Antimalarial drug concentrations in the blood: a monitoring tool for evaluating population drug usage in relation to diagnosis and treatment environment

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Summary

Malaria case management using malaria rapid diagnosis tests (mRDTs) and artemisinin-based combinations therapies (ACTs), the only effective and widely used antimalrial treatment, is a key intervention to control malaria. In Tanzania, case management policies require parasitological confirmation of malaria prior to treatment for patients of all ages. To that aim, considerable efforts have been made to improve diagnosis availability and compliance to diagnosis results and to expand access to effective antimalarials in the public and private sector. Despite that, there are concerns that many patients with malaria do not receive ACTs while others suffering from non-malaria fever do. This indicates that treatments are not always targeted to those in need.

Previous assessments of the impact of new diagnosis or treatment strategies on anti-malarial drug use relied either on longitudinal recording of drug stocks, prescription rates in health facilities, or on histories of drug intake recorded by community interviews. These measures are potentially biased and do not provide accurate estimates of overall levels of circulating drug, probably the most important driver for the development of drug resistance in pathogens.

To address this issue of public health relevance, we used a community-based approach to test the usefulness of measuring concentrations of antimalarial drugs in the blood. This tool was used to monitor population drug use and study the relationships between factors such as malaria prevalence, diagnosis and treatment practices, drug pressure and drug resistance.

In this sense, it was important to measure antimalarials using a sensitive analytical methodology adapted to field conditions. We have thus developed a liquid chromatography-tandem mass spectrometry method (LC-MS/MS) for the simultaneous quantification of 7 frequently used antimalarials and 2 active metabolites in 10-µl dried blood spots. Thanks to the good performances achieved in terms of trueness (-12.1 to +11.1%), precision (1.4 to 15.0%) and sensitivity (lower limits of quantification comprised between 2 and 20 ng/ml) with a convenient extraction procedure, the proposed assay was found to be suitable for the analysis of residual antimalarials in dried blood spots (DBS) samples collected at large scale.

The developed assay was applied within the frame of a pharmacokinetics study including 16 healthy volunteers who received a single adult dose of artemether-lumefantrine. Lumefantrine (LF) concentrations in plasma and in DBS were highly correlated at all time points (R=0.97) and LF half-life calculated from plasma and DBS measurements was similar (101.9 hours in plasma *versus* 103.6 hours in DBS). This work allowed to validate the use of DBS to measure LF concentrations in field trials conditions.

The analytical method was then used for measuring residual antimalarial drugs concentrations in 6485 DBS samples collected in a community-based cross-sectional survey rolled-out in three regions of Tanzania (Mbeya, Mtwara and Mwanza) with different levels of malaria endemicity. This study showed that amongst 6391 participants included in the antimalarial analysis, close to one fifth [20.8% (1330/6391)] had antimalarials detected in their blood and only 28.0% (372/1330) of them reported the use of antimalarials within the previous month. Furthermore, participants who did not report any fever in the previous two weeks accounted for the majority of the individuals who had residual antimalarial levels detected in their blood [71.2% (947/1330)].

There was also room for improvement with regard to diagnosis and treatment practices in case of febrile episode. Indeed, 31% of the individuals with fevers seeking care were appropriately tested (according to patient history) and 50% appropriately treated for malaria (according to the presence or absence of residual antimalarials measured in the blood).

We then used a multivariate analysis to identify factors associated with the presence of lumefantrine and/or desbutyl-lumefantrine (LF/DLF) or sulfadoxine-pyrimethamine (SP) in the blood, the antimalarials most frequently identified in the surveyed population. The most significant predictors identified were expected. Fever in the previous two weeks, young age, higher district malaria prevalence and living in a ward in which all visited drug stores had ACTs in stocks were associated with higher odds of having LF/DLF in the blood. Pregnancy, living in Mwanza region, fever in the previous two weeks and older age were associated with higher odds of having SP in the blood.

Subsequently, we investigated whether the presence of residual concentrations of LF or SP in the blood of individuals sampled in the community predicted the presence of *Plasmodium falciparum* harboring molecular markers of resistance. Indeed, the main consequence of drug pressure is the emergence of parasites resistance, which in turn threatens the sustainability of gains in malaria control. We found that having LF or SP in the blood was not associated with higher odds of carrying a parasite harboring mutations known to reduce parasites susceptibility. However, by multivariate analysis, having LF or SP in the blood at low concentrations was significantly associated with higher probability of carrying a mutant parasite.

In conclusion, our methodology of antimalarial measurement in the blood represents a reliable tool to evaluate at community level the overall impact of case management strategies on population drug pressure. This thesis provides a deep insight on the relationship between parasite prevalence and resistance, individual factors such as age and health seeking behavior,

as well as diagnosis and treatment practices. Despite efforts made to improve access to diagnostic tools, there is still a high drug pressure in the population. These results suggest that there is a need to support the use of mRDTs with proper training in the public and private sector for a better targeted use of antimalarials in favor of parasitologically confirmed malaria cases. These findings should be integrated to more advanced statistical tools to understand the effect of drug pressure on parasite resistance at population level. This could help developing urgently needed targeted strategies to stop the spread of resistance to artemisinins and partner drugs.

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ACN	Acetonitrile
ACTs	Artemisinin-based combination therapies
ADDOs	Accredited Drug Dispensing Outlets
ALu	Artemether-lumefantrine
AM	Artemether
AMFm	Affordable Medicines Facility-malaria
AQ	Amodiaquine
BMI	Body mass index
CHUV	University Hospital of Lausanne
CQ	Chloroquine
DBS	Dried blood spot
DHS	Demographic health surveys
DS	Drug store
ER	Extraction recovery
FA	Formic acid
FDA	Food and drug administration
Fe2+	Ferrous iron
HF	Health facility
HH	Household
I.S.	Internal Standrard
IDL	Instrument detection limit
IHI	Ifakara Health Institute
IPTp	Intermittent Preventive Treatment of malaria in pregnancy
IRS	Indoor residual spraying
ITNs	Insecticide-treated nets
LC-MS/MS	Liquid chromatography coupled to tandem mass-spectrometry
LF	Lumefantrine
LF/DLF	Lumefantrine and/or desbutyl-lumefantrine
LLINs	Long lasting insecticidal nets
LOD	Limit of detection
(L)LOQ	(Lower) limit of quantification
ME	Matrix effect
MeOH	Methanol

MF	Mefloquine
MIC	Minimum inhibitory concentrations
mRDTs	Rapid diagnostic tests
NMCP	Tanzania National Malaria Control Programme
OR	Odds ratio
PCR	Polymerase chain reaction
PE	Process efficiency
PK	Pharmacokinetic
PM	Pyrimethamine
Q	Quinine
QC	Quality control
SD	Sulfadoxine
SNPs	Single Nucleotide Polymorphisms
SP	Sulfadoxine-pyrimethamine
Swiss TPH	Swiss Tropical and Public Health Institute
WHO	World Health Organization
WT	Wild type
WWARN	WorldWide Antimalarial Resistance Network

1. CHAPTER I: INTRODUCTION

1.1. Malaria epidemiology and control in Tanzania

Malaria is a major public health problem in Mainland Tanzania. The entire population is at risk due to the climatic conditions that remain favorable for transmission throughout almost the entire country, which makes Tanzania the 11th largest population at risk of stable malaria in Africa [1] and the fourth dominating country in the global burden of mortality after Nigeria, the Democratic Republic of Congo and Burkina Faso [2]. It is estimated that more than 26% of all outpatient attendances are attributable to malaria, resulting in approximately 6 million confirmed and clinical malaria cases and 22'000 deaths reported in 2017 [2]. There is a large disparity in malaria prevalence among children under age five across regions, from as high as 41% in the North-Western Zone and 19% in the Lake Zone, to 1% in Northern and South-Western Highlands Zones, and more than four times higher in rural areas than in urban areas [3]. *Plasmodium falciparum* accounts for 96% of malaria infections in this country and is principally transmitted by *Anopheles gambiae* [4]. Seasonal malaria peaks occur at the end of the rainy seasons, with short rains taking place from November to January and long rains from March to May.

Tanzania enjoys one of the best annual economic growth rates in sub-Saharan Africa [5] and between 2003 and 2015, increased contributions have been used to deliver preventive and curative services. The successful implementation of insecticide-treated nets (ITNs), long lasting insecticidal nets (LLINs) and Intermittent Preventive Treatment of malaria in pregnancy (IPTp) together with consistent increase in consumption of artemisinin-based combination therapies (ACTs) [1] led to a dramatic decrease in case incidence of more than 75% between 2000 and 2015 [6]. The latest reports showed that malaria prevalence among children under age five decreased from 14.0% in 2015-2016 to 7.5% in 2017 [3].

1.2. Malaria diagnosis

Malaria is suspected clinically on the basis of fever but no signs or symptoms reliably distinguish malaria from other causes of fever [7]. This lack of specificity makes clinical diagnosis unacceptable [8]. Correct diagnosis is particularly important to reduce overuse of antimalarial medicines and to improve the diagnosis of other febrile illnesses. Therefore, since 2010, the World Health Organization (WHO) recommendations include the need to confirm a malaria infection by either microscopy or rapid diagnostic tests (mRDTs) in all settings before dispensing any antimalarial treatment [7]. When parasitological diagnosis is not possible only, a decision to provide an antimalarial should be based on the probability that the illness is malaria. mRDTs are considered to be the best option for an accurate and easy to perform diagnosis of malaria in routine practice [8]. They are rapid (can be performed in about 15

minutes), highly sensitive and specific with a high diagnostic reliability [1] and can be done by minimally trained personnel. These rapid tests are qualitative techniques based on the detection of malaria parasite antigens, relying on antibodies - antigens reactions. Due to the persistence of antigens in the blood stream after parasite clearance, the tests may remain positive for up to one month after treatment even though the patient has been cured [1]. In 2015, mRDTs accounted for 74% of all malaria testing [9].

1.3. Malaria treatments

1.3.1.Artemisinin-based combination therapies

ACTs are the only effective and widely used antimalarial treatments at the moment and their introduction was the intervention that had the second highest effect on malaria control between 2000 and 2015 worldwide [10]. In response to development of drug resistance to conventional antimalarial drugs, the WHO has been recommending ACTs as first-line therapy [7]. All countries in sub-Saharan Africa have then adopted an ACT (either artemisinin-amodiaquine, dihydroartemisinin-piperaquine or artemether-lumefantrine) as first-line drug for treatment of uncomplicated malaria [6] and thanks to the support of large initiatives and funding agencies, including The Global Fund for AIDS Malaria and Tuberculosis and the World Bank, ACTs are now being deployed intensively [11]. The therapeutic efficacy of ACTs is high in sub-Saharan Africa, with a median treatment failure rate of less than 10% [6] but resistance has been detected in South East Asia and is expected to occur in Africa in the longer term [12]. The first known case of artemisinin resistance has been identified recently in Equatorial Guinea [13]. Currently, less than 30 antimalarials are under clinical development and only a limited number of them are potential alternatives to artemisinin-based therapies. Furthermore, six candidates are endoperoxides or synthetic artemisinins, which could be subjected to cross-resistances with artemisinin-resistant parasites [12].

Artemether-lumefantrine (ALu), the currently recommended ACT in Tanzania, combines the short-acting artemisinin derivative artemether (AM) with the long-acting lumefantrine (LF). AM has a rapid onset of action resulting in prompt parasite clearance, and their short elimination half-life (2 hours) reduces the probability of development of resistance. Most of the parasites are killed during the course of the 3-days therapy (10^8 reductions in parasite biomass), most likely due to the effect of AM and its metabolite dihydroartemisinin. By contrast, LF has a slower onset of action but longer elimination half-life of 2-3 days allowing the drug to accumulate after the course of the treatment which enables the killing of the few remaining parasites (10-10^4) [14] and decreasing the relapse rate [15][14]. Both drugs protect each other from the emergence of resistance [16].

1.3.2.Malaria treatments in Tanzania

In Tanzania, delayed parasitological clearance and resistance following standard treatment courses of chloroquine (CQ) began to emerge rapidly and by 1999, many sites had documented CQ clinical failure rates in excess of 40% [1]. This advocated for a change from CQ to sulfadoxine-pyrimethamine (SP) in 2001, but in a period of five years, parasite resistance to SP had gone up to 25% on average. From 2006 to 2007, a decision for another change in favor of ALu was therefore unavoidable, thereby providing access to more effective antimalarials [17]. Moreover, sale of oral artemisinin-based monotherapies has been banned in 2006 [17]. In Tanzania, ALu's efficacy was shown to be still very high in 2011-2012 [1]. Besides ALu, artesunate plus amodiaquine (AQ) or mefloquine (MF) is also considered as an option for treatment of uncomplicated malaria in Tanzania. Quinine (Q) is the second line drug in case of treatment failure and, as well as injectable artesunate, a drug of choice for treatment of severe malaria [6]. Although SP had been abandoned as first line treatment, it was retained at lower dose for prevention purposes as the drug of choice for IPTp, because no suitable alternative to SP was available and its lower efficacy is acceptable to prevent worst effects of malaria infection during pregnancy [17].

1.4. Access to diagnosis and treatments in Tanzania

Mainland Tanzania is divided into regions and district councils, which are further divided into divisions and wards (formed by five to seven villages) [18]. The health care services are provided through a hierarchical system which includes dispensaries (primary health care), health centers (secondary health care), regional and district hospitals (tertiary health care). A dispensary serves a population of 6,000 to 10,000 people; a health centre serves 50,000–80,000; and a district hospital serves more than 250,000 people [1]. Faith-based organizations and for-profit private sector are also parts of the health system [18]. The private retail sector includes both unregistered outlets and registered Accredited Drug Dispensing Outlets (ADDOs) and Pharmacies. Registered outlets only are permitted to stock and sell ACTs [19] although a few unregistered drug stores also do [20]. ALu is free of charge in the public health facilities (HFs) and faith based organizations for children under the age of five, pregnant women, elderly and at a minimal cost for adults [21]. In 2014, Tanzania reported sufficient ACTs distributed to treat all malaria patients attending public HFs [6]. Since 2009, following WHO recommendations [7], the Tanzania National Malaria Control Programme (NMCP) case management policies require that patients of all ages should receive a diagnostic test prior to treatment [6,17], which is free of charge in the public sector [6]. Malaria mRDTs have therefore been deployed in the public sector between 2010 and 2012 and health workers have been trained in using them [6]. According to demographic health surveys (DHS) [3], more than 70% of suspected cases were

tested in 2014 in the public sector. In practice however, more than half of those seeking treatment for suspected malaria first visit the private sector (including both registered and unregistered outlets), especially in rural areas [19]. Since the Ministry of Health recognized the importance of engaging the private sector in the malaria case management [22], efforts have also been made to strengthening the delivery of medicines through retail outlets (Figure 1.1) [19].

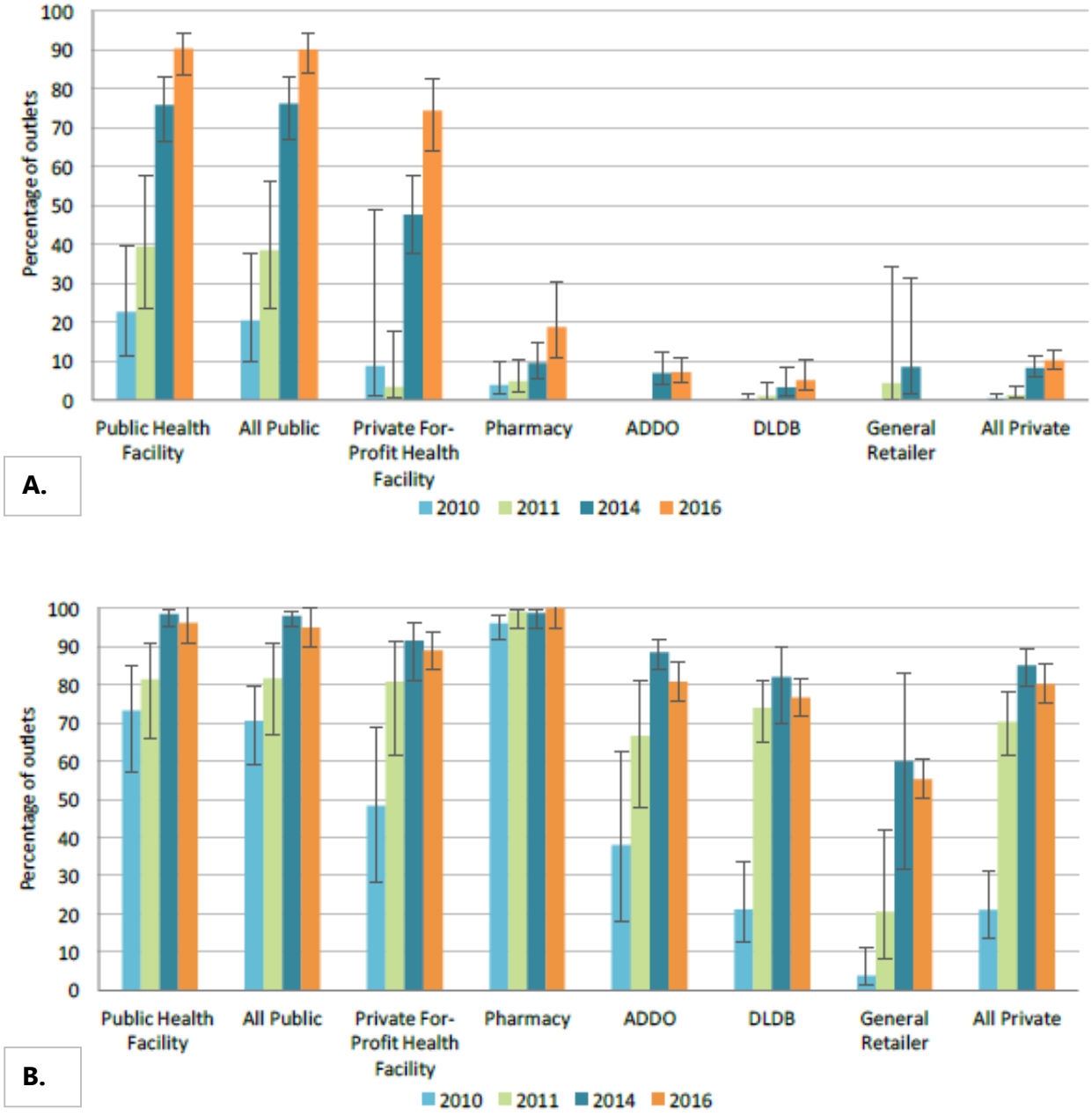

Figure 1-1: Percentage of public and private outlets with A) mRDTs and B) ACTs in stock on the day of the survey. Outlets were considered to have stocks if at least one mRDT or ACT was observed. DLDB = *Duka La Dawa Baridi,* unregistered dispensing outlet. Source: ACT Watch [23]

1.5. New diagnosis and treatment strategies

1.5.1. The aim of new diagnosis and treatment strategies

The implementation of ACTs and mRDTs has been a cornerstone in the management of fever cases. New diagnosis strategies are aimed at improving health outcome by helping in ascertaining disease etiology and better evaluating the level of severity. As ACTs are frequently used to treat causes of fever other than malaria [24,25], treatments can also be better targeted to those in need with the help of mRDTs, leading to more rational use of drugs. Such a beneficial effect has been clearly demonstrated in Dar es Salaam in 2007, where the introduction of mRDTs led to 77% reduction of antimalarial consumption [26] and 43% reduction in Senegal after deployment of mRDTs at large scale between 2007 and 2009 [27]. Such a decrease should have important implications in terms of drug pressure and occurrence of *Plasmodium* resistance [28]. Unfortunately, these new drugs policies have difficulties to be implemented sustainably. In spite of considerable efforts to increase access to essential medicines, uptake of first-line antimalarials and antibiotics is still low and/or erratic in most developing countries. Availability of diagnostic tests for malaria (microscopy, mRDTs) is improving but remains well under the estimated global need, especially in remote settings [29]. There is a mixture of underuse and overuse of antimalarials [30,31]. Underuse can be due to access problems, non-compliance of the HF to treatment guidelines, non-adherence of the patient or stock-outs. Overuse can be due to non-availability of malaria tests, variability in the quality and performance of mRDTs [25] or lack of expertise of clinicians in the management of non-malaria fevers, which has become a concern increasingly important, especially with global decrease in proportion of febrile illnesses due to malaria [24,32]. Incorrect malaria prescriptions result in wastage of medication, delays in obtaining effective treatment for the true cause of illness and important drug pressure in the population [19].

