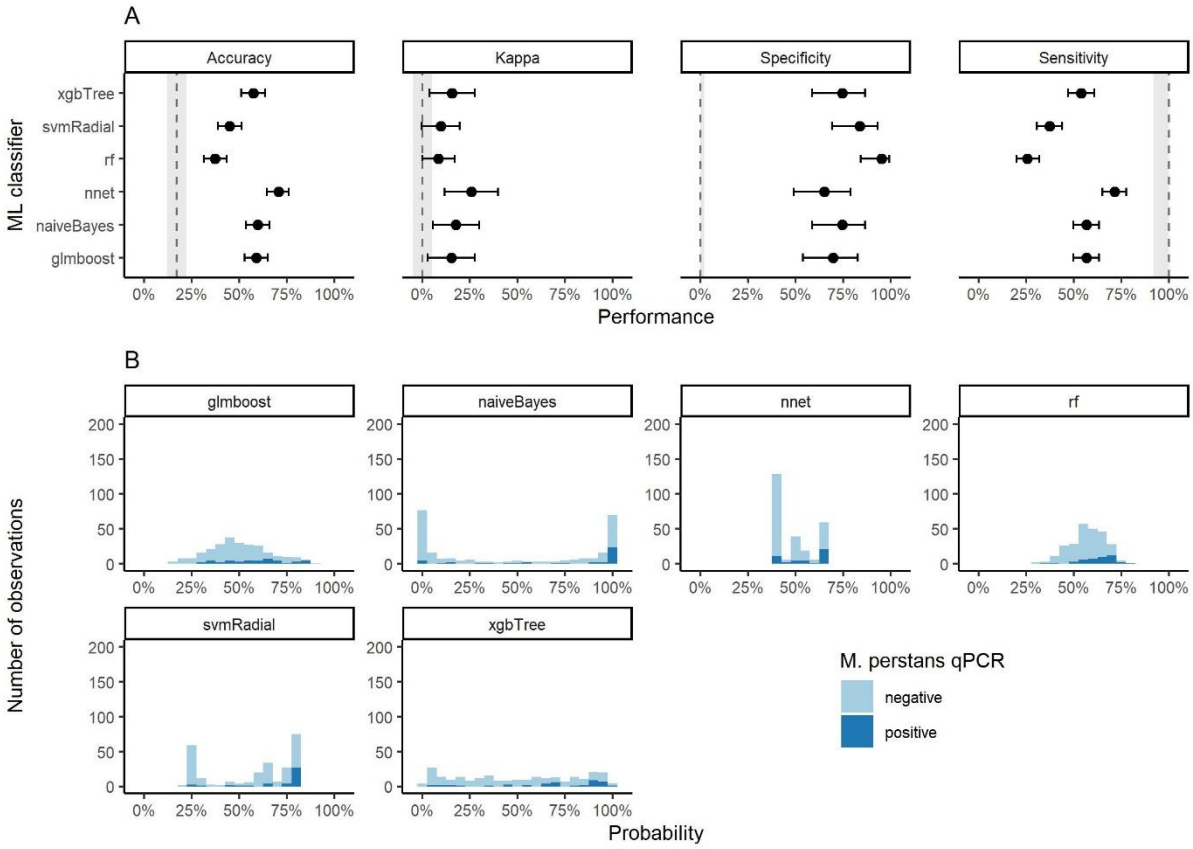

Figure 3: (A) Performance of six different ML classifiers with the MIS-2019 at-risk population dataset. Accuracy, Kappa agreement, sensitivity and specificity were calculated with their respective 95% confidence intervals. The grey dotted lines and grey shaded areas (95% CI) indicate the performance of the assumption that all men aged 15 and above and not living in Malabo are positive for *M. perstans*. (B) Probability of a sample being positive for *M. perstans* calculated by each of the six ML classifiers. Sample positive for *M. perstans* by qPCR are colored dark blue, samples negative for *M. perstans* are colored light blue.

ML predictor importance and risk factor identification

Each ML classifier ranks the features provided in the data based on their importance for classifying the *M. perstans* infection status. Next, we used the most important predictors to understand what risk factors are driving *M. perstans* positivity in our at-risk population (Figure 4). In total we identified 186 predictors from which we extracted the top 5% based on their importance. If a predictor is found in the top 5% by at least two ML classifiers, it was considered important (Figure 4A). All predictors were grouped into one of the following three categories, namely environmental/geographical, housing/infrastructure and individual/health. The category environmental/geographical contained the highest fraction of important predictors (69.2%), followed by individual/health (4.0%) and housing/infrastructure (3.4%) (Figure 4B). We calculated the relative risk for each predictor (Figure 4C). Out of the 14 important predictors as identified by the ML classifiers, six (42.9%) had a p value < 0.05 after Bonferroni correction, while only 1 ("drinks botteled water") out of 133 (0.8%) had a significant decreased risk ratio but was not considered important by our selection criteria (data not shown). These results indicate that our ML classifier selection, the models created and the identified predictors, enable us to identify risk factors among the at-risk population for *M. perstans* infection.

Environmental and geographical predictors which increase the *M. perstans* infection risk include a high tasseled cap wetness (positive values), high tasseled cap greenness (>0.1), a high enhanced vegetation index (>0.6), and a high normalized difference vegetation index (>0.6). Combined, these measures direct towards areas which can be considered as covered with tropical forests. In terms of topography, areas located above 100 meters above sea level and/or places with at least moderate steepness, seem to be associated with increased *M. perstans* positivity. Interestingly, annual average temperatures of 25 °C and less also increase the infection risk. An increased risk of *M. perstans* positivity was also observed in areas which are located more than 5 km from the next closest military camp. Predictors associated with housing and infrastructure were living in house with cement walls

(risk reduction) or in a house with the windows lacking glass (risk increase). This could be explained by both, the socio-economic status of the person or access for the vector into the house. People who owned their house had a higher risk, while if they lived in their house for more than two years the infection risk was reduced.