While a relatively large number of different drugs are now available against malaria, the parasites have progressively developed resistance against many of existing molecules. This is mainly due to inadequate uncontrolled use of drugs taken as monotherapy, but also because patients might poorly adhere to treatment and finally because of the worldwide distribution of counterfeit drugs containing insufficient drug dosage [33]. More generally, antiparasite resistance is highly dependent on the overall drug pressure, namely the quantity of circulating drugs in the general population [34].

The use of antimalarials as combination therapy is mandatory and clearly beneficial [35], but artemisinin is rapidly eliminated and the “partner drugs” persist in the bloodstream as monotherapy for weeks due to their long half-lives, particularly at subtherapeutic concentrations, selecting for newly acquired resistant parasites [10,36,37]. For example, a

recently published meta-analysis [38] showed that the principal determinant of treatment outcome with ALu is the plasma concentration of LF at day 7, with values >200 ng/mL being associated with >98% cure rates in most uncomplicated malaria patients. Lower concentrations are predictive of treatment failures and at such sub-therapeutic levels, the parasite's susceptibility to LF decreases and it is therefore a favorable environment for the development of resistance. Sulfadoxine (SD) and pyrimethamine (PM) also have long half-lives. The extensive (often inadequate [39]) use of these long-acting compounds is a critical factor contributing to drug pressure, which in turn is a potent force selecting resistant parasites [34,40]. Thus, correct use of antimalarials is now one of the five goals and recommendations of WHO Global plan for artemisinin resistance containment [12]. Renewed efforts must therefore be made overall to optimise drug use and exposure for ensuring an optimal treatment while limiting the opportunities for emergence of drug resistance [39]. Monitoring the impact of new treatment strategies in terms of use and adherence to mRDTs, use of medicines as well as dynamic of resistance are of paramount importance to evaluate the public health benefit they confer.

1.5.2. Methodologies to evaluate new diagnosis and treatment strategies and their impact on drug pressure

There are as yet no standardized methodologies for investigating access to curative health care. Most studies have used cross-sectional surveys in communities, in HFs or in the retail sector to estimate whether or not good quality drugs have been accessed. Interviews have targeted caretakers of small children and focused on health seeking and management for children who suffered from fever in the previous two weeks [41–43]. In the last decade, a great deal of research has been done on access to antimalarial medicines in relation to the changes of first-line treatment policies, mainly from CQ to SP, and then from SP to ACTs. Besides access to drugs, there is a need to evaluate new diagnosis or treatment strategies. Often, consultation processes (completeness of medical history and clinical examination, accuracy of diagnosis, appropriateness of treatment proposed etc.) are compared in intervention and control HFs, or before-and after intervention [44–46]. Exit interviews of patients are also used to check on diagnostic procedures applied and treatment prescribed. If these studies are relatively appropriate to measure effectiveness in small-scale studies, they are certainly suboptimal to assess public health impact of diagnosis or treatment strategies. Indeed, they are more likely to detect a positive effect since they are usually conducted in places where intense training and supervision have been undertaken or they are biased from the Hawthorne effect (people change their behaviour due to the fact they are observed) [47]. Also, even if a treatment has been prescribed, there is no indication on whether the prescribed treatment has been bought and ingested. Moreover, the likelihood of detecting a difference between intervention and control areas in community surveys is much lower than in studies conducted in HFs because of

the rather low prevalence rate of persons being sick and seeking treatment. The net result obtained from community surveys is however much more meaningful for health authorities who need to know the real public health benefit of specific case management strategies, and hence to decide on the relevance of their wide implementation. Cross-sectional surveys in the community have the advantage to provide data on the overall health services recourse and drug use after introduction of a new intervention. These studies are certainly informative but suffer from potential biases [48]. Recall bias is one of those, but many other factors such as fear to be judged or fear of not being appropriately cared for can give an inaccurate picture of what is really done in terms of diagnosis or treatment in the field. For all these reasons, the overall impact of the implementation of mRDTs and ACTs is difficult to assess precisely.

Two recent studies, one in Tanzania [49] and one in Cambodia [50] have shown that self-reported history is not reliable in terms of actual drug use. Indeed, 75% of patients presenting in a HF in Tanzania, and 50% in Cambodia had a detectable concentrations of antimalarial(s) in the blood, although all stated that they did not take any drug in the previous month. These studies show that population drug use can only be captured through community surveys using methodologies that rely on more objective and standardized endpoints.

Changes in diagnosis or first-line treatment strategies and/or policies should be reflected in the prevalence of individuals harboring particular drug(s) in their blood. Measuring of antimalarials concentrations in the blood of the general population is certainly the most accurate method to estimate overall drug pressure, as a result of diagnosis and treatment environment. Drug pressure can then be related to molecular markers of resistance since specific *Plasmodium* polymorphisms have been associated with reduced susceptibility to CQ, AQ, Q, SD, PM, MF, LF [51], and even more recently to artemisinin [52].

Studies investigating antimalarial consumption in community surveys using drug concentrations in bio-fluids have been searched through a literature review. Amongst the 37 studies first selected, 28 were excluded because they used drug concentrations to assess treatment adherence or because they included patients with suspected malaria presenting at a health centre or included in a clinical trial [49-50,53–78]. The 9 studies remaining are presented in Table 1.1. Most of them have been published more than a decade ago, during the CQ or SP eras and none studied ACTs consumption. The reported prevalence rates of individuals with detectable drug in urine or blood range from 0 to 91%. The most extensive study was performed in 2008 and included 3052 participants [34] and showed that between 9-91% of individual had detectable CQ levels and 0-21% PM, with a high variation between sites. Three studies showed that the presence of residual levels of CQ or PM in participants were associated with an increased proportion of mutant parasite isolates [79,80] and one showed that mutant

parasite isolates were only observed at high concentrations of CQ [81]. Only one mutation was less frequent in children with CQ than in children without CQ detected [80]. Among other studies including malaria patients (not presented in the table), Talisuna *et al.* showed that drug use was inversely related to parasite prevalence, and that, in areas of low transmission, drug pressure was the critical factor for parasite resistance, which was not the case in high transmission places [69].

Table 1-1: Studies investigating antimalarial consumption in community surveys using drug concentrations in bio-fluids

Number and reference	Publication Year	Antimalarial	Country	Survey type and population	Number of participants	Analytical method	Proportion of antimalarials detected	Relationship with parasite resistance
1. [80]	2012	CQ and PM*	Rwanda	Household survey, children < 5	749	ELISA** in plasma	CQ in 17.6% of participants, PM in 0%.	*Pfmdr1* Y86 mutation less frequent in isolates of children with CQ levels than of children without CQ levels (16.7 vs 44.0%). *Pfcrt* T76 more frequent in isolates of children with CQ levels than of children without CQ levels (83.3% vs. 73.8%, p = 0.39).
2. [34]	2008	CQ	Senegal, Burkina Faso, Cameroon	Household survey, all ages	3052	ELISA and HPLC-UV*** in urine	CQ and PM ranged from 9% to 91% and from 0% to 21%, respectively.	NA
3. [81]	2003	CQ	Nigeria	Schools and health posts, children	228	HPLC-UV in blood	CQ in 31% of participants.	Prevalence of the *Pfcrt* T76 increased with blood levels of CQ (P < 0.0001). K76 allele more frequent in individuals without detectable CQ levels. *Pfcrt* T76 only observed in children with CQ levels > 150 nmol/L.
4. [82]	2001	CQ	Nigeria	Schools and health posts, children	405	HPLC-UV in blood	CQ in 52% of participants.	NA
5. [79]	2001	CQ and PM	Ghana	Pregnant women presenting for antenatal care in a hospital	172	ELISA in urine and serum	CQ and PM in 45% and 12% of the participants, respectively.	Resistance markers to CQ more prevalent in samples with residual CQ.
6. [83]	2000	CQ and PM	Ghana	Pregnant women presenting for antenatal care in a hospital	530	ELISA in urine	CQ or PM in 65% of the participants.	Residual levels of CQ or PM associated with a reduced prevalence of *P. falciparum* infections and raised proportion of submicroscopic parasitaemia.
7. [84]	1999	PM	Nigeria	Schools and vaccination programs, children	146	ELISA in blood	PM in 4% of the participants.	NA
8. [40]	1999	CQ and SD*	Kenya	Household survey, children	318	ELISA in blood	CQ and SD in 37% and 4% of the participants, respectively.	NA
9. [85]	1994	CQ	Tanzania	Schools, children	163	HPLC-UV in blood	CQ in 78% of the participants	Drug levels were not sufficient to affect parasites.

*CQ = chloroquine, PM = pyrimethamine, SD = sulfadoxine

**ELISA = enzyme-linked immunosorbent assay

***HPLC-UV = liquid chromatography coupled to UV detection

These varying results illustrate the complex interplay between health system, drug pressure, immunity and individual behaviour (Figure 1.2). They call for more research in the field, using sensitive and reliable methods for drug concentration assessment, and careful analysis integrating all possible determinants.

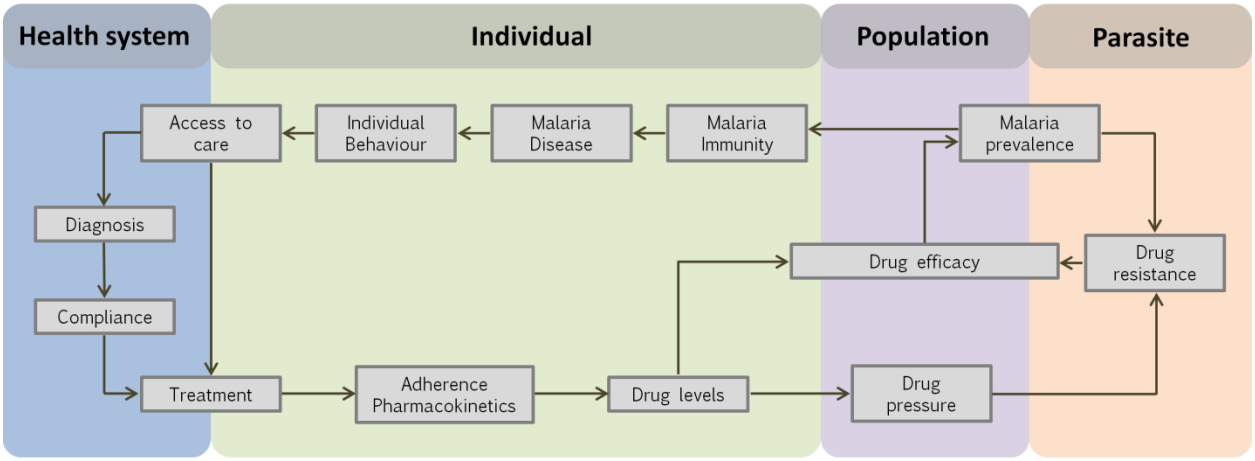

Figure 1-2: Relationship between malaria prevalence, diagnosis and treatment strategies, drug use and drug resistance.

1.6. Analysis of antimalarials in dried blood spots

The main constraints in research field trials at the population level in low-income areas are the issues of difficult sample handling, limited storage and transportation facilities. The dried blood spot (DBS) sampling technique can overcome part of these problems and samples solid state may improve stability of the drugs. This technique is also less invasive because it allows avoiding venipuncture by sampling capillary blood using a simple fingerpick [86]. Some analytical advantages are also to take into consideration: the current sampling standard for drugs quantification allows the measurement of the drugs levels in plasma, whereas the site of action of antimalarials is mostly within erythrocytes [87,88]. For that reason, the DBS technique provides the advantage of measuring total levels in whole blood (i.e. comprising erythrocytes) [89]. The concentration of antimalarial drug measured in whole blood may thus better reflect the exposure at the site of antiparasitic activity.

However, the many logistical and practical advantages of DBS over conventional venous sampling are potentially offset by errors related to volume of blood applied onto the filter paper card, haematocrit (especially with patient infected by malaria wherein protozoan multiplication induce significant red blood cells lysis) [86,90], sample collection (e.g expression of interstitial tissue fluid [91]) and drug distribution (some drugs have complex venous-capillary and blood-plasma concentration relations). Due to the limited volume of blood on the filter paper cards (typically less than 30µl), extreme analytical sensitivity is required for

accurate and precise drug levels measurements. This can be provided by the liquid chromatography coupled to tandem mass-spectrometry (LC-MS/MS) technique, the gold standard for the quantification of drugs in bio-fluids. Furthermore, for the analysis of a large sample size, as encountered in cross-sectional surveys, the LC-MS/MS is a method of choice due to the minimal sample preparation required. In our study, the already existing LC-MS/MS method for the quantification of 14 antimalarials in plasma [89] needed to be adapted and fully validated according to requirements inherent to the DBS sampling technology [92,93].

Artemisinin derivatives have not been analysed in this project because of their very short half-life, typically 2 hours. Also, when using the DBS sampling method, they are rapidly degraded in the presence of ferrous iron (Fe2+) which is released during red blood cells haemolysis. Their known high reactivity with ferrous ion is necessary for their antimalarial activity but creates serious problems for measurement in biological samples [94].

1.7. Main molecular markers of resistance to antimalarials

Decreased susceptibility to CQ and AQ has been associated with the presence of the *pfcrt* 76T and *pfmdr1* 86Y mutations [51]. Inversely, decreased susceptibility to LF has been associated with the wild *pfcrt* K76 allele [95,96] and on the *pfmdr1* gene, gradually acquired tolerance to LF has been suggested, starting with the wild N86 allele, followed by the combination of the N86 and D1246 wild types and finally the triple combination of the N86, the 184F mutation and the D1246 [97]. Increased *pfmdr1* copy number has been correlated with treatment failure following MF and LF [51,98]. Increased treatment failure to SP has been associated to the *pfdhfr* single 108N and triple 51I, 59R and 108N mutations [95]. The *pfdhfr-pfdhps* quintuple mutant (the combination of the *pfdhfr* 51I, 59R and 108N triple mutations with the *pfdhps* 540E, 581G double mutations) correlated with high level resistance to SP and occurs after the *pfdhfr* triple mutations. More recently, a mutation on the propeller region of the *Kelch* gene (known as K13 marker) has been identified as causing resistance to artemisinin [52].

1.8. Rationale for this study

The impact of new diagnosis or treatment strategies on drug use has previously been evaluated by longitudinal recording of HF drugs stocks and/or their prescription rates in intervention and control areas, or before and after an intervention implementation. Alternatively, information on recent history of fever, diagnosis and drug intake was gathered through community surveys. But the biases potentially affecting these measured are numerous and these study might therefore not reflect accurately the amount of drugs circulating among the population. This latter variable is probably the most important determinant to the development of pathogen resistance to drugs.

We therefore propose to evaluate such a community-based approach to test the usefulness of measuring concentrations of antimalarial drugs in the blood to monitor population drug use and study the relationships between factors such as malaria prevalence, diagnosis and treatment conditions and practices, drug pressure and drug resistance (see Figure 1.2).

The present work complements the efforts made in previous projects that assessed management of childhood illnesses, quality of care, access and rational use of drugs in Tanzania [8,25,26,99,100] and explores new perspectives never used to assess drug pressure at large scale. The performances of the LC-MS/MS and the molecular diagnosis platforms have already been shown in previous studies [49,50,89,101,102] led by our research group at the Swiss Tropical and Public Health Institute (Swiss TPH). A strong collaboration with the Ifakara Health Institute (IHI) had already been set up on the above issues. Furthermore, a project aimed at exploring the impact of introducing mRDTs and a highly subsidized ACT in the private retail sector in Tanzania (IMPACT2) conducted jointly by the IHI, the Center for Disease Control and the London School of Hygiene and Tropical Medicine [20,103] served as a basis for the design of our study and facilitated the implementation of our project.

2. CHAPTER II: GOAL AND OBJECTIVES

2.1. General goal

To use the measurement of antimalarials blood concentrations as an objective and reliable tool to assess drug pressure in the general population and to understand how levels of residual antimalarial drugs in community surveys interrelate with malaria prevalence, access to care, diagnosis and treatment practices as well as drug resistance.

2.2. Specific objectives

A) To develop and validate a LC-MS/MS assay for the simultaneous analysis in dried blood spots (DBS) of the main antimalarial drugs used in combination with artemisinin derivatives in Tanzania. (CHAPTERS IV)

B) To measure blood concentrations of residual antimalarial drugs [primarily lumefantrine (LF) and sulfadoxine-pyrimethamine (SP)] in representative samples of people in malaria endemic areas and to compare these results with self-reported history of drug use. (CHAPTER V)

C) To use antimalarials blood measurements in community surveys to assess diagnosis and treatment appropriateness in case of fever episode at population level. (CHAPTER V)

D) To use antimalarials blood measurements in community surveys to evaluate the actual state of population drug pressure and to identify the factors associated with the presence of LF or SP in the blood. (CHAPTER VI)

E) To investigate whether the presence of residual LF or SP in the blood of individuals sampled in the community predicts the presence of *Plasmodium falciparum* harbouring molecular markers of resistance. (CHAPTER VII)

3. CHAPTER III: METHODOLOGY

Preliminary comment

To answer objective A, the *DBS-ALU* project has been carried out in Switzerland. To answer objectives B-E, the *AMACO* project has been conducted in Tanzania.

3.1. Objective A

3.1.1.Collaboration

The LC–MS/MS assay development and the DBS sample analysis were carried out in the Laboratory of Clinical Pharmacology of the University Hospital of Lausanne (CHUV), Switzerland. The pharmacokinetic (PK) study was carried out in the service of Clinical Pharmacology of the CHUV.

3.1.2.Study design and population

To assess the relationship between LF concentrations measured in plasma and in DBS samples, a PK study was conducted in Switzerland in June 2016. This study was a prospective open-label one-group interventional trial. Sixteen healthy male and female subjects aged between 18 and 45 years with a body mass index (BMI) between 18 and 29 kg/m^2 were included. The specific eligibility criteria are presented in Appendix 1.

All the participants received a single adult dose of Riamet® (artemether-lumefantrine, Novartis), followed by 6 to 10 capillary and venous blood sampling, from hour 0 to hour 336 after drug intake. All visits were ambulatory.

3.1.3.Laboratory analysis

An LC-MS/MS assay for the multiplex analysis of 7 antimalarials and 2 active metabolites in DBS was developed. This essay was used to measure antimalarial concentrations in DBS samples collected during the PK study in Switzerland. This methodology was adapted to measure LF concentrations in erythrocytes and whole venous blood and a previously developed method [89] was used to measure LF concentrations in plasma.

3.1.4.Ethics

This PK study was approved by the "*Commission cantonale d'éthique de la recherche sur l'être humain*" of Canton de Vaud (CER-VD), Switzerland. Written informed consents were obtained from all participants.

3.2. Objectives B-E

3.2.1.Collaboration

One cross-sectional survey was carried out in Tanzania in collaboration with the IHI of Dar es Salaam, Tanzania. The assessment of Single Nucleotide Polymorphisms (SNPs) related to parasite resistance to drugs was done in the Laboratory of Molecular Parasitology and Epidemiology of the Swiss TPH, Basel, Switzerland.

3.2.2.Study area, design and population

The survey took place from May to August 2015, after the rainy season in three regions of Tanzania: Mtwara, Mwanza and Mbeya with populations of 1'270'854, 2'772'509 and 2'707'410 respectively [104]. The regions are located in South-Eastern, North-Western and South-Western zones of mainland Tanzania, respectively (Figure 3.1) and cover a range of malaria endemicity, different geographic and ecological zones, different settings (rural, urban) as well as different levels of access to diagnosis and treatment facilities. The choice of the regions in this study was based on the IMPACT2 project [20], which reported that the level of malaria prevalence amongst all age groups in 2012 was moderately high for Mtwara and Mwanza (17.4% and 16.1% respectively), and low for Mbeya (2.3%). According to the latest DHS survey [3], malaria prevalence among children under age five in 2015 was 20% in Mtwara, 15% in Mwanza and 1% in Mbeya (Figure 3.1).

The cross-sectional survey included three types of surveys conducted concurrently in randomly selected wards:

1) HH-based surveys: up to six members were randomly selected amongst all consenting individuals of the HH. The exclusion criteria were individuals under three months of age and those with a severe illness requiring immediate referral. Interviews were conducted with a questionnaire in Swahili including demographic characteristics, information on time to the closest HF and closest pharmacy or drug retailer, history of fever in the previous two weeks as well as history of antimalarial use in the previous months. In addition, capillary blood spots were collected from finger prick to assess the presence of malaria antigens by mRDT and applied onto filter paper for further drug concentrations measurements by LC-MS/MS.

2) drug outlet-based surveys: all private and public drug dispensing outlets surrounding and serving the selected villages were visited. Details about antimalarials stocks and diagnostic tools (mRDTs and microscope) available at the time of the visit were recorded.

3) exit interviews in HF-based surveys: in each ward, the main HFs serving the surveyed villages were selected. After completion of their consultation with HF staff and after visiting the HF pharmacy for possible treatment procurement, patients who consulted for fever were interviewed to collect information on demographics, malaria diagnostic test performed, test result and drug obtained.

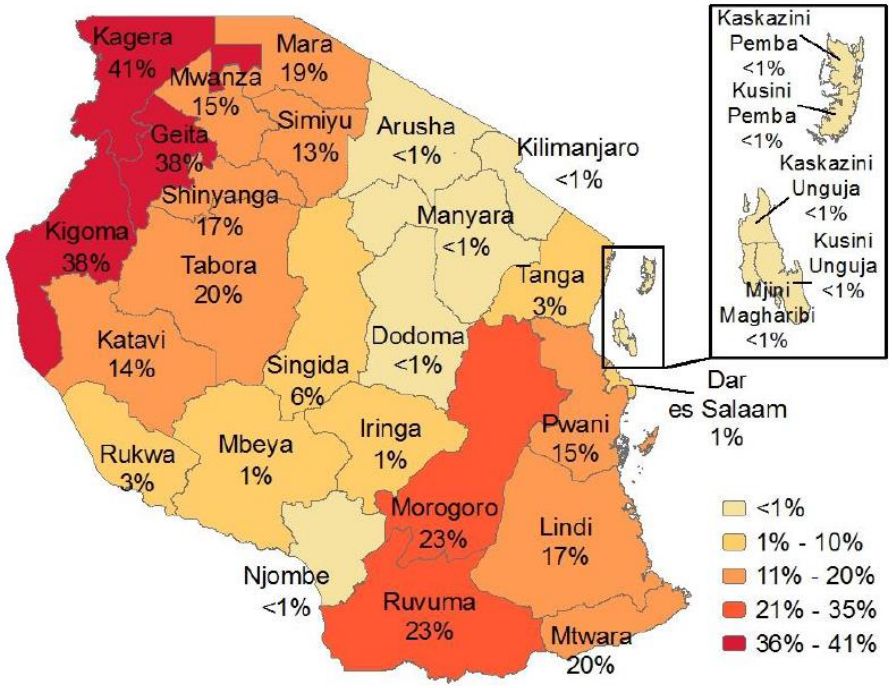

Figure 3-1: Malaria prevalence in Tanzania in children under age five, by region (assessed by mRDTs) in 2015 Source: DHS surveys [3]

3.2.3.Laboratory analysis

The LC-MS/MS assay has been used for the multiplex analysis 7 antimalarials and 2 active metabolites in DBS samples collected during the cross-sectional survey in Tanzania. SNPs related to parasite resistance to drugs were analysed by polymerase chain reaction (PCR) followed by sequencing in DBS samples of individuals tested positive for malaria during the cross-sectional survey in Tanzania (Objective E). To assess resistance to LF, the *pfmdr1* mutations N86Y, Y184F, the *pfcrt* mutations C72S, M74I, N75E, K76T/I/N as well as the number of copy of the *pfmdr1* gene were analysed. To assess resistance to SP, the *pfdhfr* mutations A16V/S, N51I, C59R, S108N/T, I164L and *pfdhps* mutations S436A/F, A437G, K540E, A581G, A613T/S were analysed.

3.2.4.Ethics

This cross-sectional study was approved by three different Ethics Committees (Swiss Ethics Committees on research involving humans, Institutional Review Board of the Ifakara Health

Institute and National Institute for Medical Research in Tanzania). Written informed consents were obtained from all participants or their responsible caretaker.

4. CHAPTER IV: ANTIMALARIAL DOSAGE IN DRIED BLOOD SPOTS

4. A) LC-MS/MS method for the simultaneous analysis of seven antimalarials and two active metabolites in dried blood spots for applications in field trials: analytical and clinical validation

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4.1. A) Abstract

In epidemiological studies, antimalarials measurements in blood represent the best available marker of drugs exposure at population level, an important driver for the emergence of drug resistance. We have developed a liquid chromatography-tandem mass spectrometry method (LC-MS/MS) for the simultaneous quantification of 7 frequently used antimalarials (amodiaquine, chloroquine, quinine, sulfadoxine, pyrimethamine, mefloquine, lumefantrine) and 2 active metabolites (N-desethyl-amodiaquine, desbutyl-lumefantrine) in 10-µl dried blood spots (DBS). This sampling approach is suitable for field studies wherein blood samples processing, transportation and storage are problematic. Sample preparation included extraction from a 3 mm-disk punched out of the DBS with 100-µl of methanol + 1% formic acid containing deuterated internal standards for all drugs. Good performances were achieved in terms of trueness (-12.1 to +11.1%), precision (1.4 to 15.0%) and sensitivity, with lower limits of quantification comprised between 2 ng/ml (sulfadoxine) and 20 ng/ml (chloroquine, quinine, pyrimethamine, mefloquine, lumefantrine and desbutyl-lumefantrine). All analytes were stable in DBS kept for 24h at room temperature and at 37°C. The developed assay was applied within the frame of a pharmacokinetics study including 16 healthy volunteers who received a single dose of artemether-lumefantrine. Lumefantrine concentrations in plasma and in DBS were highly correlated (R=0.97) at all time points, confirming the assumption that lumefantrine concentrations determined in DBS confidently reflect blood concentrations. The blood/plasma ratio of 0.56 obtained using the Bland-Altman approach (and corresponding to the slope of the linear regression) is in line with negligible penetration of lumefantrine into blood cells. This sensitive multiplex LC-MS/MS assay enabling the simultaneous analysis of antimalarials in DBS is suitable for epidemiological studies in field conditions.

4.2. A) Introduction

While a relatively large number of different drugs are now available against malaria, the emergence of parasites resistant against current, frequently used antimalarial agents appears inexorable [105]. One of the leading causes of drug resistance to antimalarials is the frequently unrecognized intake of doses without prescription, often in insufficient amounts or over insufficient durations. Such poorly structured use of treatments produce residual drug levels in blood that remain below target minimal inhibitory concentrations for prolonged periods of time, thus exerting a strong selective pressure on parasites. Renewed efforts must therefore be encouraged to optimise drug use and exposure while decreasing the risk of drug resistance development [39].

To determine overall drug exposure and resulting selection pressure in a population, interviewing individual patient is possibly the most straightforward approach. However it only represents an indirect surrogate, likely to be flawed by numerous confounding factors. Indeed, interviewees reports have been previously shown not to give a comprehensive and accurate picture of drug use in a population [49,50,106]. Direct measurement of antimalarials concentrations in blood samples might represent a more accurate method to estimate overall drug pressure in the general population.

In that perspective, blood samples collection onto filter paper cards using the dried blood spot (DBS) approach has attracted much interest. It actually appears to be the most convenient approach for collecting blood specimens in field conditions, wherein samples handling, transportation and available storage facilities are well recognized issues. However, this approach raises a number of bioanalytical challenges [107] and because of the limited whole blood volume applied onto the paper cards (i.e. less than 30 µl), extreme analytical sensitivity is required for accurate and precise drug levels measurements.

Liquid chromatography coupled to mass spectrometry (LC-MS) has progressively become the reference bioanalytical method because of its unsurpassed sensitivity, selectivity and the extremely low limits of quantification this technique can provide. While a number of analytical methods have been developed for measuring antimalarials in DBS [108–127] only three, to the best of our knowledge, have used mass spectrometry combined with liquid chromatography (LC-MS) [113,128,129]. In fact, the LC-MS technique has so far been applied for quantifying antimalarials mostly in plasma or in whole blood samples [130–134,134–146]. Moreover, amongst all published methods that use liquid chromatography (LC), very few allow the analysis of multiple antimalarials simultaneously: sulfadoxine, pyrimethamine, chloroquine, amodiaquine and N-desethyl-amodiaquine in whole blood by HPLC and UV detection [147];

as much as 14 antimalarials and their metabolites in plasma using triple stage quadrupole tandem mass spectrometry (LC-MS/MS) [131]; and quinine, mefloquine, sulfadoxine, pyrimethamine, lumefantrine and chloroquine in DBS by LC-MS with ion trap detection [113]. The latter, despite using mass spectrometry, did not allow reaching very low limits of quantification, which would preclude its general application for screening samples for the presence of low residual levels.

The aim of the present work was to develop and validate a LC-MS/MS method for the simultaneous sensitive analysis in DBS of the major antimalarial agents quinine (Q), chloroquine (CQ), amodiaquine (AQ), sulfadoxine (SD), pyrimethamine (PM), mefloquine (MF) and lumefantrine (LF) [and their active metabolite desbutyl-lumefantrine (DLF), N-desethyl-amodiaquine(DAQ)], most of them used at present in combination with artemisinin derivatives. Those latter however undergo complex chemical reactions in the presence of ferrous iron (Fe^{2+}) [94], thus precluding their direct analysis in the lysed whole blood matrix of DBS paper cards. The proposed method is a powerful tool for determining antimalarials levels in individuals from a large population. Since all pharmacokinetic/pharmacodynamic (PK/PD) studies for lumefantrine [38] have been as yet based on plasma concentrations, a required validation step necessitated to study the strength of the correlation between lumefantrine (the most widely used antimalarial in combination with artemisinin derivates at present) measured in DBS and in plasma. A clinical validation to translate DBS measurements into plasma concentrations should always be performed when developing any DBS sampling [92]. In addition, we have examined the concentrations of lumefantrine found *in vivo* in red blood cells, the site of erythrocytic stages of *Plasmodium falciparum*.

4.3. A) Materials and methods

4.3.1.A) Chemicals and reagents

CQ diphosphate and Q hydrochlorine were purchased from Sigma-Aldrich (Buchs, Switzerland) and Novartis Pharma AG (Basel, Switzerland), respectively. AQ, DAQ, SD, PM, MF, LF and DLF were obtained from Toronto Research Chemicals (TRC, Ontario, Canada). The internal standards (I.S.) CQ-D_4 diphosphate, Q-D_3, AQ-D_{10}, DAQ-D_5, PM-D_3, SD-D_3, MF-D_9 hydrochloride, LF-D_9 and DLF-D_9 were purchased at TRC (Ontario, Canada). Ultrapure water was prepared using a Milli-Q® Advantage A10 apparatus (Millipore Corp., Burlington, MA, USA). Chromatography was performed using HPLC-grade acetonitrile (ACN), methanol (MeOH), isopropanol and formic acid 98% (FA) and ammonium acetate, all purchased from Merck (Darmstadt, Germany). Whole blood used for the preparation of calibration and control

samples as well as for the determination of matrix effect was obtained from blood withdrawn from patients with Vaquez Disease at the occasion of their regular phlebotomy at the CHUV (Lausanne, Switzerland). This blood was immediately stored at -20°C and consequently haemolysed.

4.3.2.A) LC-MS equipment

The liquid chromatography system used consisted of Rheos 2200 quaternary pumps and a HTS PAL autosampler used for sample injection (CTC Analytics AG, Zwingen, Switzerland) controlled by the Janeiro-CNS software (Flux Instruments, AG, Thermofischer Scientific Inc., Waltham, MA). The LC-MS/MS system comprised an XSelect® UPLC HSS T3 2.5 µm; 75 x 2.1 mm analytical column (Waters, Milford, MA, USA) used for the chromatographic separation coupled to a triple stage quadrupole (TSQ) Quantum Ion mass spectrometer (MS) from Thermofischer Scientific, equipped with an electrospray ionization (ESI) interface operated in the positive mode.

4.3.3.A) Internal standards, calibration standards and quality controls (QCs) preparation

Primary stock solutions of 1 mg/ml (except 2.5 mg/ml for sulfadoxine-D_3) prepared using each pure internal standards compound dissolved in MeOH were further diluted with MeOH to obtain 1µg/ml stock solutions (free form). A single sample extraction solution was subsequently prepared with MeOH containing 1% formic acid and all I.S. at 5 ng/ml (AQ-D_{10} and DAQ-D_5) and 50 ng/ml (CQ-D_4, DLF-D_9, SD-D_3, PM-D_3, Q-D_3, LF-D_9 and MF-D_9). All solutions were stored at -20°C.

Standard stock solutions of antimalarial drugs and metabolites (chemical structures depicted in Figure 4.1a) were prepared in suitable solvents reported in Table 4.1.a and stored in polypropylene tubes at -20°C. Serial dilutions of stock solutions with MeOH/H_2O (1:1, v/v) were performed to obtain working solutions at concentrations suitable for the subsequent preparation of calibration and quality control samples.