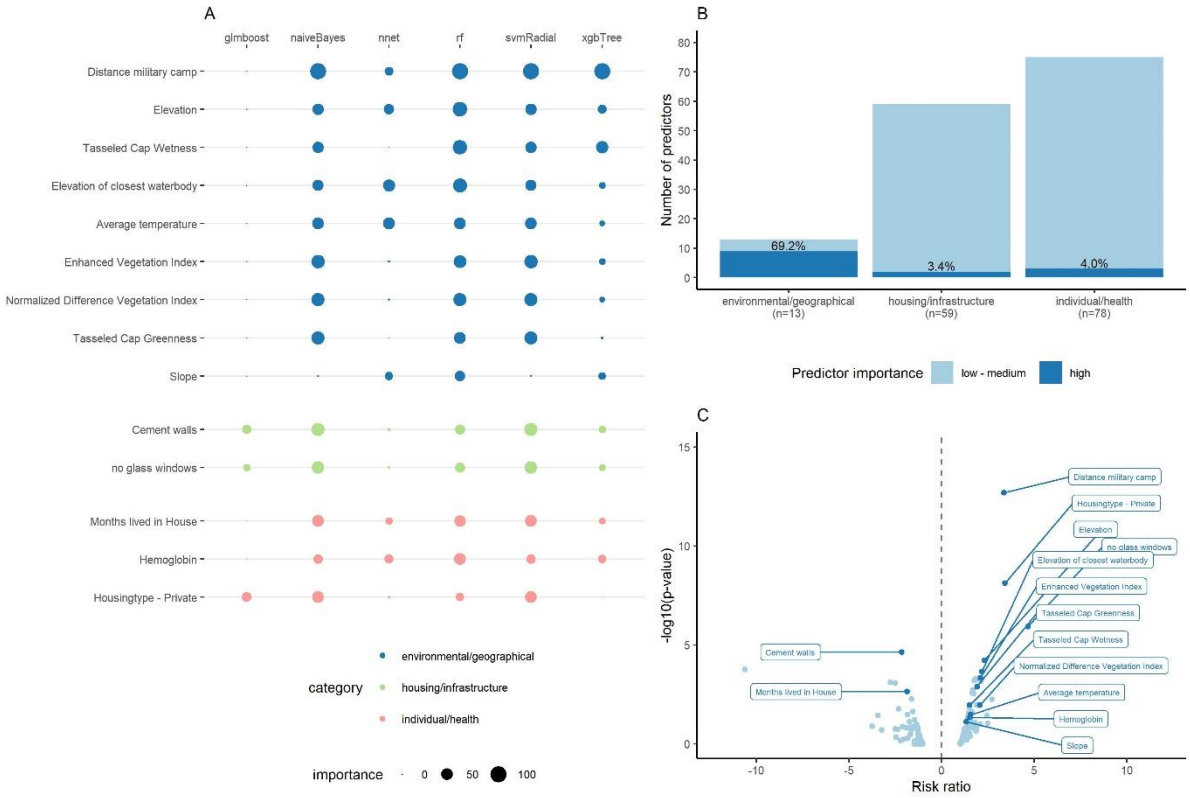

Figure 4: (A) Calculated importance of the top 5% ML predictors of each ML classifier shared between two or more ML classifiers. High importance is depicted by large circle sizes. ML predictors are grouped and colored by ML category. (B) Number of important ML predictors for each category. Highly important ML predictors are colored dark blue. ML predictors with low to medium importance are colored light blue. (C) Volcano plot showing the risk ratios for *M. perstans* positivity for all ML predictors. Highly important ML predictors are colored dark blue. ML predictors with low to medium importance are colored light blue.

Discussion

We present here to our knowledge the first report on using ML approaches to define predictors for *M. perstans* infection based on a combination data derived from the molecular diagnosis of the infection, individual socio-economic and behaviour data collected in the MIS questionnaire together with fine-grained environmental data sets of the island. Although few reports have been published on *M. perstans* epidemiology in Africa, our ML enabled to decrypt the contribution of geographical, occupational, environmental, and socio-economic factors that help drive transmission of this filarial parasite on Bioko Island.

Our findings confirm previous reports that being male, of older age and living in a rural environment significantly increases the odds of being infected with *M. perstans*.[1,7] However, our data reinforce that *M. perstans* is a vector borne disease dependent on the presence of the transmitting vector, *Culicoides* spp. Previous reports described the environmental factors that might favour the distribution and abundance of this vector. *Culicoides* spp. are attracted to livestock including cattle, horses and sheep that causes them to disperse quite widely and quickly in any agricultural environment.[15–17] In Uganda, Stensgaard and colleagues identified cattle densities as significant co-variate for *M. perstans* infection in school children.[3] However, on Bioko Island, cattle breeding is very limited to one or two farms and therefore, we could not recapitulate these observations from Uganda in our setting. The association of *M. perstans* infection to presence of aquatic and forested areas and banana plantations has been linked to decomposing wood, tree holes, and forest floor cover that are suitable breeding sites for *Culicoides* spp.[18,19]

In combination, the predictors identified by the ML classifiers direct towards the specific occupational group of cacao farmers as a very high-risk group for carrying *M. perstans* infection. The satellite remote sensing data indicated that isolated, rural areas with moist tropical forest located 100 meters and more above sea level are the region on Bioko Island where *M. perstans* positivity is the

highest. On Bioko Island these are exactly the settings in which cacao plantations are found. These predictors were confirmed in our qPCR-based *M. perstans* infection data in that the infection was higher in individuals who reported to work in agriculture or fisheries in the MIS questionnaire. Interestingly, *Culicoides* spp. represent the most common pollinators of cultivated varieties of cacao.[20] Historically, Bioko Island is considered as one of the most ideal settings for cacao plantations endowed with perfect climate, soil, and rainfall levels to grow cacao.

Although, it has been reported that midges from the *Culicoides* species can fly up to 5 km, most disperse just a few hundred meters from their breeding sites.[15] This could delineate the probable dispersion of the infection where people carrying the microfilariae drive disease transmission. Here, ML enables to define and predict the dispersion of *M. perstans* infection among the rural population using environmental information provided by satellite remote sensing. Currently, no specific drug treatment exists for Mansonellosis. Our data strongly support that vector control measures would seem to be a highly efficient intervention for this disease since the highest predictors were in the environmental/geographical category. Clearly, control of *Culicoides* spp. must consider the crepuscular biting habit of adult *Culicoides* spp. and that bed nets used for malaria control seem to be less efficient for these vectors.[16]

ML is increasingly being used for data mining in epidemiological studies, infectious disease diagnosis, risk factor identification or prediction of disease outbreaks.[21] Here, we demonstrate for the first time that ML is suitable to identify the major predictors driving the distribution of a highly neglected tropical disease by piggy-bagging on data collected during malaria surveys.

Conclusion

We have used ML to analyse data sets collected from cross-sectional MIS studies in 2018 and 2019 together with environmental, socio-economic and occupational data from a rural population living in

Central Africa. Our data indicate that environmental factors supporting the breading and sustaining of *Culicodes* spp. populations are best predictors for *M. perstans* infections. Control measures targeting these vectors in population with highest risk like Cacao farmers might have the biggest impact on *M. perstans* control in the absence of drug treatment measures.

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Chapter VI

Molecular epidemiology of malaria and filarial infection in pregnant women on Bioko Island, Equatorial Guinea

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Keywords: pregnancy, *Plasmodium* spp , bed nets, *Mansonella perstans*, *Loa loa*, Equatorial Guinea

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Abstract

In Malaria endemic areas, pregnant women are at high risk to cope with multiple parasitic infections including filarial helminths. *M. perstans* is one of the most prevalent filariosis parasite among all filarial infections, particularly in Sub-Saharan Africa. However, the statistics of filarial infection in pregnant women remains elusive due to their exclusion during mass treatment regimen. The aim of this report is to describe the positivity rate of *M. perstans* infections in pregnancy in Equatorial Guinea. The molecular examination of used malaria RDTs collected from 293 pregnant women showed that 3.7 % were infected with *M. perstans* and that these women resided mostly in poorer rural areas. Although mild anaemia were observed among most of infected pregnant women, anaemia in pregnancy could not be associated with *M. perstans* infection. Malaria and Loaisis were as well prevalent with 8.1% and 1.4%, respectively. Parasite co-infections were solely observed between *L. loa* and either *Plasmodium* spp. or *M. perstans*.

Introduction

Helminth infections constitute a serious public health problem that occurs mainly in communities living in resource-deprived settings (Flügge et al., 2020). Helminth infections during pregnancy impacts negatively on mother and child health resulting in neonatal prematurity, low birth weight, increased maternal morbidity and mortality (Christian et al., 2004). Prenatal exposure to pathogen antigens might favour foetal tolerance or sensitization to the antigens, leading to the possibility of mounting limited protective immune responses against vaccination or infection (Ndibazza et al., 2013). Pregnant women in these communities are at risk to be either mono- or co-infected with parasitic infections including filarial helminths and malaria because of their significant geographical overlap and potential immunological interplay in West Africa (Drame et al., 2016, Flügge et al., 2020).

Mansonellosis is a parasitic infection caused by three species of the genus Mansonella that reside in humans as their primary definitive hosts where microfilariae are circulating in peripheral blood or in the skin (Ta-Tang et al., 2018). It has been estimated that around 100 million people may be infected by *M. perstans* and that 600 million people live at high risk of contracting an infection in Sub-Saharan Africa (Ta-Tang et al., 2018). *M. perstans* infection during pregnancy might induce poor response to BCG tuberculosis vaccination and tetanus toxoid (Elliott et al., 2010). Interestingly, *M. perstans* infection has been associated with higher malaria parasitemia (Muhangi et al., 2007) and prevalence (Hillier et al., 2008). This study investigated the molecular epidemiology of *Plasmodium* spp, *M. perstans* and *L. loa* among pregnant women in Bioko Island by using malaria RDTs collected during the MIS2018 and MIS2019.