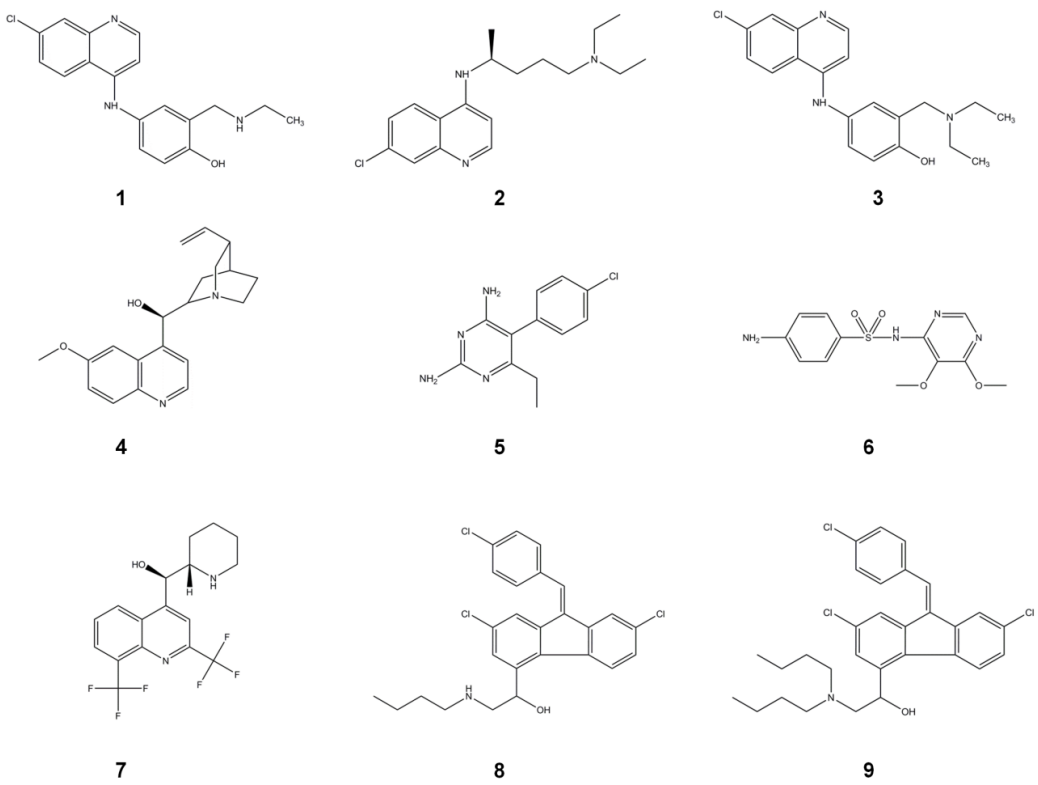

Figure 4-1.a: Chemical structures of antimalarials/metabolites. (1: N-desethyl-amodiaquine (DAQ) 2: chloroquine (CQ), 3: amodiaquine (AQ), 4: quinine (Q), 5: pyrimethamine (PM), 6: sulfadoxine (SD), 7: mefloquine (MF), 8: desbutyl-lumefantrine (DLF), 9: lumefantrine (LF)). Drawn with ChemDraw software (version Prime 16.0).

Each working solution was diluted in blank haemolysed whole human blood to obtain calibration samples and the corresponding 3 quality control samples at low (L), medium (M) and high (H) levels (Table 4.1.a) and kept at -20°C prior to analysis. Blood haemolysis had been carried out by freezing human whole blood at -20°C followed by a thawing cycle. DBS calibration and QC samples were prepared on the day of analysis by spotting 10-µl aliquots of each thawed calibration and quality control samples onto FTA DMPK-B cards (Whatman®, Maidstone, UK). The blood spots were then allowed to dry for at least 2 hours at room temperature prior to DBS processing.

Table 4-1.a: Preparation of calibration standards and quality control samples.

Compound	Concentration (ng/ml) and solvent of stock solutions	Calibration range in haemolyzed whole blood (ng/ml)	QCs controls (ng/ml)
N-desethyl-amodiaquine	1 (in MeOH)	6-1500	18, 120, 1200
Chloroquine	10 (in H_2O)	20-5000	60, 400, 4000
Amodiaquine	5 (in MeOH)	15-3750	45, 300, 3000
Quinine	10 (in H_2O)	20-5000	60, 400, 4000
Sulfadoxine	1 (in MeOH)	20-5000	60, 400, 4000
Pyrimethamine	1 (in MeOH)	2-500	6, 40, 400
Mefloquine	4 (in MeOH)	20-5000	60, 400, 4000
Desbutyl-lumefantrine	1 (in MeOH/DMSO 4 :1 +1%FA)	20-5000	60, 400, 4000
Lumefantrine	1 (in MeOH/DMSO 4 :1 +1%FA)	20-5000	60, 400, 4000

4.3.4.A) LC-MS/MS analysis conditions

The mobile phases used for the chromatography consisted of 2 mM ammonium acetate + 0.1% FA in ultra-pure water (mobile phase A) and acetonitrile + 0.1% FA (mobile phase B). The mobile phase A was also used for the dilution of organic DBS extracts prior to their HPLC injection (see below).

The chromatographic program comprised 3 steps, namely a 11-min separative step followed by a 4-min washing with mobile phase B and isopropanol (mobile phase C) and a 4-min column re-equilibration step. The mobile phase was delivered using a stepwise gradient elution according to the sequence reported in Table 4.2.a. The injection volume was 20 µl and the autosampler injection syringe and injection valve were rinsed 5 times automatically after each sample injection using 2 solutions, i.e. MeOH/H_2O (1:1, v/v, weak wash) +1% FA and ACN + 1% FA (strong wash) to reduce sample carry-over (see below).

Table 4-2.a: LC-gradient elution program.

Time (min)	Mobile phase A* (%)	Mobile phase B* (%)	Mobile phase C* (%)	Flow rate (µl/min)
0.0	98	2	0	300
10.5	10	90	0	300
11.0	2	98	0	600
11.5	2	98	0	600
12.0	0	0	100	500
13.5	0	0	100	500
14.0	2	98	0	600
14.5	2	98	0	600
15.0	98	2	0	300
19.0 (total analysis time)	98	2	0	300

*Mobile phase A: 2mM ammonium acetate + 0.1% FA in MeOH
Mobile phase B: acetonitrile + 0.1% FA
Mobile phase C : isopropanol 100%

Mass spectrometry acquisition has been set using the Selected Reaction Monitoring mode using 3 consecutive time segments with ESI in the positive mode. The selection of MS/MS transitions for antimalarials and their corresponding I.S. have been performed by continuous infusion of a solution of each pure standard at 1 µg/ml in MeOH directly into the mass spectrometer. The selected MS/MS transitions, the tube lens and collision energy for each analyte and I.S. are reported in Table 4.3.a. The Xcalibur software package (version 2.0) (ThermoQuest, Thermofischer Scientific Inc., Waltham, MA) was used to control the instruments and chromatographic data acquisition, peak integration and quantification.

Table 4-3.a: MS/MS conditions and retention times for antimalarial drugs, metabolites and internal standards.

Compound	Precursor ion (*m/z*)	Product ion (*m/z*)	CE* (eV)	Tube Lens (V)	Segment n°	Retention time (min)
N-desethyl-amodiaquine	328.3	283.0	26	60	1	3.58
N-desethyl-amodiaquine-d5	333.0	282.9	27	60	1	3.58
Chloroquine	322.2	142.2	27	74	1	3.65
Chloroquine-d4	326.1	146.2	24	72	1	3.64
Amodiaquine	357.3	284.1	16	73	1	3.75
Amodiaquine-d10	366.1	282.9	26	72	1	3.75
Quinine	325.1	307.1	24	89	2,3	4.16
Quinine-d3	328.1	310.1	26	60	2,3	4.15
Sulfadoxine	311.0	155.9	28	68	3	5.28
Sulfadoxine-d3	314.5	156.0	25	63	3	5.24
Pyrimethamine	249.1	233.0	38	87	3	5.28
Pyrimethamine-d3	253.1	234.0	38	87	3	5.28
Mefloquine	380.0	361.0	26	94	4	6.91
Mefloquine-d9	389.0	371.0	26	94	4	6.90
Desbutyl-lumefantrine	472.1	454.0	22	82	5	9.50
Desbutyl-lumefantrine-d9	481.3	463.1	22	82	5	9.49
Lumefantrine	531.1	512.1	29	95	5	10.49
Lumefantrine-d9	539.0	521.1	34	95	5	10.46

*CE=collision energy

4.3.5.A) Blood sample collection for antimalarials measurements

Comparative PK study of DBS versus plasma, in healthy subjects

A PK study with 16 healthy volunteers monitored over 14 days after a single adult dose of artemether-lumefantrine was conducted at the University Hospital of Lausanne (Switzerland) for a formal head-to-head comparison of concentrations of LF measured in DBS and simultaneously in plasma and red blood cells. It also helped to determine the time during which LF could still be detected in DBS after a single antimalarial drug dose. Venous and capillary blood samples were obtained at 11 time points in 16 volunteers after administration of a single adult dose of artemether-lumefantrine (Riamet®, Novartis, Basel, Switzerland), consisting of 4 tablets containing each 20 mg artemether and 80 mg LF, together with light standardized food (one glass of milk and a croissant). Venous blood samples (10 ml, as two 4.9-ml aliquots) were collected on EDTA anticoagulant, just before treatment initiation and at

various time points on Day 0, Day 1, Day 2, Day 6 and Day 13. Venous blood tubes were then centrifuged at 2000 *g* for 10 min at 4°C and the collected plasma stored at -80°C prior to analysis. After discarding the residual plasma and buffy coat at the upper layer interface of red blood cells pellets, the packed erythrocytes from the first tube were directly placed at -80°C. The erythrocytes from the second tube were subjected to a quick single washing step with 5 ml NaCl 0.9% and this suspension was centrifuged again. After removing the supernatant rinsing solution, the erythrocytes were stored at -80°C.

At each time point, capillary blood was also obtained in parallel by skin puncture at a fingertip with a lancet, and collected in a graduated heparin-coated capillary tube. Three separate 10-µl drops of capillary blood were applied with the capillary tube onto DBS cards. The cards were allowed to dry for at least 2 hours at room temperature before being sealed in a zip-lock bag with desiccant and stored at -80°C for long term storage (up to 2 months) until analysis. On the first blood sampling time and on the last visit, one drop of blood was used to measure the volunteer's haematocrit using a micro-haematrocrit centrifuge (HAEMATOKRIT 200®, Hettich Zentrifugen, Lauenau, Germany).

DBS samples collection in field conditions

Within the frame of a cross-sectional survey initiated by the Swiss Tropical Institute (Basel, Switzerland) in collaboration with the Ifakara Health Institute (Tanzania), a total of 6485 DBS samples were collected in Tanzania in 2015 for the detection of the 9 antimalarials/metabolites using the present analytical method. During the survey, 4 drops of capillary blood were collected from every consenting individual, aged 3 months to 100 years and sampled as DBS.

4.3.6.A) Sample processing

DBS extraction procedure

Several extraction solutions were tested: MeOH, ACN and MeOH/H_2O mixtures. The influence of pH on extraction yield has also been examined by adding 1% FA or 1% ammonia into the extracting solvent. The extraction solution that provided the best recovery rates and suitable calibration curves for the considered drugs overall was found to be a solution of MeOH with 1% FA.

A 3-mm diameter disk was punched out from the centre of the DBS and transferred into a 1-ml polypropylene tube. A 100-µl aliquot of extraction solution (MeOH + 1% FA) containing the 9 labelled I.S. and the sample was vortex-mixed for 10 seconds prior to incubation into an ultrasonic bath for 10 min without heating (Fischerbrand®, Thermofischer Scientific, United Kingdom). The suspension was centrifuged for 10 min at 14'000 rpm (19'000 *g*) with a

benchtop centrifuge (Mikro 200R, Hettich Zentrifugen, Lauenau, Germany) in order to remove filter paper fibres and other remnant solid materials. A 70-µl aliquot of the supernatant was finally reconstituted in 70 µl of 2 mM ammonium acetate + 0.1% FA buffer into a glass HPLC vial prior the injection of a 20-µl sample into the LC-MS/MS system for the analysis. Vials were maintained at +10°C in the thermostated autosampler rack during the entire analysis.

Lumefantrine measurements in plasma, DBS and erythrocytes

For the comparative study with healthy subjects, concentrations of LF were measured in plasma, erythrocytes and capillary blood spotted onto DBS cards. Capillary blood DBS samples were analysed with the above-method. Plasma samples were analysed using an optimized method previously developed in our lab for the simultaneous determination of 14 antimalarial drugs and their metabolites in human plasma [131], which has been further improved with the use of stable isotopically labelled I.S. The LF concentrations were measured in erythrocytes using an adaptation of the methodology applied to DBS samples analysis. Briefly, after complete thawing, 100 µl of haemolyzed erythrocyte suspension was mixed with 900 µl of phosphate buffered saline solution (pH 7.4) and ground using a Precellys®24 bead mill homogenizer. A 100-µl aliquot of this suspension was extracted with 400 µl of MeOH + 1% FA containing the I.S. The same procedure used for DBS extraction was applied thereafter (i.e. 10 min incubation in an ultrasonic bath, centrifugation, reconstitution (1:1, v/v) of the supernatant with 2 mM ammonium acetate + 0.1% FA before injection of 20-µl into the LC-MS/MS).

4.3.7.A) Analytical method validation

The LC-MS/MS method validation was based on the *FDA* guidelines [148] as well as on the approach of Matuszewski et al. [149] and on the recommendations of Li et al. for quantitative analysis of small molecules in DBS [93]. Comprehensive characterization of the analytical method includes selectivity, matrix effect, extraction recovery, process efficiency, calibration curve, trueness and precision, sensitivity and carry-over as well as drugs stability in the considered biological matrix in various conditions. The influence of punching position, blood spot volume and haematocrit on DBS sampling was also assessed.

Selectivity

To ensure method selectivity, DBS extracts from 6 different blank blood sources were analysed with and without I.S. and screened for potential interfering endogenous matrix components and cross-talk interferences.

Matrix effect, extraction recovery and process efficiency

The matrix effect (ME) was first evaluated qualitatively by a post-column infusion of a neat clear solution containing 1 µg/ml of all the 9 antimalarial drugs and all I.S. in MeOH into the MS/MS detector during the chromatographic analysis of blank DBS extracts from 6 different blood sources. The LC-MS signal of each drug was visually examined in order to identify any perturbation (drift or shift) at the analyte's retention time. Additionally, ME was evaluated quantitatively (as well as its variability) according to Matuszeswky methodology [149]. 3 series of QC samples at L, M and H concentrations were prepared as following:

A) Matrix-free solution of analytes and I.S. in MeOH + 1%FA;
B) Blank DBS extracts samples spiked with antimalarials/metabolites and I.S. *after* extraction;
C) Complete procedure, i.e. antimalarials spiked in whole blood samples that are spotted onto filter paper, dried for 2 hours and subjected to the previously described extraction procedure.

For the series *B* and *C*, each of the 3 concentrations were measured in DBS from whole blood from 6 different sources and serie *A* was measured in triplicate. The 3 series of samples were diluted 1:1 (v/v) in 2 mM ammonium acetate buffer + 0.1% FA prior to their injection onto the column. For the series *A* and *B*, the amount of analytes and I.S. spiked in the MeOH + 1% FA solution and in the blank extracts were calculated in order to obtain the total amount (100%) of drugs theoretically recovered after extraction in series *C*. The volume of whole blood contained a in 3-mm filter paper punched disk was obtained by the rules of three, as the ratio of the mean spot area of a series of 10 µl whole blood applications, versus the calculated ($\pi \cdot r^2$) area of the 3-mm punched disk. The resultant volume was then 2.1 µl, which was similar to the one previously reported in the literature [150].

The MEs were expressed as the ratio of the mean peak areas of analytes added into blank whole blood extracts spiked *after* extraction (*B*) to the mean peak areas of pure analytes solubilised in MeOH + 1%FA (*A*) (*B/A* ratio in %). The extraction recovery (ER) of each antimalarial drug/metabolite and I.S. were measured as the ratio of the mean peak areas of analytes in processed DBS samples (i.e spiked *before* extraction) (*C*) to the mean peak areas of analytes added into blank whole blood extracts spiked *after* extraction (*B*) (*C/B* ratio in %). The process efficiency (PE) of antimalarial drugs/metabolites and I.S. were calculated as the ratio of the mean peak areas of analytes in processed DBS samples (i.e spiked *before* extraction) (*C*) to the mean peak areas of pure analytes solubilised in MeOH + 1%FA (*A*) (*C/A*

ratio in %). Internal standard-normalization of the parameters was assessed by using ratios of analyte peak areas to the corresponding I.S. peak area (i.e. A2, B2 and C2 ratios).

Calibration curve

According to the *FDA* [148], a minimum of 75% of the matrix-based standard calibration samples have to fall within ±15% (LLOQ: ±20%) of the nominal value when back-calculated. In addition, the regression coefficient (R^2) should be at least ≥0.99. An eight levels calibration curve was calculated by quadratic log-log regression of the peak area ratio of the drug to corresponding I.S. versus the concentration of the drug. Different regression models were tested and the quadratic log-log regression was the one with lowest total bias across the whole calibration range, due to the large range of concentrations measured and the large range of areas resulting. The log-log approach has been extensively studied on the antimalarial piperaquine, as reported by Singtoroj, *et al.* [151]. The calibration range was selected to cover concentrations previously reported in pharmacokinetic studies (see Table 4.1.a). Each level of calibration was measured twice, i.e. at the beginning and at the end of the analytical run, with satisfactory concentrations/responses relationships (R^2>0.99).

Trueness and precision

The 3 concentrations of the low (L), medium (M) and high (H) quality control samples were chosen to encompass the calibration ranges and the low (L) QC sample corresponded to 3 times the respective lower limit of quantification (i.e. lower calibration level), according to *FDA* [148]. Trueness and precision were determined by the analysis of 5 different QCs replicates (n=5) during a single run for the intra-assay, and by triplicate analysis of the 3 different QCs replicates repeated on 3 different days (n=9) for the inter-assay. All samples (calibration standards and QCs) were spotted on the filter paper cards on the same day. The intra- and inter-days precision correspond to the coefficient of variation (CV%) within a single run and between different days, respectively and should be ≤ 15%. The trueness was expressed as the bias or percentage of deviation between nominal and back-calculated concentrations. The analytical runs were considered valid if mean bias for QCs were within ±15%.

Sensitivity and carry-over

The lowest levels of the calibration curves (i.e. also the lower limits of quantification) were selected initially to reflect the residual concentrations, based on previously published pharmacokinetics studies measuring plasma exposure. However, the low volume of blood spotted onto the paper card (10 µl) hampers to achieve concentrations as low as those reported for plasma analysis for all compounds. The concentration of the lowest calibration

standard had thus to be increased for achieving a bias that would be comprised within ±20%, in accordance to the *FDA* recommendations [148]. The limit of detection (LOD) was calculated using the instrument detection limit (IDL), which is the minimum amount of analyte required producing a LC-MS signal that is statistically distinguishable from the background noise level within a specified confidence level (99%). This approach is based the following well-established statistical formula: IDL = t × (RSD/100 %) × amount measured, where the confidence factor t is determined using Student t-distribution with a 99 % confidence level and the RSD (relative standard deviation) = standard deviation/mean value. This formula gives more reliable LOD values as it takes into account the signal intensity and its consistency [152]. Carry-over effect was assessed by injecting series of blank DBS extracts just after the highest calibration standard and determining the peak area of each analyte at their respective expected retention time. The residual peak area was expressed as percentage of the absolute peak area of the highest calibration standard.