Material and Methods

Participants were recruited during two successive annual malaria indicator surveys (MIS) that were conducted in 2018 and 2019 to assess progress of malaria control on the island. The survey including the here reported molecular investigations were approved by the Ministry of Health and Social Welfare of Equatorial Guinea and the Ethics Committee of the London School of Hygiene & Tropical Medicine (Ref. No. LSHTM: 5556). After providing written informed consent, pregnant women were enrolled and subjected malaria testing using malaria rapid diagnostic tests (mRDTs), and asked to fill in an extensive questionnaire(Cook et al., 2018, Guerra et al., 2018). The questionnaire consisted of demographic information, haemoglobin measurements and malaria prevention parameters in pregnancy. The molecular detection of *Plasmodium* spp., *M. perstans* and *L. loa* was based on the extraction of nucleic acids from used mRDTs as described (Guirou et al., 2020). A multiplex RT-qPCR enabled the detection of *Plasmodium* spp. based on amplification of the Pan-Plasmodium 18S rDNA sequence (Pspp18S) and the *P. falciparum*-specific acidic terminal sequence of the var gene family (Guirou et al., 2020, Hosch et al., 2021). Filariasis was identified by qPCR based on amplification of the 18S ribosomal RNA of *M. perstans* (Bassene et al., 2015) and the hypothetical protein LLMF72 of *L. loa* (Fink et al., 2011) in a multiplex qPCR assay (Yoboue et al., 2022). The human RNase P gene (HsRNaseP) was used as a control for successful nucleic acid extraction (Schindler et al., 2019).

Results

In total, 293 pregnant women were included during the MIS 2018 and 2019 surveys with a majority of 79 % (n=233) residing in Malabo, the capital city on Bioko Island (Table 1). The mean age was 26.2 years (standard deviation (SD) = 6.3 years). Women aged between 15–24 years were mostly

represented and there was no febrile case reported among this population. Infection with *Plasmodium* spp. were recorded in 8.2% women (n=24). This prevalence was higher among women presenting with mild and moderate anaemia and in women between 15-24 years of age. Importantly, pregnant women that were found positive (13/24) for *Plasmodium* spp. reported of not sleeping under insecticide-treated bed nets (Table 2). A total of 5.1% (n=15) pregnant women were infected with either *M. perstans* (3.7 %, n=11) or *L. loa* (1.4%, n= 5). The prevalence of *M. perstans* infections was higher in Luba district (n=6) while all four women infected with *L. loa* came from Malabo. Women with a lower socio-economic status were at higher risk to be infected with *M. perstans*. Mild anaemia was observed among women with *M. perstans* infection (Table 2). Malaria prevention measures taken during pregnancy did not seem to have an effect on the prevalence of *M. perstans* infections (Table 2). In two pregnant women, co-infections were observed with *L. loa* (n=1) and *M. perstans* (n=1) and *Plasmodium* spp.

Table 1. Prevalence of *Mansonella perstans*, *Loa loa* and *Plasmodium* spp. in pregnant women

Characteristics	***Plasmodium spp. N=24 (8.2%)***	***L. loa N=5 (1.4%)***	***M. perstans N=11 (3.7%)***	***Total***
Age (years)				N =293
15-24	16 (66 %)	2 (50 %)	4 (36 %)	132 (45 %)
25-34	4 (17 %)	2 (50 %)	5 (46 %)	128 (44 %)
35-47	4 (17 %)	-	2 (18 %)	33 (11 %)
District				N =293
Malabo	19 (79 %)	4 (100 %)	3 (27 %)	233 (80 %)
Baney	5 (21 %)	-	2 (18 %)	44 (15 %)
Luba	-	-	6 (55 %)	9 (3 %)
Riaba	-	-	-	7 (2 %)
Months of pregnancy				N=282*
1st trimester	9 (41 %)	2 (50 %)	2 (18 %)	80 (28 %)
2nd trimester	7 (32 %)	-	6 (55 %)	100 (36 %)
3rd trimester	6 (27 %)	2 (50 %)	3 (27 %)	102 (36 %)
Socio-economic status				N= 288*
1 lowest	5 (22 %)	2 (50 %)	6 (54 %)	42 (15 %)
2 second lowest	5 (22 %)	1 (25 %)	2 (18 %)	57 (20 %)
3 middle	3 (13 %)	1 (25 %)	1 (9 %)	70 (24 %)
4 second highest	6 (26 %)	-	2 (18 %)	58 (20 %)
5 highest	4 (17 %)	-	-	61 (21 %)
Level of education				N =293
do not know	3 (13 %)	1 (25 %)	3 (27 %)	71 (24 %)
none	-	-	-	2 (1 %)
primary	1 (4 %)	-	3 (27 %)	22 (8 %)
secondary	18 (75 %)	2 (50%)	5 (46 %)	141 (48 %)
post-secondary	2 (8 %)	1 (25 %)	-	57 (20 %)

N: number of pregnant women respondents,

*missing data due to limited amount of NA extracted from RDT

Table 2. *Plasmodium spp*. and *M. perstans* prevalence and malaria prevention measures