Stability of antimalarials and their metabolites

The stability experiments included freeze-thaw cycles, bench- and long- term stabilities. To investigate the freeze-thaw cycles stability, whole fresh blood was spiked at L, M and H QCs levels and separated into 3 aliquots. One set of whole blood aliquots was directly employed as DBS, extracted and analysed in triplicate while the 2 others were stored at -80°C and subjected to 1 or 2 freezing-thawing cycles (i.e. kept at -80°C for 2 hours followed by thawing at room temperature for 30 min) before being spotted onto DBS cards. Analytes were considered to be stable when mean measured values did not deviate by more than 15% from the nominal values, with a CV ≤15%. For the bench stability experiments, L, M and H QCs were spotted in triplicate onto the DBS cards. After drying, the DBS cards were placed in a plastic bag with desiccant and either kept at laboratory temperature (24-25°C) or in an incubator at 37°C with 80% humidity for 24 hours, before being processed and analysed using freshly spotted DBS calibrators. Information on medium to long-term stability of DBS samples were obtained by storing series of DBS cards at -20°C and -80°C for 1 month. Concentrations variations were expressed as percentage of the concentration measured at T=0.

Influence of punching position, blood spot volume and haematocrit on DBS sampling

The influence of the actual position of the punched paper disk within the blood spot was examined, as blood cells together with soluble analytes are likely to interact differently with the solid cellulose support of DBS card, leading to chromatography-wise repartition during spot deposit and heterogeneous levels from central to peripheral areas within the same spot (also called "chromatographic" effect) [93]. The importance of such potential intra-spot

heterogeneity was ascertained by the quantitative analysis of three 3-mm diameter disks punched one at the centre, intermediate and outer zone of blood spot of DBS samples containing L, M and H QC concentrations from 6 different sources of blood. These samples were analysed with a standard set of calibration samples rigorously punched at the centre. The chromatographic effect was considered negligible if the intra-spot trueness at L, M and H QC concentration levels lie within the acceptable (≤15%) limits.

We also examined the effect of blood volume variability applied on filter paper on analytical performance: 5-, 10- and 15-µl aliquots of whole blood at L, M and H QC concentrations (spiked in 6 different sources of blood) were spotted onto the filter paper and a standard 3-mm disk was punched out from the centre of all DBS. Results were expressed as the relative difference between concentrations measured with the 10-µl aliquot (used for calibration and considered as the reference volume) and the 5- and 15- µl aliquot volumes.

Haematocrit is known to affect blood rheological properties (i.e. viscosity) [93] and therefore affects blood distribution on the card during application, leading to erratic volume of blood per punch and hence drug concentrations. The effect of the haematocrit on analytical performance was investigated by measuring L, M and H QCs spiked in 6 different sources of blood adjusted with plasma to obtain values at 25, 30, 35, 40, 45 and 50% haematocrit that were analysed with calibrators prepared in blood at standard fixed 40% haematocrit.

4.3.8.A) Statistical analysis

An ordinary linear regression was performed to test for the significance of the haematocrit effect on the concentrations of antimalarials measured, using p-values with significance defined as $p < 0.05$. The correlation between LF levels measured in parallel in DBS and venous plasma was calculated by linear regression using the approach proposed by Bland-Altman [153]. Calculations were done with Microsoft Office Excel (version 2010) and RStudio (version 3.4.0).

4.3.9.A) Ethical approval

The epidemiological study in Tanzania has been approved by three different Ethics Committees (Swiss Ethics Committees on research involving humans, Institutional Review Board of the Ifakara Research Institute and National Institute for Medical Research in Tanzania). The clinical study was approved by the "*Commission cantonale d'éthique de la recherche sur l'être humain*" of Canton de Vaud (CER-VD), Switzerland. Written informed consents were obtained from all volunteers.

4.4. A) Results

4.4.1.A) Selectivity

The chromatographic profiles of the 9 antimalarial drugs extracted from a DBS sample and their corresponding deuterated analogues simultaneously analysed by LC-MS/MS are shown in Figure 4.2.a, whereas analytes retention times and selected MS/MS transitions are reported in Table 4.3.a. No cross-talk interferences were observed, notably between AQ and its metabolite DAQ and their respective I.S., despite the same product ions used. Of note, a minor interfering peak was observed on the LC-MS/MS trace for DAQ at a retention time corresponding to that of Q and Q-D_3 (Figure 4.2.a). Its peak area was constant in all calibration levels, suggesting cross talk interference between DAQ and Q-D_3, due to the identical *m/z* value of their respective precursor ions (328.3 for DAQ and 328.1 for Q-D_3). Yet, thanks to the efficient chromatographic separation, the accurate quantification of DAQ and Q would not be compromised.

No co-eluting peaks could be detected at the corresponding retention time of antimalarials/metabolites, when blank DBS are processed either with or without I.S. In good agreement with *FDA* guidelines, the signal-to-noise ratio at LLOQ was at least 5:1 and 20:1 of the mean response of the I.S. for all analytes at their respective retention times. A chromatogram of a DBS extract containing all analytes at LLOQ superimposed on a blank DBS extract are presented in Figure 4.3.a. The method is therefore sufficiently selective at the selected *m/z* transitions without significant contribution and signal cross-talks due to isotopic composition from I.S. to its respective analyte.

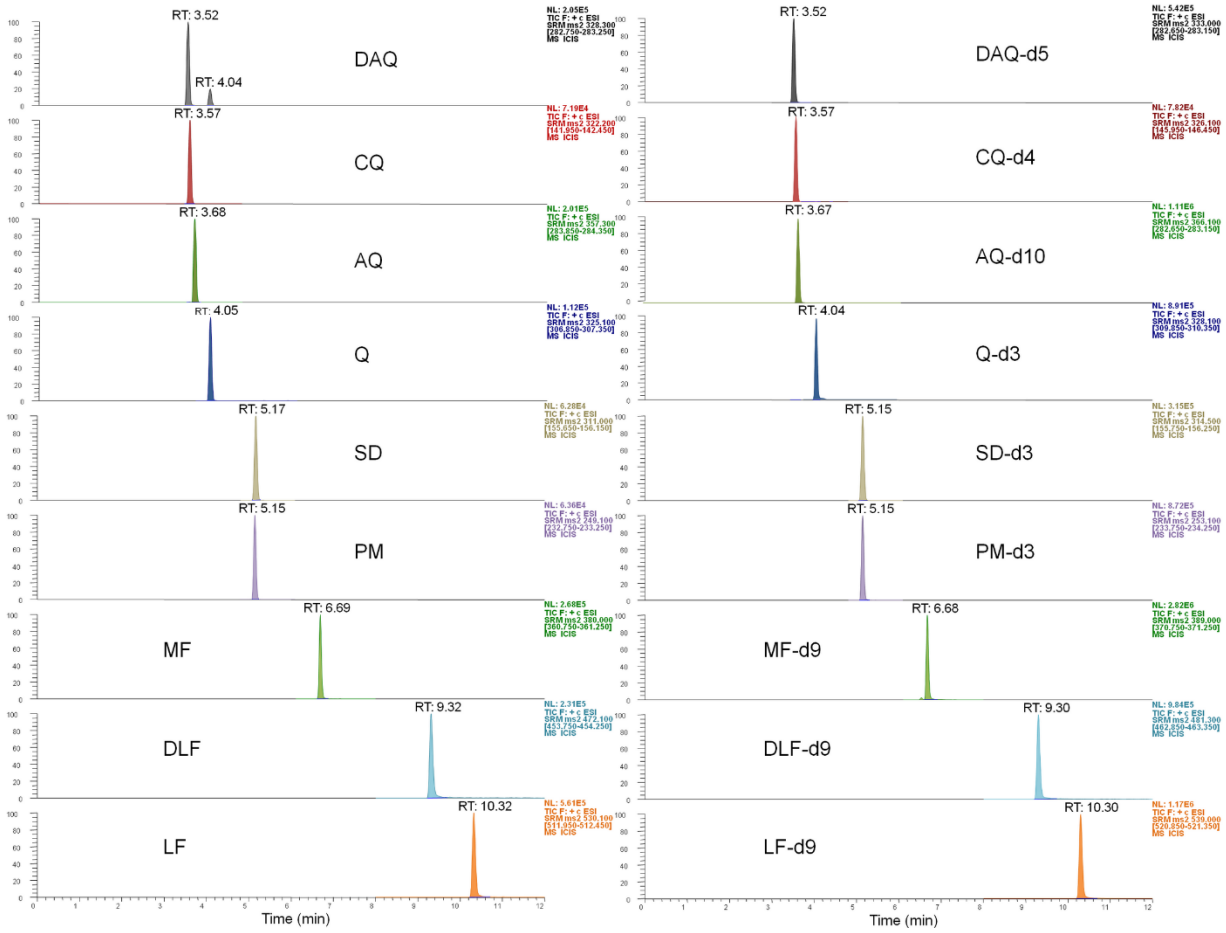

Figure 4-2.a: Example of a LC-MS/MS chromatogram of an extracted DBS calibration standard (one quarter of the highest QC, see Table 4.1.a) containing each antimalarial drug and their respective internal standards at the chosen concentrations (see section 4.3.3.A). Details of the LC gradient program and MS/MS detection are reported in Table 4.2.a and 4.3.a, respectively.

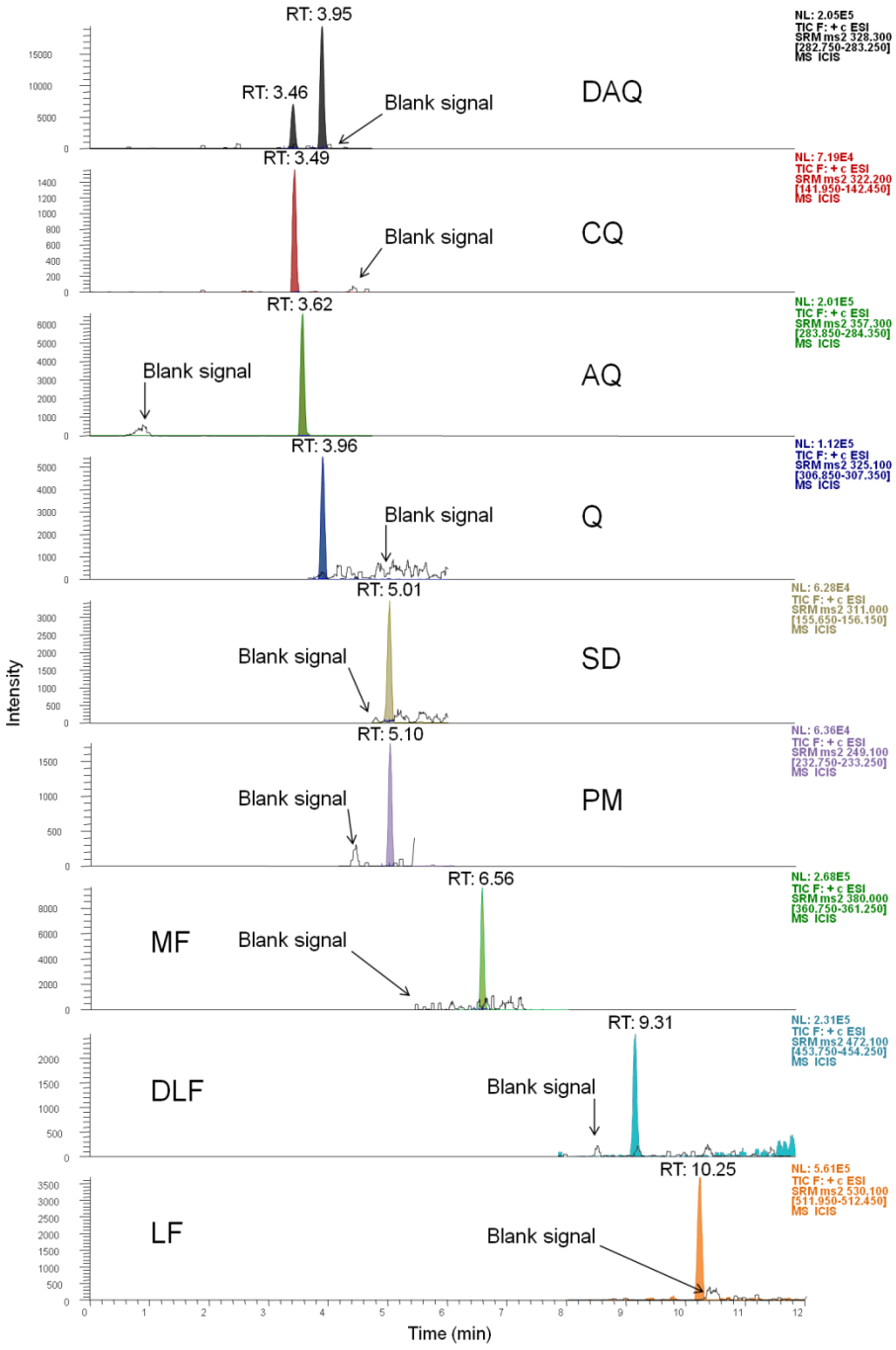

Figure 4-3.a: LC-MS/MS chromatogram of a DBS extract containing all analytes at LLOQ superimposed on a blank DBS extract. The concentrations of each analytes at LLOQ are the following: DAQ=6ng/ml; CQ=20 ng/ml; AQ=15 ng/ml; Q=20 ng/ml; SD=20 ng/ml; PM=2 ng/ml; MF=20 ng/ml; DLF=20 ng/ml; LF=20 ng/ml.

4.4.2.A) Matrix effect, extraction recovery and process efficiency

Due to the complex nature of whole blood matrix and the generic sample preparation employed, most attention focused to characterize and control the matrix effects (MEs). Indeed, a number of endogenous components from whole blood (e.g. proteins, phospholipids, glucides or salts) potentially co-eluting with target analytes are likely to influence the

ionization efficiency of the compounds and consequently their signal intensities. The MEs were first examined qualitatively (Figure 4.4.a). No significant matrix effects (drift or shift of the signals) were noticeable for all antimalarials, with limited variabilities, as all traces at the retention time of analytes were superimposable. Exception were observed for Q and CQ, and to a lesser extent LF and DLF, whereby the signal intensity of one out of the 6 different blank extracts differ, underscoring the uselfulness of isotopically labelled I.S. The good chromatographic retention developed for antimalarial drugs was necessary to avoid massive signal suppression coming from early polar components contained in whole blood matrix. Conversely, lipophilic endogenous compounds – most probably phospholipids – that were eluted between 8 and 9 min appeared to perturb DLF and LF signals. An enhancement of the signal was also observed just after the retention times of these two compounds but due to the reproducibility of this effect over the 6 DBS extracts batches, no impact on quantitative measurements should be expected. Indeed, associated co-eluting I.S. will be affected in the same way and peak area ratios of the analytes to I.S. will correct for the matrix effect.

The quantitative ME, extraction recovery (ER) and process efficiency (PE) are reported in Table 4.4.a. Regarding ME, values above or below 100% indicate an ionization enhancement or suppression, respectively, due to the co-eluting matrix components. An important positive matrix effect for CQ and AQ was observed, whereas a negative matrix effect for Q, SD, MF, DLF and LF was assessed, especially at lower concentrations. Most importantly however, the I.S.-normalization for all compounds (B2/A2 ratio in %) was comprised between 94.3% and 118.1% indicating a successful correction of DBS-related matrix effect.

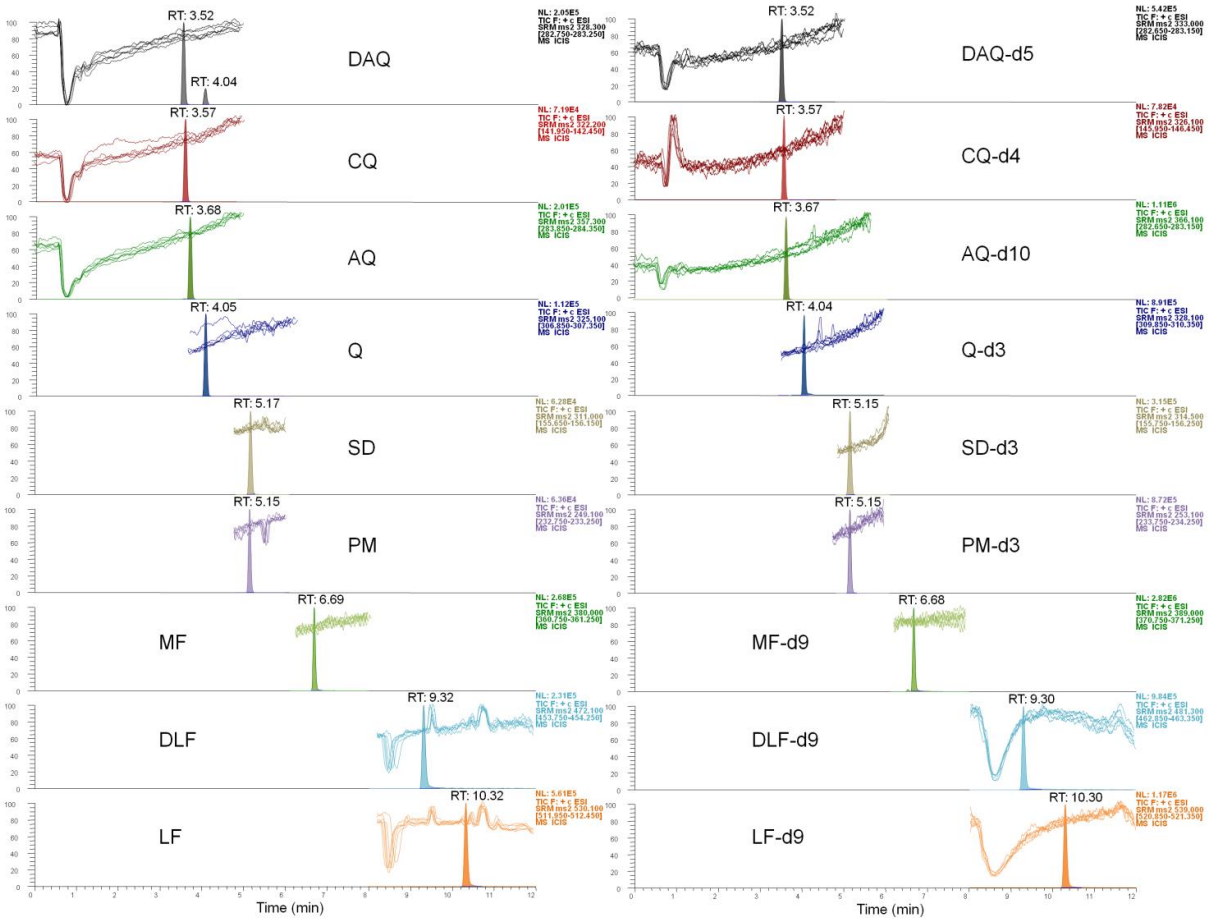

Figure 4-4.a: Qualitative evaluation of the matrix effect. LC-MS/MS chromatograms of 6 blanks extracts obtained during post-column infusion of a matrix-free (MeOH) solution containing 7 antimalarial drugs and 2 metabolites (each at 1µg/ml) and their respective I.S.

Table 4-4.a: Matrix effect (ME), extraction recovery (ER) and process efficiency (PE) assessment for antimalarials and metabolites.

Compound	$C_{Nominal}$ (ng/ml)	Mean peak area			Mean peak-area ratio			ME (%)	CV (%)	ME (%)	Extraction ER %	CV (%)	Analysis ER (%)	CV (%)	PE %	CV (%)
		A* (n=6)	B (n=6)	C (n=6)	A2*	B2	C2	B/A		B2/A2	C/B		C2/B2		C/A	
DAQ	18	82’625	90’505	28’978	0.062	0.068	0.025	109.5	5.9	110.0	32.0	13.4	37.3	12.8	35.1	13.8
	120	542’363	532’958	206’896	0.384	0.412	0.163	98.3	3.7	107.3	38.8	6.2	39.6	10.7	38.1	7.5
	1200	7378’557	6’613’487	3’214’987	3.604	4.128	1.922	89.6	7.7	114.5	48.6	12.7	46.6	4.1	43.6	10.0
CQ	60	5’432	12’463	14’362	0.068	0.080	0.087	229.4	6.6	118.1	115.2	18.8	108.7	12.9	264.4	15.6
	400	39’161	89’572	109’004	0.481	0.495	0.564	228.7	5.5	102.9	121.7	4.9	114.0	14.0	278.3	5.9
	4000	534’410	1’097’129	1’318’823	4.538	5.067	5.614	205.3	6.7	111.6	120.2	10.3	110.8	14.4	246.8	5.9
AQ	45	120’824	154’284	61’106	0.027	0.028	0.012	127.7	6.4	104.4	39.6	10.8	43.1	7.7	50.6	8.9
	300	733’633	1’033’774	458’737	0.151	0.176	0.079	140.9	4.6	116.8	44.4	8.9	44.9	10.7	62.5	11.3
	3000	9’392’344	11’013’241	5’819’910	1.516	1.749	0.874	117.3	6.6	115.4	52.8	11.3	49.9	5.0	62.0	8.0
Q	60	69’428	33’572	29’192	0.032	0.030	0.031	48.4	11.3	94.5	87.0	22.4	104.4	13.5	42.0	14.2
	400	450’461	213’563	230’789	0.185	0.192	0.207	47.4	7.7	103.6	108.1	11.1	107.9	9.5	51.2	8.3
	4000	5’866’036	2’634’814	3’030’599	1.661	1.870	2.016	44.9	6.5	112.6	115.0	14.6	107.8	9.5	51.7	10.0
PM	60	90’255	81’396	82’330	0.017	0.016	0.017	90.2	9.5	97.0	101.1	11.3	107.2	8.4	91.2	6.0
	400	407’602	392’467	435’589	0.093	0.103	0.111	96.3	10.4	110.1	111.0	12.0	108.1	10.7	106.9	10.7
	4000	4’416’292	3’999’055	4’616’256	0.869	1.002	1.089	90.6	6.0	115.3	115.4	14.4	108.7	8.6	104.5	9.1
SD	6	50’925	26’791	18’069	0.033	0.033	0.030	52.6	12.0	99.5	67.4	14.0	89.9	16.7	35.5	7.6
	40	334’718	140’774	136’743	0.190	0.203	0.200	42.1	10.2	107.3	97.1	7.7	98.4	14.6	40.9	6.6
	400	3’426’490	1’342’894	1’354’144	1.751	2.013	1.916	39.2	4.2	115.0	100.8	8.2	95.2	15.2	39.5	4.7
MF	60	13’023	8’574	7’953	0.007	0.007	0.008	65.8	13.4	96.9	92.8	14.8	117.4	13.6	61.1	11.4
	400	94’897	55’572	65’364	0.044	0.046	0.053	58.6	9.1	104.7	117.6	15.6	115.1	15.7	68.9	14.6
	4000	829’761	451’452	592’871	0.422	0.456	0.523	54.4	4.4	108.0	131.3	13.9	114.6	13.4	71.5	15.3
DLF	60	173’997	104’097	60’693	0.072	0.068	0.053	59.8	14.0	94.3	58.3	7.7	77.6	10.7	34.9	7.7
	400	1’115’653	475’867	490’227	0.412	0.409	0.363	42.7	12.6	99.4	103.0	16.3	88.7	7.5	43.9	7.7
	4000	14’962’544	6’069’955	7’562’842	3.929	4.172	4.053	40.6	10.8	106.2	124.6	12.4	97.2	7.2	50.5	5.7
LF	60	599’395	471’095	298’327	0.061	0.057	0.040	78.6	11.6	94.3	63.3	11.0	69.6	11.4	49.8	6.7
	400	4’685’718	3’042’437	2’759’016	0.357	0.364	0.269	64.9	8.4	101.8	90.7	8.9	73.8	19.4	58.9	4.1
	4000	33’605’099	18’124’918	22’392’317	3.651	3.671	3.404	53.9	12.1	100.6	123.5	12.8	92.7	10.5	66.6	11.9

*A = Matrix-free solution of analytes and I.S. in MeOH + 1%FA. A2 = internal standard-normalization using ratios of analyte peak areas to the corresponding I.S. peak area B = Blank DBS extracts samples spiked with antimalarials/metabolites and I.S. *after* extraction. B2 = internal standard-normalization using ratios of analyte peak areas to the corresponding I.S. peak area C = Complete procedure. C2 = internal standard-normalization using ratios of analyte peak areas to the corresponding I.S. peak area

The ER *(C/B ratio)* was comprised between 32.0% and 131.3% and was always decreased for low QC samples.

The PE *(C/A ratio)* takes into account the extraction recovery and the matrix effect. The PE was especially high for CQ mostly due to the contribution of high matrix effect.

More generally, this is not the matrix effect *per se* that constitutes an analytical issue, but rather its variability. Overall, the observed variability (CV%) for the ME, ER and PE were rather limited with only few values above the recommended ±15%. Thus, both the use of isotopically-labelled I.S. and whole blood matrix-matched calibration samples were found to correct for most of these matrix effects, which overall would only limitedly affect calibration, trueness and precision.

4.4.3.A) Calibration curve, trueness and precision

Over the chosen ranges of concentrations (reported in supplementary Table 4.1.a), an excellent relationship with measured analyte/I.S. peak area ratios was observed by applying quadratic log-log regression. With this model, a regression coefficient (R^2) greater than 0.99 was always obtained and the back-calculated concentration of calibration samples were within the ±15% (±20% at LLOQ) margins.

As shown in Table 4.5.a, the intra-assay precision for all antimalarial drugs and metabolites for all QC levels was within ±14.3% and inter-assay dispersion was ≤15.0% overall. Globally, the intra-assay trueness ranged from -12.1 to +11.1%, whereas from -5.2 to 9.9% for inter-assay measurements.

Table 4-5.a: Intra- and inter-assay trueness (bias%) and precision (CV%) for the analysis of 9 antimalarials and 2 metabolites in DBS calculated for QC samples at low (L), medium (M) and high (H) concentrations.

Compound	$QC_{Nominal}$ (ng/ml)	Intra-assay (*n=5*)		Inter-assay (*n=9*)	
	L M H	$C_{Measured}$ (ng/ml)	Trueness bias (%) (CV (%))	$C_{Measured}$ (ng/ml)	Trueness bias (%) (CV (%))
N-desethyl-amodiaquine	18	18.0	0.1 (10.4)	18.1	0.5 (10.6)
	120	118.5	-1.2 ±(5.2)	124.6	3.9 (11.3)
	1200	1326.4	10.5 (3.6)	1229.2	2.4 (10.2)
Chloroquine	60	58.8	-2.0 (10.6)	57.3	-4.6 (11.3)
	400	389.7	-2.6 (5.8)	416.8	4.2 (11.7)
	4000	4227.4	5.7 (2.3)	4200.6	5.0 (5.4)
Amodiaquine	45	43.4	-3.8 (12.6)	49.0	8.9 (14.4)
	300	304.4	1.5 (8.7)	311.9	4.0 (9.4)
	3000	3334.1	11.1 (3.2)	3117.4	3.9 (10.8)
Quinine	60	59.7	-0.5 (4.7)	62.7	4.5 (9.11)
	400	396.2	-0.9 (2.6)	414.4	3.6 (8.0)
	4000	4103.0	2.6 (2.4)	4056.6	1.4 (5.9)
Sulfadoxine	60	61.2	1.9 (6.7)	63.6	6.1 (15.0)
	400	390.0	-2.5 (5.3)	432.7	8.2 (13.0)
	4000	4122.3	3.1 (2.0)	4097.5	2.4 (6.3)
Pyrimethamine	6	5.5	-9.6 (7.7)	5.7	-5.2 (13.6)
	40	38.6	-3.5 (5.5)	41.8	4.5 (13.4)
	400	400.9	0.2 (1.4)	401.0	0.3 (4.2)
Mefloquine	60	61.6	2.6 (14.3)	65.9	9.9 (14.9)
	400	419.4	4.8 (8.2)	428.8	7.2 (10.5)
	4000	4023.6	0.6 (5.5)	4012.8	0.3 (8.0)
Desbutyl-lumefantrine	60	57.5	-4.3 (3.3)	61.4	2.3 (6.0)
	400	407.7	1.9 (4.9)	413.9	3.5 (5.9)
	4000	4140.7	3.5 (2.9)	4058.2	1.5 (5.0)
Lumefantrine	60	53.5	-12.1 (6.1)	58.3	-2.9 (9.2)
	400	389.7	-2.6 (5.2)	392.7	-1.8 (5.1)
	4000	3878.4	-3.0 (2.1)	3869.9	-3.2 (3.2)

4.4.4.A) Sensitivity and carry-over

The LLOQs correspond to the concentrations of the lower calibration standard, given in Table 4.1.a. The LLOQ of PM, DAQ and AQ were 2, 6 and 15 ng/ml, respectively, and 20 ng/ml for all other antimalarials. The LOD (calculated according to the IDL and the injection volume) were the lowest for DAQ and PM at 1 ng/ml, followed by AQ, Q and CQ at 2, 5 and 7 ng/mL respectively. The LOD was 8 ng/ml for LF, DLF and SD, and 12 ng/ml for MQ. The carry-over

produced by the highest calibration sample on the following injected blank DBS extract was found non negligible for DLF and LF, even with a prolonged rinsing step, with residual signals corresponding to a carry-over of 0.64% and 0.35% of the highest calibration level, respectively. This 0.64% carry-over for DLF corresponds to a signal higher than the LLOQ signals peak areas. In the subsequent injection of blank extracts, residual DLF and LF signals decreased down to 0.15% and 0.11%, respectively, of the peak areas of highest calibration level. For this reason, at least two blank samples should be programmed after the highest calibration sample prior to the subjects' samples analysis series.

4.4.5.A) Stability of antimalarials/metabolites in whole blood and in DBS

The stability of the antimalarials/metabolites in whole blood subjected to freeze-thaw cycles was found to be satisfactory in whole blood calibration samples. After 2 freezing-thawing cycles, the mean concentrations did not deviate by more than ±15% from the nominal values for the L, M and H QCs, indicating that when repetitive analyses of samples are considered, the spiked haemolysed whole blood can be frozen and thawed at least twice (data not shown).

Results of the short-term stability experiments for antimalarials in DBS samples (assessed to reflect field trial conditions whereby DBS frequently cannot be stored at negative temperatures) are given in Table 4.6.a. For the L, M and H QC levels, the stability was satisfactory with variations up to 24h not exceeding ±15.0% of the initial concentrations, except for SD (-17.6% at L QC). These results indicate that, taking into account the analytical variability, analytes can be considered sufficiently stable for at least 24h at room temperature. A slight decrease in concentrations was nevertheless noticed overall and for this reason, DBS samples should not allowed be left at ambient temperature for more than 24h. Variations of antimalarials concentrations in DBS stored at -20°C and -80°C (Table 4.6.a) were mostly comprised within the acceptable limits (±15.0%) and compounds stabilities could be hence considered satisfactory after 1 month, except for AQ , its metabolite DAQ and SD. Of interest, storage of DBS cards at -80°C instead of -20° appeared to limit the sample degradation.

Table 4-6.a: Stability of antimalarials and metabolites in DBS stored for 24h at room temperature (RT) and 37°C and for 1 month at -20°C and -80°C. Variations of QC concentrations (at L, M and H levels) are expressed as percentage of the concentration measured at *T*=0.

	24 hours					
	RT			37°C		
	QC			QC		
	L	M	H	L	M	H
	Variation (%) from *T=0*			Variation (%) from *T=0*		
N-desethyl-amodiaquine	-1.5	-10.2	9.0	-6.6	8.2	5.5
Chloroquine	10.9	4.6	12.2	-3.6	7.6	1.5
Amodiaquine	-8.6	0.1	11.4	-15.4	5.6	0.1
Quinine	7.6	3.1	13.1	-16.3	10.0	4.7
Sulfadoxine	17.6	1.5	10.1	14.8	11.6	9.5
Pyrimethamine	12.0	0.3	10.7	3.7	6.8	5.7
Mefloquine	4.6	2.8	16.7	-7.3	13.7	7.7
Desbutyl-lumefantrine	0.0	-5.4	12.8	11.3	10.9	12.1
Lumefantrine	4.7	-0.9	5.6	6.7	8.6	3.9
	1 month					
	-20°C			-80°C		
	QC			QC		
	L	M	H	L	M	H
	Variation (%) from *T=0*			Variation (%) from *T=0*		
N-desethyl-amodiaquine	-27.5	-5.0	-18.7	-27.8	-13.5	-13.7
Chloroquine	10.0	9.0	12.0	9.8	5.5	-0.5
Amodiaquine	-24.4	-12.4	-10.6	-3.6	-4.7	-6.0
Quinine	15.2	-12.1	8.9	7.4	9.8	12.8
Sulfadoxine	-21.7	-19.6	-18.8	-9.6	-19.4	-8.8
Pyrimethamine	2.7	-3.7	-8.4	3.8	0.7	-1.4
Mefloquine	8.5	-7.4	4.9	1.2	8.9	16.5
Desbutyl-lumefantrine	14.8	6.3	-9.1	-0.6	-5.9	3.2
Lumefantrine	-13.7	6.4	7.7	-6.4	5.8	13.8

4.4.6.A) Influence of punching location, blood spot volume and haematocrit on DBS analysis

Overall, the bias between concentrations (mean of 6 values) measured in the paper disk punched out from the centre, intermediate, and outer zone of DBS did not exceed ±15.0 % with the exceptions of DAQ (-17.9%), AQ (-17.6%) and MF (17.5%) at low QC level and LF (-21.4%) at high QC level, when measured in the border of blood spot. Hence, for minimizing analytical variability, it is recommended to rigorously punch out the paper disk in the centre of DBS.

The volume of blood spotted onto the DBS cards impacted the measured concentrations. For each analyte, the concentrations measured with 5 µl were systematically lower, with a bias ranging from -3.6% (low QC for CQ) to -24.6% (medium QC for LF). Conversely, the 15-µl blood spots resulted in higher concentrations with a bias ranging from +0.5% (high QC for Q) to +35.7% (low QC for CQ).

As reported in Table 4.7.a, the measured concentrations of all analytes increased significantly with increasing haematocrit (*p<0.05* for all QCs, except for H QC of DAQ, M QC of AQ and MF and L QC of DLF). The concentrations of all antimalarials/metabolites deviated by more than ±15% across the entire considered haematocrit range, using 40% as reference haematocrit.

Table 4-7.a: Influence of haematocrit values on the measured DBS concentrations. Differences are expressed as the bias (%) between the DBS concentrations measured with each haematocrit value and those measured with a 40% haematocrit, used as reference. *P-values* were measured by linear regression.