Characteristics	***Plasmodium spp.***	***M. perstans***	***Total***
Anaemia			N= 288*
none	7 (30 %)	1 (9 %)	152 (53 %)
mild	8 (33 %)	9 (82 %)	79 (27 %)
moderate	8 (33 %)	1 (9 %)	54 (19 %)
severe	1 (4 %)	-	3 (1 %)
Iron pills supplement			N =283*
do not know	-	-	3 (1 %)
no	9 (41 %)	3 (27 %)	105 (37 %)
yes	13 (59 %)	8 (73 %)	175 (62 %)
Folic acid supplement			N =283*
do not know	-	-	4 (2 %)
no	9 (40.9 %)	3 (27.3 %)	103 (36 %)
yes	13 (59.1 %)	8 (72.7 %)	176 (62 %)
Sickness within the last two weeks			N =293
No	19 (79.2 %)	9 (81.8 %)	258 (88 %)
yes	5 (20.8 %)	2 (18.2 %)	35 (12 %)
Antenatal care attendance			N =283*
no	9 (41 %)	1 (9 %)	76 (27 %)
yes	13 (59 %)	10 (91 %)	207 (73 %)
Fansidar treatment			N =212*
do not know	1 (7 %)	-	5 (2 %)
no	4 (29 %)	3 (30 %)	67 (32 %)
yes	9 (64 %)	7 (70 %)	140 (66 %)
Intermittent preventive treatment sulfadoxine / pyrimethamine			N =293
do not know	13 (54 %)	3 (27 %)	131 (44.7 %)
none	-	-	1 (0.3 %)
less than three	6 (25.0 %)	2 (18 %)	64 (22 %)
more than three	5 (20.8 %)	6 (55 %)	97 (33 %)
Insecticide-treated bed nets using			N =250*
no	13 (65.0 %)	3 (30.0 %)	102 (41 %)
yes, always	7 (35.0 %)	6 (60.0%)	115 (46 %)
Yes, sometimes	-	1 (10.0 %)	33 (13 %)
Household sprayed			N =293
do not know	6 (25 %)	1 (9 %)	50 (17 %)
no	10 (42 %)	4 (36 %)	160 (55 %)
yes	8 (33 %)	6 (55 %)	83 (28 %)

Discussion

We report to our knowledge for the first time the positivity rate of *M. perstans* and *L. loa* among pregnant women living on Bioko Island and their correlation with widely used malaria intervention tools. Malaria RDT collected during MIS 2018 and 2019 facilitated the collection and preservation of peripheral blood at room temperature. These RDTs were used as source for extraction of NA that enabled the qPCR based detection of *Plasmodium* spp., *M. perstans* and *L. loa* (Guirou et al., 2020). Pregnant women were likely to have higher exposure to *M. perstans* when residing in poorer, rural areas like Luba district and belonging to lower socio-economic status. Our finding is consistent with the previous reports that *M. perstans* infection are more prevalent in rural populations (Asio et al., 2009) (Yoboue et al., 2022). In contrast, *Plasmodium* spp. in pregnant women was found to be independent of geographical location on the island. The risk of *Plasmodium* spp, infection is positively associated with self-reported lack of insecticide treated bed net usage. This finding underscores the relevance of sleeping under bed nets as successful malaria prevention tool that is particularly of relevance for pregnant women and their offspring (Eisele et al., 2012). *M. perstans* were more prevalent among women with mild anaemia, although most of them (73 %) were treated with oral iron supplementation. Anaemia in pregnancy does not seem to be associated with filarial worm infections and could also be cofounded by poorer nutritional status (Muhangi et al., 2007). We could not establish a positive association between *M. perstans* and *Plasmodium* spp. infection as was stipulated in previous studies (Hillier et al., 2008). While our study is limited in sample size, it confirms that *M. perstans* infection is mainly encountered in rural areas on Bioko Island in populations with lower socio-economic status. While Momo-Ngoma et al., reported on the prevalence of *L. loa* in pregnant women in Gabon (Mombo-Ngoma et al., 2015), the molecular epidemiology of filarial infection among pregnant women, particularly in Sub-Saharan Africa, remains largely elusive (Bal et al., 2018). This work constitute a baseline study on epidemiology of filarial worms during

pregnancy in Bioko Island and can be used to initiate further epidemiologic and clinical investigations of the potential impact of these parasites in pregnant women and their off-springs.

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Chapter VII

Discussion

7.1 High-throughput screening of *Plasmodium* spp. based on used malaria RDTs and relevance in malaria surveillance

Several studies have recognized that dried blood captured on used mRDTs represent a valuable source of DNA and RNA, that can be successfully used for downstream molecular diagnosis of pathogens (Morris et al., 2013, Zainabadi et al., 2017, Robinson et al., 2019). However, most studies focused on demonstrating the feasibility of extracting NAs rather than fitting this approach as a molecular surveillance tool for malaria at larger scale (Robinson et al., 2019, Guirou et al., 2020). The cost, time, final template volume and the purpose of NAs extraction should be considered when choosing an extraction method (Morris et al., 2013). In this study, we presented the development of a high-throughput approach for large-scale molecular surveillance of malaria parasites based on extraction of NAs from used mRDTs (Chapter 2). During the development of the extraction protocol of NAs from mRDTs which we labelled as the ENAR protocol, special attention was given to evaluation of its reproducibility, the impact of long-term storage of mRDTs before extraction on stability and quality of NAs yield and resulting quantitative measurement results of *Plasmodium* spp. (Guirou et al., 2020). The protocol was optimized with the development of a software for sorting and identification of mRDT and a significant reduction in processing time by using the entire mRDT strip enabling to analyze samples collected from more than 20% (2,690) of the total number of MIS 2018 participants. The extraction protocol was able to co-extract DNA and RNA molecules from very small (5 µL) volumes of dried blood retained on the mRDTs. Using RT-qPCR, the lower limit of parasite detection of this method was calculated as 1 parasite/µL of blood applied to the mRDT representing approximately a 100 times higher sensitivity compared to mRDT-based malaria diagnosis (Guirou et al., 2020). Furthermore, we found a high proportion (28.4%) of false-positive mRDT among the 1,065 malaria positive mRDTs screened which is most likely related to PfHRP2 antigen persistence in blood following recent antimalarial treatment (Chapter 3). That might lead to an overestimation of the malaria prevalence in regions with good access to antimalarial treatment. People in such areas would

be unnecessarily exposed to antimalarial drug treatments resulting in associated danger of parasite drug resistance development. Our group has already detected presence of *P. falciparum* strains carrying non-synonymous mutations in the propeller region of *kelch13* gene as one of the molecular markers for artemisinin resistance (Guirou et al., 2020). The conduct of the ENAR protocol takes around three hours, including two hours for the incubation period of mRDT test strip in the extraction buffer. In addition, the cost of the extraction of NA was estimated to be $3 per sample including reagents and consumables. Interestingly, the same aliquot of extracted NAs can be used for other molecular investigations in parallel including filarial worms detection, making this approach highly cost efficient, particularly in settings where malaria and filariosis is co-endemic. The ENAR protocol might provide a powerful method for molecular disease surveillance allowing a large number of samples screened not only for presence or absence of blood dwelling pathogens but also for more in-depth molecular analysis including genotyping for improved policy making decisions.

7.2 Importance of molecular tools and application of Machine learning for filarial worms control and elimination

Diagnostic tools are important for filariasis elimination programs because these data affect decision making regarding (i) where and how often to distribute drugs through mass drug administration strategies, (ii) how to measure the intervention effect, (iii) how to define targets and endpoints for stopping mass drug administration and (iv) how to monitor populations for resurgence of disease transmission following suspension of MDA (Weil and Ramzy, 2007). However, the current diagnostic toolboxes for filarial parasite detection is fairly limited (Bennuru et al., 2018). Until NAs detection and recombinant antigen production became technically feasible, diagnosis of filariasis largely relied on classical parasitological methods like microscopy or serology based on testing of serum reactivity with crude parasite extracts (Bennuru et al., 2018). The traditional method of

diagnosing filarial infections is to examine blood or skin samples for the presence of mf by microscopy and for many settings this is still the standard procedure today (Walther and Muller, 2003). When targeting disease elimination, , a higher number of blood samples need to be tested over extended time periods to be sure that transmission has been interrupted (Peeling and Mabey, 2014). Moreover, as the prevalence of infections falls, so does the positive predictive value of a diagnostic test, and highly specific tests are required to evaluate elimination progress (Peeling and Mabey, 2014), especially in areas where multiple filarial species are co-endemic (Bennuru et al., 2018).

NA-based diagnostic tools usually offer better sensitivity and specificity compared to direct parasitological or serological methods (Bennuru et al., 2018). In this study, we adapted the ENAR protocol using mRDTs to characterize the epidemiology of blood-dwellings filarial worms. The advantages for filariasis assessment are multiple: (i) mRDTs are widely deployed across all malaria endemic settings, (ii) the volume of blood required is small (5µL) minimizing discomfort and inconvenience to the volunteer, (iii) there are minimal storage requirements in terms of temperature control, and (iv) NA extracted from blood stored on mRDT has been shown to be suitable for qPCR detection up to one year post-collection (Robinson et al., 2019, Guirou et al., 2020). *M. perstans* and *L. loa* were targeted in our study because their mf can be found in peripheral blood and mf circulation is less restricted by time collection during the day in contrast to *Wuchereria bancroti* that needs to be investigated in blood collected from 10PM to 2AM. A multiplex qPCR assay was developed named LLMP-qPCR assay, based on well-defined DNA sequences including the LLMF72 for *L. loa* (Fink et al., 2011) and 18S ribosomal RNA gene for *M. perstans* (Bassene et al., 2015).

This study described for the first time in larger scale the infection rate of these parasites in people living on Bioko Island. *M. perstans* and *L. loa* infections are preferably found among poorer communities living in rural areas on Bioko Island. Both parasites are predominately encountered in Riaba and Luba districts that might present an environment enabling vector expansion and therefore parasite transmission. *L. loa* vectors have been recorded mainly in that part of the island (Cheke et

al., 2003) whereas *Culicoides* spp. are more associated with aquatic environments, banana and plantains stems (Wanji et al., 2019b) but the detailed ecology of both vectors remains elusive on Bioko Island. We found that *M. perstans* infection is eight times more prevalent than *L. loa* with higher infection intensity among people older than 40 years and living in rural areas including Riaba, Luba and Baney districts (Chapter 3). Male might be more likely to be exposed to vector bites which could be linked either to their professional occupations or alternatively to the physiological differences between female and male (Whittaker et al., 2018). However, detailed knowledge about the factors that may contributed for the differential distribution of *M.perstans* is not well defined (Simonsen et al., 2011, Wanji et al., 2016).

Therefore, we aimed to investigate the factors that might drive the transmission of *M. perstans* infection in Bioko Island through an approach based on artificial intelligence or Machine Learning (ML). Currently**,** ML is extensively used for epidemiological data analysis and disease diagnosis (Kondeti et al., 2019) for identifying patterns in database systems enabling for instance to determine risk factors for infection or make predictions on disease outbreaks (Wiens and Shenoy, 2018). Here, we have used the opportunity of the MIS conducted in 2018 that resulted in the collection of individual socio-economic, geographic and occupational data combined with data from the *M. perstans* qPCR results in combination with environmental information to address this question (Chapter 4). Environmental and geographical factors constitute the major predictor for the occurrence of *M. perstans* on Bioko Island. Previous reports associated *M. perstans* infection to some environmental factors including aquatic and forested areas and banana plantations that is suitable for the distribution and abundance of vectors *Culicoides* spp. (Wanji et al., 2019b, Sick et al., 2019, Ta-Tang et al., 2018). In addition, we found that the infection was more present in individuals who work in the agricultural or fishery sectors. These observations support that vector control measures would seem to be the best intervention for Mansonellosis since no specific and effective drug treatment exists currently for this disease. However, control of *Culicoides* spp. must consider the crepuscular

biting habit of adults (Wanji et al., 2019) and that bed nets used for malaria control seem to be less efficient for these vectors.