Compound	QC level	Haematocrit value											p-value*
		40% (reference)	25%		30%		35%		45%		50%		
		$C_{Measured}$ (ng/ml)	$C_{Measured}$ (ng/ml)	Bias (%)	$C_{Measured}$ (ng/ml)	Bias (%)	$C_{Measured}$ (ng/ml)	Bias (%)	$C_{Measured}$ (ng/ml)	Bias (%)	$C_{Measured}$ (ng/ml)	Bias (%)	
DAQ	L	29.7	21.3	-28.3	23.6	-20.6	25.7	-13.3	32.7	10.3	33.4	12.6	<0.001
	M	193.7	132.4	-31.7	149.2	-23.0	198.7	2.6	188.7	-2.6	195.7	1.1	0.049
	H	1576.0	1127.6	-28.4	1425.2	-9.6	1258.1	-20.2	1511.9	-4.1	1558.0	-1.1	0.052
CQ	L	73.8	57.8	-21.7	54.5	-26.1	59.5	-19.4	74.3	0.8	81.5	10.5	0.007
	M	407.6	357.8	-12.2	308.5	-24.3	344.8	-15.4	412.1	1.1	458.1	12.4	0.027
	H	7276.6	6315.6	-13.2	6219.1	-14.5	7484.5	2.9	7777.7	6.9	8297.2	14.0	0.006
AQ	L	47.5	41.8	-12.0	35.5	-25.3	35.8	-24.7	50.3	5.8	54.1	13.7	0.049
	M	293.3	271.2	-7.5	236.8	-19.3	223.0	-24.0	310.1	5.7	342.4	16.8	0.076
	H	5304.0	4630.4	-12.7	4455.6	-16.0	5059.1	-4.6	5569.7	5.0	5710.4	7.7	0.003
Q	L	66.0	53.1	-19.6	51.9	-21.4	56.1	-15.1	69.9	5.9	75.2	13.9	0.002
	M	420.8	362.5	-13.8	308.2	-26.8	357.7	-15.0	436.0	3.6	480.8	14.3	0.019
	H	7345.4	6209.7	-15.5	6104.4	-16.9	7306.5	-0.5	7664.4	4.3	8242.4	12.2	0.004
PM	L	6.6	5.9	-11.1	5.6	-14.8	6.7	1.5	7.1	7.6	7.9	19.2	0.006
	M	42.1	32.4	-23.0	35.0	-16.9	41.1	-2.2	43.7	3.8	43.7	3.9	0.007
	H	414.5	321.4	-22.5	365.7	-11.8	351.5	-15.2	434.9	4.9	439.1	6.0	0.004
SD	L	66.0	56.8	-13.9	54.9	-16.8	59.9	-9.3	68.6	4.0	80.5	22.0	0.006
	M	434.2	387.9	-10.7	322.2	-25.8	363.3	-16.3	440.3	1.4	471.1	8.5	0.050
	H	7348.7	6225.6	-15.3	6281.2	-14.5	7097.1	-3.4	7540.4	2.6	8056.9	9.6	<0.001
MF	L	68.9	59.7	-13.4	55.7	-19.1	66.5	-3.5	71.7	4.0	74.1	7.4	0.009
	M	428.6	387.3	-9.6	321.3	-25.0	357.7	-16.5	419.6	-2.1	465.4	8.6	0.069
	H	7425.4	6587.3	-11.3	6520.0	-12.2	7375.8	-0.7	7449.7	0.3	8027.5	8.1	0.007
DLF	L	77.2	71.4	-7.5	69.9	-9.5	77.3	0.1	91.3	18.3	78.5	1.6	0.120
	M	532.3	454.0	-14.7	481.0	-9.6	541.1	1.7	578.0	8.6	543.0	2.0	0.030
	H	4692.2	3840.5	-18.2	4230.2	-9.8	4214.9	-10.2	4909.1	4.6	4782.1	1.9	0.006
LF	L	94.4	86.1	-8.8	85.8	-9.2	91.2	-3.4	105.9	12.1	97.5	3.2	0.034
	M	551.6	462.1	-16.2	494.3	-10.4	553.2	0.3	582.3	5.6	547.7	-0.7	0.042
	H	5117.8	3942.7	-23.0	4401.0	-14.0	4505.8	-12.0	5229.5	2.2	5221.0	2.0	0.003

4.4.7.A) Lumefantrine measurements in plasma, DBS and erythrocytes in healthy volunteers

The relationship between concentrations of LF measured in DBS and in plasma was examined at different time-points after the administration of a single oral adult dose of artemether-lumefantrine. As shown in Figure 4.5.a, LF concentrations measured in plasma and in DBS over all time points were highly correlated (R=0.97). This excellent correlation was especially found for concentrations below 600 ng/ml (close-up insert in Figure 4.5.a), which were measured in the latest samples (i.e. taken up to 72 to 336 hours after single drug dose intake). In the Bland-Altman analysis plot (Figure 4.6.a), the geometric mean ratios of LF concentration in DBS versus plasma was 0.57 (95% Confidence Interval: 0.55-0.59), which was also supported by the linear regression slope (Figure 4.5.a). The percentage ratios were generally consistent across concentrations and had an acceptable variation.

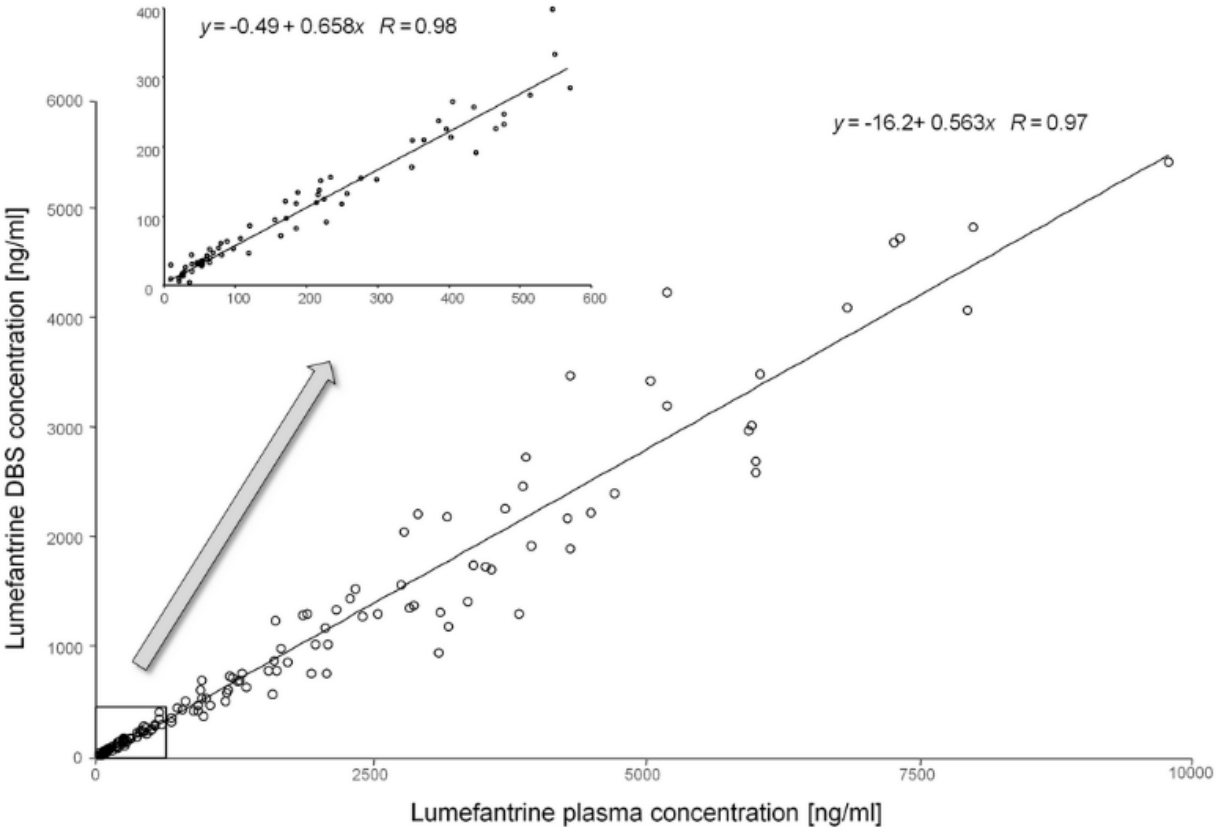

Figure 4-5.a: Relationship between total plasma and DBS concentrations of LF simultaneously measured in healthy volunteers over 14 days after drug intake. A zoom on the lower concentration range (0-600 ng/ml in plasma) is reported.

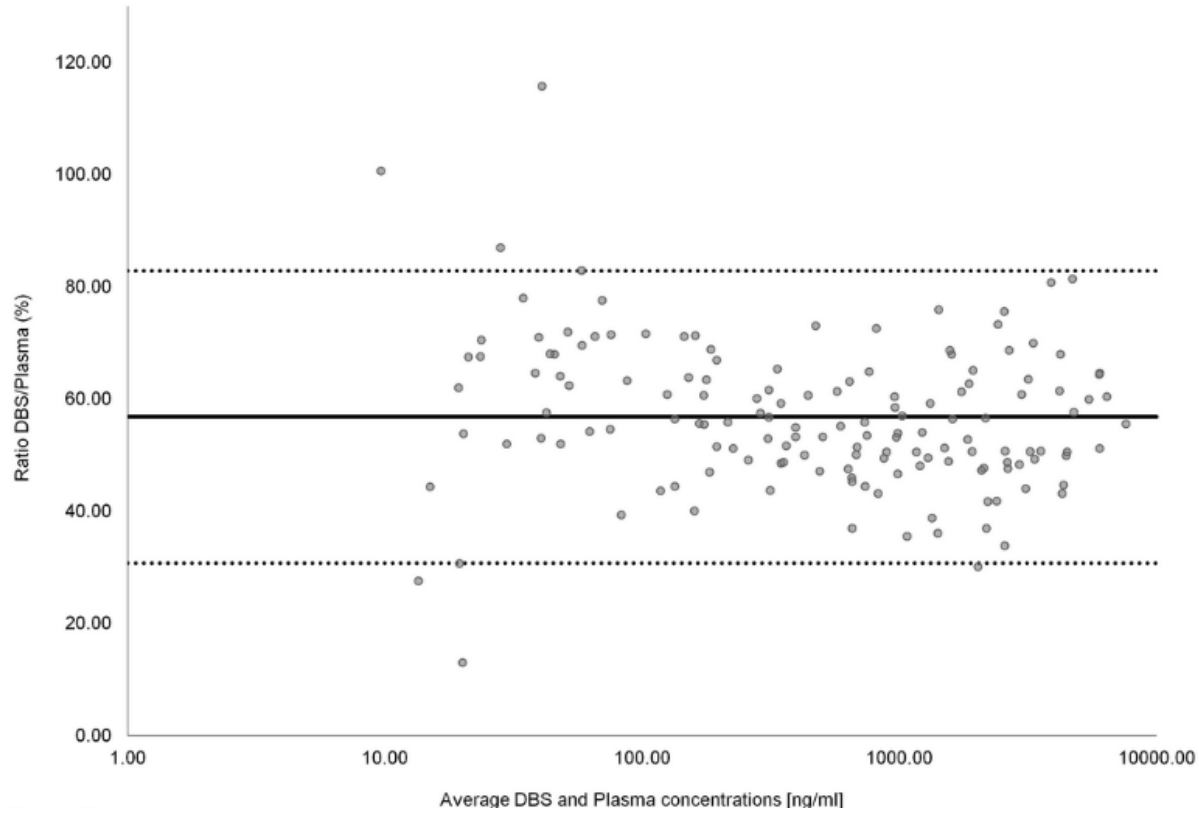

Figure 4-6.a: Bland-Altman plot for total plasma and DBS concentrations of LF on a logarithmic scale, simultaneously measured in healthy volunteers over 14 days after drug intake. The solid line represents the mean ratio, while the dotted lines the 95% CI of agreement (mean ratio ± 1.96 x SD).

The concentrations of LF measured in erythrocytes were less well correlated either with DBS (R=0.88) and with plasma (R=0.87) (data not shown). We found that the washing procedure significantly impacted LF concentrations results in red blood cells, which corresponded to 3.6% and 9.0% of plasma levels, respectively, when erythrocytes were subjected or not to the NaCl 0.9% washing step. Figure 4.7.a shows the chromatographic profiles of LF and DLF determined in DBS and plasma samples collected from one healthy volunteer who participated in the clinical pharmacokinetic study in Switzerland 24 hours after receiving a single adult dose of artemether-lumefantrine (Riamet®). Measured concentrations in DBS were 1161 ng/ml and 50 ng/ml, for LF and DLF, respectively (Figure 4.7.a, left), and 2059 ng/ml and 10 ng/ml for LF and DLF, respectively, in plasma (Figure 4.7.a, right). Of note, because only LF and DLF were measured in this pharmacokinetic study, the chromatographic program has been adapted to speed-up the chromatographic separation step (7 min instead of 12).

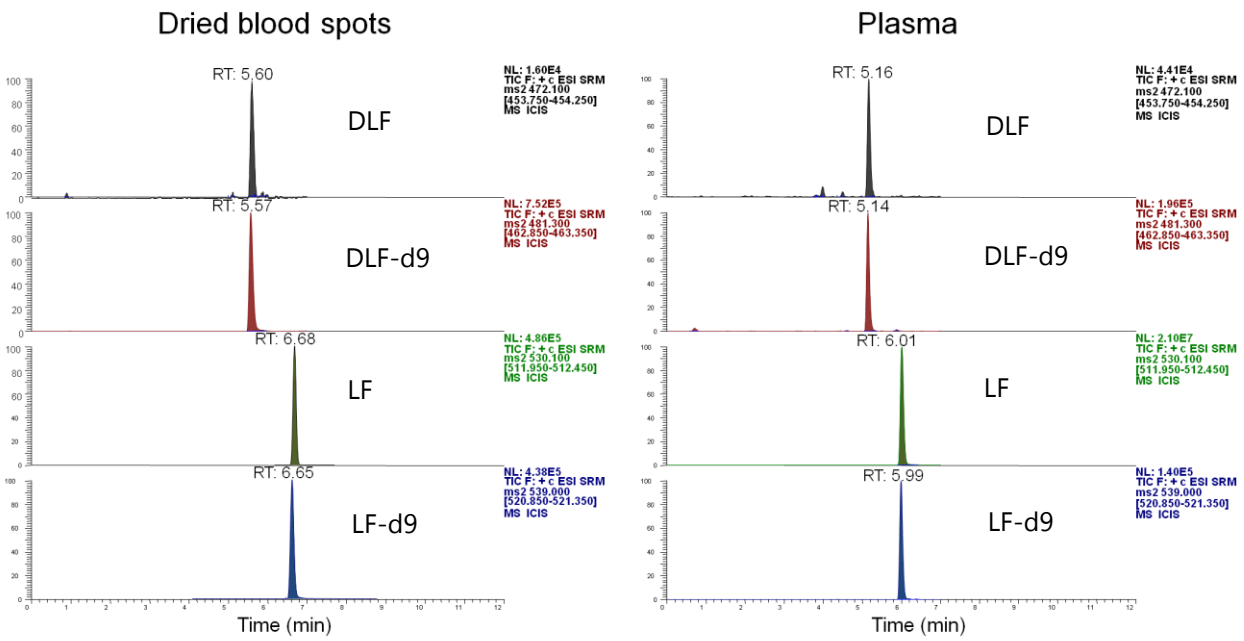

Figure 4-7.a: LC-MS/MS chromatogram of a DBS sample (left) and the corresponding –associated - plasma sample (right) from a volunteer receiving artemether and lumefantrine (as Riamet®; details in the manuscript).

4.4.8.A) Epidemiological applications

This analytical method was applied in a cross-sectional survey for measuring residual antimalarial drugs concentrations in 6485 DBS samples collected in a field trial in Tanzania. Only concentrations higher than LLOQ were considered positive for the epidemiological analysis. Figure 4.8.a shows representative examples of chromatograms from positive DBS samples extracts collected during the field study (through household surveys) in which PM, SD, DLF, LF and DAQ, CQ, AQ, Q, PM, SD, MF were detected. The full results and data analysis of the epidemiological study will be reported elsewhere (CHAPTER V, VI).

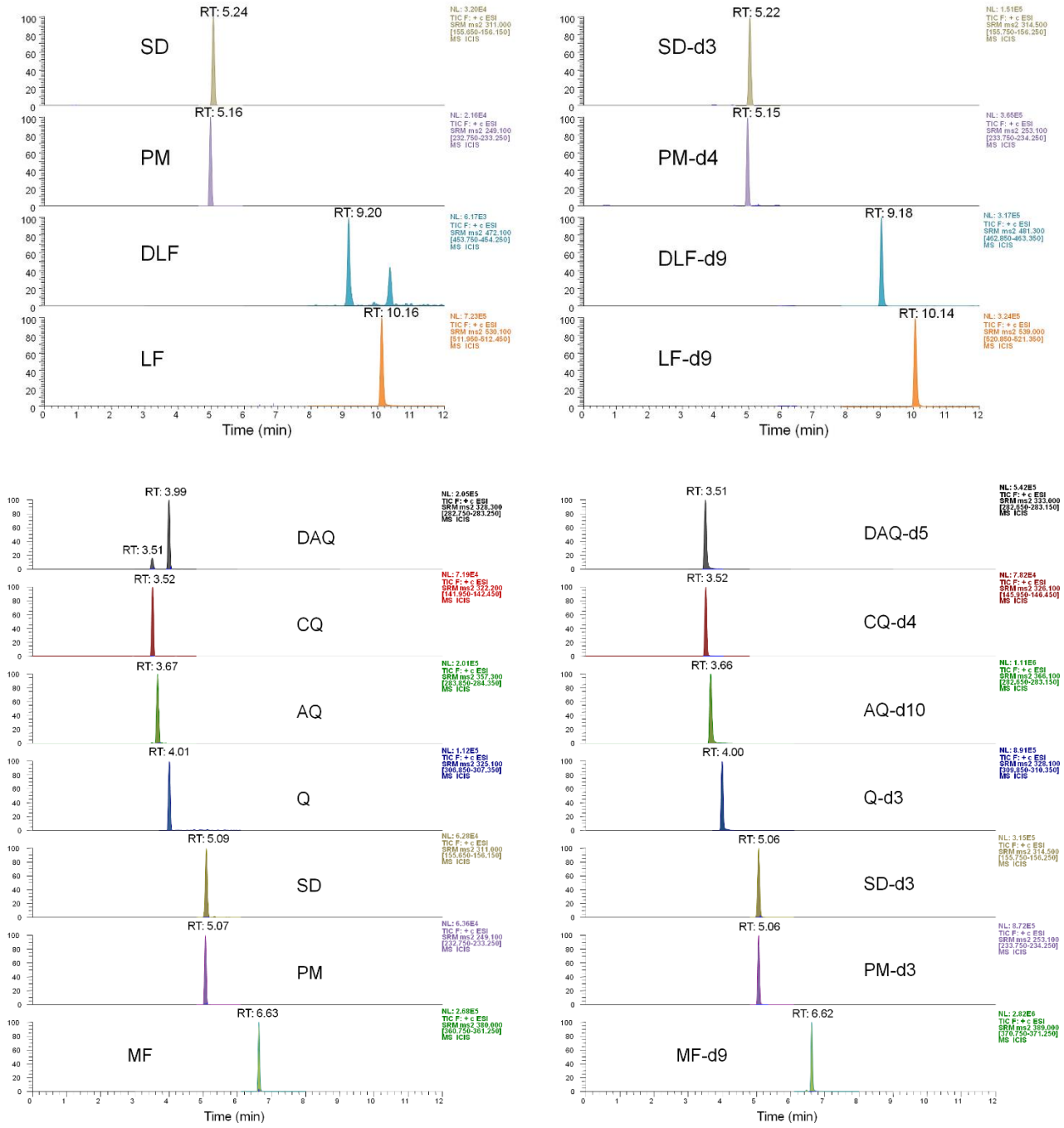

Figure 4-8.a: LC-MS/MS chromatogram of a processed DBS sample collected during a field trial in Tanzania containing A) SD (3812 ng/ml), PM (22 ng/ml), DLF (23 ng/ml) and LF (1560 ng/ml) (left) and their corresponding internal standards (right) and B) DAQ (7 ng/ml), CQ (32 ng/ml), AQ (18 ng/ml), Q (40 ng/ml), SD (43 ng/ml), PM (4 ng/ml) and MF (35 ng/ml) (left) and their corresponding internal standards (right).