7.3 Pregnancy in malaria-filarial worms co-infection

We extended our investigation on filarial worms and co-infection with malaria on pregnant women. In total, 293 pregnant women were enrolled during two successive MIS surveys in 2018 and 2019 on Bioko Island (Chapter 5). During the survey, pregnant women were interrogated on the implementation of malaria prevention measures that include their attendance to antenatal care clinics during pregnancy and intermittent preventive malaria treatment, the use of insecticide-treated mosquito nets (ITNs) and other vector controls measures. This study revealed the importance of the use of ITNs on malaria prevention. On Bioko Island, wide spread use of bed nets were introduced in 2008, following several mass dissemination campaigns coupled with other communication channels (e.g. antenatal care (ANC) clinics, and schools) as recommended by WHO. Since insecticide treated bed nets (INT) were automatically distributed to every pregnant women attending ANC facilities, more effort is needed to encourage women to use ITNs. We could not establish a positive association between *M. perstans* and *Plasmodium* spp. co-infection even though previous studies stipulated that maternal infection with *M. perstans* increases the risk of malaria infection during pregnancy (Hillier et al., 2008, Ndibazza et al., 2013, Blackwell, 2016). Interestingly, *L. loa* is the predominantly encountered co-infection despite its low infection rate in the general population. Mild and moderate anemia were observed among pregnant women infected with either *Plasmodium* spp. or *M. perstans* although most of them (72.7 %) were treated with iron pills. Like other reports (Muhangi et al., 2007), our study would likely show that anemia in pregnancy does not seem to be associated with filarial worm infections and could also be cofounded by poorer nutritional status based on socio-economic status or infection with intestinal helminths like hookworms (Blackwell, 2016). One strong limitation

of our study is the small sample size and the low number of infected women identified that prevent us from drawing firm conclusions about the relevance of this disease in pregnancy. However, we describe for the first time the rate of *M. perstans* infections in pregnant women residing on Bioko Island. Clearly, women from poorer, rural areas like Luba district and having a lower socio-economic status carry a higher burden of *M. perstans* infection, thus contributing to the local spread of *M. perstans* by acting as a potential reservoir for this vector borne-disease.

Chapter VIII

Conclusion and Outlook

Conclusion and Outlook

Chronic filarial infections like onchocerciasis, LF and loiasis can have serious, negative socio-economic impact on affected communities. Few reports have investigated Mansonellosis since it is difficult to detect this infectious disease using standard diagnostics measures (Ta-Tang et al., 2021). Hence, Mansonellosis burden remains largely unknown since limited research funding is available, making it the filarial infection that is the most neglected (Ta-Tang et al., 2021). In this thesis, we adopted a novel approach and software tools to investigate filarial worms epidemiology and risk factors that might favor these infections on Bioko Island. We based our approach on repurposing used mRDTs from regularly conducted MIS in Equatorial Guinea. Malaria RDTs are widely used in malaria endemic regions that have a large geographical overlap with *M. perstans* and *L. loa* endemic areas. Previous studies showed that human DNA is fixed and preserved on the mRDTs (Guirou et al., 2020), thus our approach could be used to investigate other blood-borne pathogens without the need of collecting more blood samples. The ENAR approach and molecular assays can be realized in public health laboratories where qPCR machines are usually available. In addition, the experiment is simpler and less expensive compared to existing methods, in terms of sample collection, storage, and processing, making this approach useful for a molecular epidemiological studies for blood-borne neglected parasites including *M. perstans* and *L. loa*. The ability to use this small amount of dried blood sample contained in mRDT for ultrasensitive detection of *M. perstans* will facilitate to characterize and assess the importance of this infection in endemic or co-endemic areas. Pregnant women, for instance, that are usually overlooked in filarial infection studies could be taken into account based on better integration of national programs of Malaria and filariasis. We plan to establish a standardized measure that will allow us to quantify mf to better guide drug interventions and possible novel drug testing. Future evaluations are needed to investigate the presence of *Mansonella sp* "DEUX" on Bioko Island. Some studies suggested that subpopulations of *M. perstans* that do not

harbor the endosymbiont *Wolbachia*, exist limiting certain drug treatment options (Grobusch et al., 2003). Molecular characterization and parasite genotyping based on mf circulating in peripheral blood could be an essential step to understand the parasite population dynamics opening also new avenues of better insight into the immunological interplay between humans and *M. perstans* infections or superinfections

Here, the combination of different datasets enabled to provide meaningful information to characterize *M. perstans* infection using artificial intelligence. ML is currently used for data mining in epidemiological studies, infectious disease diagnosis, risk factor identification or prediction of disease outbreaks**.** For diseases highly neglected like Mansonellosis, the combination of the ENAR approach, ML based data mining of metadata collected during MIS for each individual included might be a good opportunity to answer outstanding research questions in a disease field suffering from good funding support.

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Curriculum Vitae

Name: **Charlene Aya YOBOUE**
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Academic qualifications

2017: PhD student at University of Basel/Swiss Tropical and Public Health Institute, Switzerland

2013: Master II in Sciences of Nature at Nangui Abrogoua University of Abidjan, Côte d'Ivoire

2009: Master I in Sciences of Nature at Nangui Abrogoua University of Abidjan, Côte d'Ivoire

2008: Bachelor in Sciences of Nature at Nangui Abrogoua University of Abidjan, Côte d'Ivoire

Work experience

2017: PhD project
Molecular epidemiology of Mansonella perstans on Bioko Island (Equatorial Guinea): identification of risk factors, co-infection with malaria and impact in pregnant women at Swiss Tropical and Public Health Institute and the University of Basel.

2015: Associated-PhD student to the DOLF-RCT project
"Alternative chemotherapies for the treatment and elimination of lymphatic filariasis (LF) in Africa, Côte d'Ivoire."

2014: Technician on the project entitled:
"*Small-scale study to compare the efficacy and residual activity of K-Othrine Polyzone and the K-250 WG Othrine in indoor residual spraying, Elibou, southern Côte d'Ivoire"*

2013: Master II thesis
"*Susceptibility to Pyrethroids of Culex spp and Anopheles spp from two different types of environments in a context of massive use of insecticides in agriculture, Tiassale, southern Côte d'Ivoire.*".

2010: Master I
"*Evaluation of culicidae density and malaria transmission after 18 months of Long-Lasting Insecticide treated Nets using (LLINs) in Djekanou*".

Research and competence areas

Human filariases and malaria based studies including:

- Clinical trial
- Immunology tests (human cells and parasite culture, antigen preparation, Enzyme-Linked Immunosorbent Assay (ELISA), multiplex detection immunoassays)
- Molecular diagnostic (Polymerase Chain Reaction (PCR), LAMP (Loop-mediated isothermal amplification), sequencing)
- Medical Entomology: vector ecology and testing vector control tools (collection and identification of mosquitoes, Bioassays, indoor residual spraying)
- Insectary management (Breeding of susceptible Kisumu and wild mosquitoes).

Conferences and presentations

Charlene Aya Yoboue, Salome Hosch, et al: Molecular epidemiology of filarial nematodes on Bioko Island using nucleic acids extracted from malaria RDTs. **ASTMH 2021 Annual Meeting, 17-21 November 2021 (oral), Virtual Meeting**.

Charlene Aya Yoboue, Salome Hosch, et al: Multi-parasitism of *Plasmodium* spp., *Mansonella perstans* and *Loa loa* in clinically healthy children, adults and elderly people living on Bioko Island. **Colloque International RTAS 2021, 9-11 November 2021 (Poster), Abidjan (Côte d'Ivoire)**

Charlene A. Yoboue, Sarah Frieschmann, et al: Cytokine signatures associated with microfilaria clearance following single dose of Ivermectin, Diethylcarbamazine and Albendazole therapy for lymphatic filariasis in Côte d'Ivoire. **68th Annual Meeting ASTMH (American Society of Tropical Medicine and Hygiene), 20- 24 November 2019 (Poster), Gaylord National Resort and Convention Center, National Harbor, MD**

Charlene A. Yoboue, Chouaibou M. Seydou, et al: Susceptibility to deltamethrin of *Culex sp* and *Anopheles sp* in a context of intensive use of pesticides, Tiassalé (Ivory Coast). **3rd Scientific Symposium on Malaria; April 22th-23th 2015 (Oral), Abidjan (Côte d'Ivoire)**

Charlene A. Yoboue, Chouaibou M. Seydou, et al: Susceptibility to Pyrethroids of *Culex spp* and *Anopheles spp* from two different types of environments in a context of massive use of insecticides in agriculture, Tiassale, southern Côte d'Ivoire. **First Pan African Mosquito Control Association Conference (PAMCA), October 06th-08th 2014 (Poster), Nairobi (Kenya)**

Charlene A. Yoboue, Chouaibou M. Seydou, et al: Susceptibility to Pyrethroids of *Culex spp* and *Anopheles spp* from two different types of environments in a context of massive use of

insecticides in agriculture, Tiassale, southern Côte d'Ivoire. **First Pan African Mosquito Control Association Conference (PAMCA), October 06th-08th 2014 (Poster), Nairobi (Kenya)**

Awards and grants

2021 Leading House Africa (SwissTPH)-Research Partnership Grant I (15,000CHF)
Project: Molecular epidemiology of filarial nematodes in Côte d'Ivoire using locally established next generation sequencing capacities (Co-applicant: Dr Tobias Schindler (SwissTPH))

2019 Travel Award for the ASTMH 68th Annual Meeting, to be held November 20-24 at the Gaylord National Resort and Convention Center, National Harbor, MD

2017 Recipient of Swiss Government Excellence Scholarships granted by the State Secretariat for Education, Research and Innovation

List of publications

1- **Yoboue CA**, Hosch S, Donfack OT, Guirou EA, et al. (2022) Characterising co-infections with Plasmodium spp., Mansonella perstans or Loa loa in asymptomatic children, adults and elderly people living on Bioko Island using nucleic acids extracted from malaria rapid diagnostic tests. PLoS Negl Trop Dis 16(1): e0009798. https://doi.org/10.1371/journal.pntd.0009798

2- Hosch S, **Yoboue CA**, Donfack OT, et al. (2022) Analysis of nucleic acids extracted from rapid diagnostic tests reveals a significant proportion of false positive test results associated with recent malaria treatment. Malaria journal, 21(1), 23. https://doi.org/10.1186/s12936-022-04043-7

3- Guirou EA, Schindler T, Hosch S, Donfack OT, **Yoboue CA**, et al. (2020) Molecular malaria surveillance using a novel protocol for extraction and analysis of nucleic acids retained on used rapid diagnostic tests. Sci Rep 10, 12305. https://doi.org/10.1038/s41598-020-69268-5