4.5. A) Discussion & conclusion

We developed a sensitive LC-MS/MS assay for the simultaneous analysis of 7 antimalarials and 2 active metabolites in DBS. Thanks to the excellent chromatographic separation, to the high selectivity of tandem mass spectrometry detection and to the availability of stable isotopically-labelled I.S., this analytical method offered the convenience of a single extraction procedure from a 3-mm diameter disk paper card with MeOH + 1% FA. To our knowledge, this is the first method that considers the multiplex analysis of 9 antimalarials/metabolites simultaneously in DBS with the required selectivity, trueness and precision.

Overall, both precision and trueness of the lowest calibration standard (corresponding to the LLOQ) were within the ±20% limit recommended by the *FDA* for all antimalarials. These LLOQ values were generally higher than those reached in plasma [131], but still remained lower than those from the single previously published LC-MS/MS method employing ion-trap MS detection for multiple antimalarials in DBS [113]. The proposed assay was found suitable for the analysis of residual antimalarials in DBS samples collected in a large-scale cross-sectional survey in Tanzania. The high sensitivity of the method was a key element for the assessment of residual antimalarial drug exposure at a population level in the context of epidemiological studies. Indeed, very low residual concentrations are encountered in a large number of samples, which could be analysed without the need of tedious sample preparation.

The good stability of antimalarials in whole blood after 2 freeze-thaw cycles indicates that haemolysed whole blood is suitable for preparation and storage of calibration curves, even though some authors have recommended to use fresh whole blood for the preparation of matrix-matched calibration samples, not always available on demand [93,127,154]. Short and long term stability assays indicated that DBS samples can be kept at room temperature for up to 24h and should be transferred without delay at -80°C, where they can be stored for a prolonged time prior to analysis.

In contrast with previous examples reported in the literature [155], the matrix effect in our hands appeared to depend on the matrix-to-analyte concentration ratio, explaining why the signal enhancement found for CQ and AQ was especially important at low concentrations. The recovery appeared to be lower than previously reported for plasma [131] with a similar extraction method. Irreversible adsorption of analytes onto the solid filter paper support might be possibly incriminated [156], as the recovery assessment in method validation implied the addition of antimalarials into *liquid* blank DBS extracts (i.e. spiked *after* DBS extraction). Yet, the present recovery rates were satisfactory given the large number of drugs extracted simultaneously and the convenience of the process. Adsorption phenomenon onto the filter

paper appears to be a saturable process as it especially affects low QC levels while having less impact on high concentration QCs. The repeatability of extraction recoveries between all QC levels was improved after I.S.-normalization. Of note, extraction recoveries for CQ, Q, PM, MF, DLF and LF were found in some instances to exceed 100%. A potential explanation could derive from calculations of nominal drugs concentrations in solutions *B,* which were based on the extrapolated volume of whole blood contained in the 3-mm filter paper punched disk, assuming that blood and analytes were evenly distributed within the 10-µl DBS. However, as shown in section 3.7, there was a heterogeneous drug distribution within the blood spot, and the concentrations spiked after extraction might therefore have been underestimated. It must be acknowledged that since the I.S. were not spotted onto the DBS card with the blood sample but were only contained in the extracting organic solvent, they did not confidently reflect *every* step of DBS solid state extraction of target analytes from paper cards. Yet, their use remains key element to normalize matrix effect variability that potentially detrimentally affects performance analysis of particularly complex biological extracts.

The DBS sampling technique offers many advantages including facilitated logistics especially suitable for field trials. However, its advantages must be weighed against the degree of potential uncertainty associated with DBS sampling methods [157]. In our assay, the concentrations in DBS were shown to be influenced by the volume of blood spotted onto DBS cards and the so-called "chromatographic" effect occurring after blood spot deposition. In fact, the studied compounds appeared to interact with the solid DBS card material during the transfer of the drop of whole blood. These results underscore the need of precise sample pipetting when collecting blood as DBS for accurate measurements. For the sake of standardisation, it is therefore recommended to punch paper disk rigorously at the same location within DBS (ideally, the centre). While those parameters can be controlled during blood collection and analysis, the impact of haematocrit on drug concentrations, although apparently more trivial, is not a factor that can be easily normalized during field trials. Haematocrit normally ranges from 41-51% for men and 37-47% for women [158]. Yet particular attention should be paid to individuals suffering from malaria, as they are likely to have lower haematocrit values, due to significant red blood cells lysis after protozoan multiplication. For example, an emblematic symptoms of malaria is anaemia with haematocrit <30% [159]. Consequently, blood viscosity and hence the rate and extent of diffusion of blood onto the filter paper after blood drop deposition can be affected. This in turn would mean that smaller amounts of blood per punched disk will be available for analysis, leading to spuriously low measured drug concentrations. Ideally, haematocrit value should be measured and recorded for each enrolled subject. For more accurate measurements, the haematocrit value of DBS calibration samples should be standardized according to study population.

Moreover, the haematocrit effect could be circumvented by analysing the entire DBS, as long as a defined and reproducible volume of blood is spotted on the filter paper card [92].

A critical part of the method optimisation was the reduction of carry-over, a current issue when analysing basic lipophilic compounds such as DLF and LF because of their tendency to get strongly adsorbed onto various components of the LC system (i.e. injection valve and syringe) as well as onto the reverse-phase chromatography packing of the column. The efficiency of the washing program of the injection syringe was hence finally improved by adding 1% of FA into the two different washing solutions (MeOH/H_2O 1:1 + 1% FA and ACN + 1% FA). The carry-over was mainly reduced by programming a prolonged rinsing step of the LC-MS system with 1 min of ACN (1000 µl/min) followed by 2 min of isopropanol (500 µl/min) and again 1 min of ACN (1000 µl/min) at the end of each analysis. However, the level of carry-over currently remains an important limitation and it implies that future samples following highly concentrated samples should be systematically re-analysed if concentration of DLF show higher than the LLOQ in blank analysis. Alternatively, when the analysis of DBS samples collected over an entire dosing interval (i.e. for PK studies) will be considered, it will be advised to program the analytical sequence in reverse chronological order (i.e. by injecting the late -low levels- samples first) for overcoming carry-over issue. The long analytical time – at the current UHPLC (ultra-high performance liquid chromatography) era – due to the prolonged but necessary rinsing step represents another limitation of the present method.

A comparison between simultaneous plasma and DBS concentrations is recommended as part of the assay method validation, and a good correlation between both measurements is required before giving trust to the quantitative analysis [93]. An excellent correlation was actually found between LF concentrations measured in DBS and those determined in parallel in plasma samples. However, the concentrations measured in both matrices were not identical (i.e the slope was not equal to 1.0). Our observations indicate that LF is predominantly distributed in the plasma, as LF concentrations measured in separated red blood cells pellets were remarkably low, while differing significantly (i.e. 3.6% *vs* 9.0% of plasma levels) depending on whether erythrocytes were subjected or not to a washing procedure with NaCl 0.9% (to remove interstitial remnant plasma). These results were in line with a prior *in vitro* study using radio-labelled tracers, in which the fraction of LF in erythrocytes was around 10% of plasma using the erythrocyte partitioning method (no wash of erythrocytes) [160,161]. We found previously that the volume of residual plasma trapped between red blood cells after a single centrifugation corresponds to 11.2 ± 2.7% of the total volume of the erythrocytes [162]. Thus, the concentration of LF measured in erythrocytes collected without washing in our healthy subjects was elevated essentially due to LF emanating from residual plasma present in the

erythrocytes layer. In our study, the mean haematocrit value for healthy volunteers was 41% (i.e. a 10-µl whole blood spotted onto DBS card would correspond to 4.1 µl of erythrocytes and 5.9 µl of plasma), while LF levels in DBS were about 0.6 times lower than those measured in plasma, confirming that plasma contributes for essentially all the LF measured in DBS. Our results are thus in good accordance with the formula suggested by Eyles *et al.* for correlating analytes concentrations between plasma and DBS [163]. This suggests that the fraction of LF that reaches its site of action – expected to occur mostly inside cells wherein the drug inhibits the intraerythrocytic development of the Plasmodium [87,88] – is remarkably low but yet sufficient to inhibit parasites growth. Of note, LF circulates in two forms in plasma: a fraction bound (99.7%) to plasma proteins and the remainder unbound (free). Total plasma LF levels have been determined in our study, while only the unbound LF fraction is likely to diffuse into tissues equilibrate with cells to exert its pharmacological activity. Our in vivo study focused on LF because it is the most widely antimalarial used in combination with artemisinin derivates in Tanzania. A literature review presented all analytical strategies to determine antimalarials in bio fluids [164] but none of the studies included in this article compared plasma and DBS concentrations. One [127] showed a good correlation between Q concentrations measured in DBS and in whole venous blood and one [116] showed a good correlation between AQ and DAQ concentrations measured in whole venous blood and whole venous blood spotted onto filter paper cards. Another older study showed that MF concentrations in serum where significantly higher than in whole blood with a mean ratio of 1.28 and a good correlation between both type of measurements [165].

This sensitive LC-MS/MS method, enabling the simultaneous analysis of 7 antimalarial drugs and 2 active metabolites in DBS is suitable for measuring drug exposure in a population. Furthermore, to the best of our knowledge, no PK/PD studies have yet been realized using LF concentrations measured in DBS. The present study provides therefore useful information to perform an indirect extrapolation based on DBS/venous LF levels comparison. A recently published meta-analysis from Study Group of the WorldWide Antimalarial Resistance Network (WWARN) showed that the principal determinant of treatment outcome is the plasma concentration of LF at day 7, with plasma values >200 ng/ml being associated with >98% cure rates in most uncomplicated malaria patients [38]. Lower concentrations are predictive of treatment failure. Moreover, at such sub-therapeutic levels, the parasite's susceptibility to LF decreases, therefore offering favourable conditions for the development of resistance. When tested *in vitro*, 99% of *P. falciparum* isolates from Tanzania were inhibited at 60 ng/ml and 100% at 159 ng/ml [166]. This additional level of complexity brought by protein binding has also to be addressed when considering general comparisons between *in vivo* PK and *in vitro* sensitivity tests results.

In conclusion, knowledge of the excellent correlation and of the ratio between plasma and DBS levels of LF is of importance for field trials aiming at assessing actual circulating concentrations of this antimalarial at a population-wide scale, and their potential relationship with the emergence of parasite resistance.

Acknowledgements

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4. B) Lumefantrine pharmacokinetics using plasma and dried blood spots: a head-to-head comparison in healthy volunteers

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This manuscript is a working paper

4.1. B) Introduction

The current sampling standard for drug measurement is blood withdrawn by venous puncture, that needs to be centrifuged prior to plasma collection and storage generally in the frozen state. In comparison, the use of the dried blood spots (DBS) sampling strategy can make some aspects of field trials conditions more convenient, for example easier sampling technique, lighter transportation, and cheaper storage of samples. A recently published meta-analysis including 21 studies on the pharmacokinetics (PK) and pharmacodynamic relationships of artemether-lumefantrine, showed that the principal determinant of treatment outcome is the plasma concentration of lumefantrine (LF) at day 7, where values >200 ng/ml have been associated with >98% cure rates in most uncomplicated malaria patients [167]. Conversely, lower concentrations are predictive of treatment failures and through the exposition to such sub-therapeutic concentrations for a prolonged period, parasite's susceptibility to LF decreases opening the way to resistance development. In CHAPTER IVa, a good correlation, albeit not identity, was observed between LF concentrations measured in plasma and in DBS. These results called for more detailed characterization of the PK profile of LF in DBS in order to explore the impact of DBS sampling on LF PK parameters. This will help assessing to what extent this sampling method could be used as a good surrogate to plasma measurements in efficacy studies or in the frame of population PK of antimalarials in field studies.

4.2. B) Materials and methods

The present investigation was a prospective open-label one-group interventional study. The clinical study was approved by the "Commission cantonale d'éthique de la recherche sur l'être humain" of Canton de Vaud (CER-VD), Switzerland. A total of 16 healthy male and female subjects aged 18-45 years and with a body mass index (BMI) within 18-29 kg/m2 range were included in the study. Detailed inclusion and exclusion criteria are detailed in Appendix 1. Written informed consent was obtained from all subjects before enrolment in the study. All the participants received a single adult dose of artemether-lumefantrine [Riamet® (Novartis Pharma Schweiz AG)], corresponding to 4 tablets of 20 mg artemether and 120 mg LF. Capillary and venous blood sampling was made in parallel at 11 time points including the blood collection at time 0 and up to hour 336 (14 days) after drug intake. Venous blood was collected by venous puncture in 4.9 ml tubes containing citrate as anticoagulant. The tube was centrifuged at 2000g for 10 min at 4°C and the collected plasma kept at -80°C. Capillary blood was obtained by fingerprick and collected into a graduated heparin-coated capillary tube. Three 15 µl drops of capillary blood were applied next to each other onto a DBS card which were allowed to dry at least 2 hours at room temperature before being stored at -80°C. LF concentrations in plasma and in DBS were measured within the next month using a liquid

chromatography coupled to tandem mass spectrometry (LC-MS/MS) assay described in CHAPTER IVa.

The PK analysis of LF in plasma and DBS was performed separately using a nonlinear mixed-effects modelling approach (NONMEM®, version 7.4.1). This approach allows to estimate population means and variances of the PK parameters and to identify factors influencing them [168]. Neither of the previously published models was found to be suitable to depict our data as they either used a one-compartment model, included children or pregnant women with malaria, or were not sufficiently described [168–174]. One- and two-compartment PK structural models were tested. The half-life and AUC of LF were calculated using the final model parameters estimates. We then simulated 1) predicted LF concentrations in DBS and in plasma over 336 hours for 1000 individuals after a single dose intake and 2) predicted LF concentrations in DBS over 1080 hours (45 days) for 1000 individuals after a full artemether-lumefantrine course (6 doses at hours 0, 8, 24, 36, 48 and 60). RStudio (version 3.4.0) was used to output descriptive PK analysis and plots. Covariate analysis, including testing body weight, hematocrit and plasma protein binding, will be performed at a later stage.

4.3. B) Results and discussion

In total, 8 males and 8 females aged from 24 to 36 years were included in our study. The two-compartment model was chosen as it described most adequately the data [170,171]. The final parameters estimated by the model were systemic apparent clearance (CL/F), inter-compartmental apparent clearance (Q/F), apparent central volume of distribution (Vc/F), apparent peripheral volume of distribution (Vp/F) and apparent absorption rate constant (ka). One volunteer out of the 16 was excluded from the present analysis due to outlying apparent central volume of distribution (Vc/F). At this step of the analysis, we decided to construct the model without this individual as the difference in central volume of distribution could be explained by the influence of a covariate which hindered the fit of our structural model. As shown in Figure 4.1.b, DBS and plasma half-life of LF at individual level (for each of the 15 healthy volunteers) showed a strong correlation (R=0.97) *and a* good agreement between LF half-life in plasma and in DBS *(slope of the regression=0.98)*.

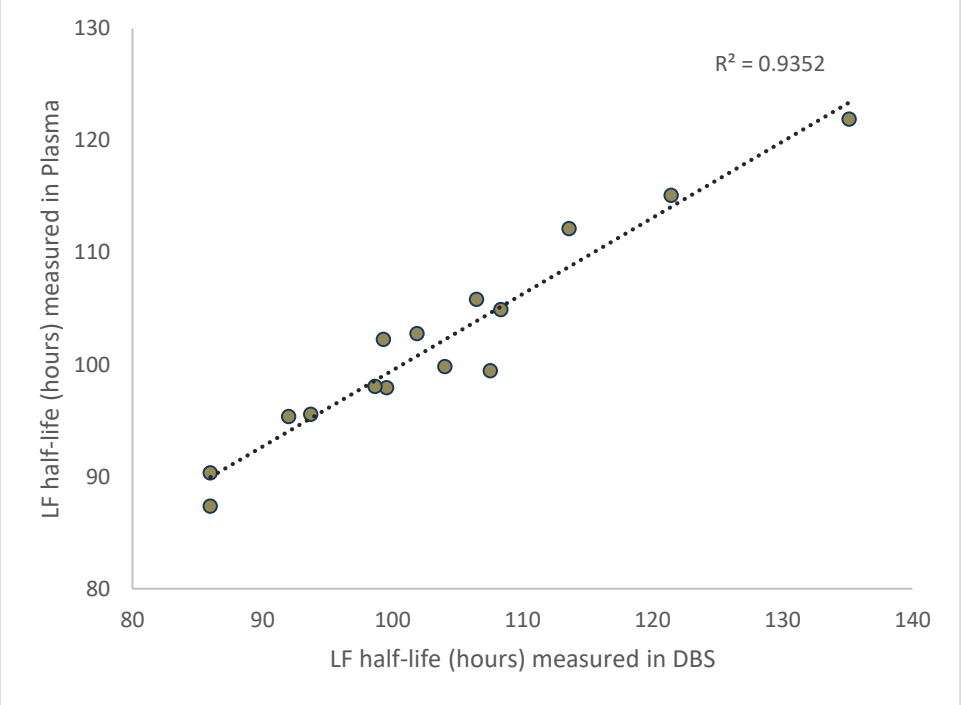

Figure 4-1.b: Relationship between LF half-life in DBS and in plasma calculated at individual level (in 16 heathy volunteers) using parameters estimated with NONMEM®.

The average Cmax (maximum concentration), AUC (area under the curve) and half-life for LF in DBS and in plasma as well as a simulated PK profile after a single adult dose intake are presented in Table 4.1.b and Figure 4.2.b, respectively. Cmax measured in plasma (4111.2 ng/mL, 95% CI: 3858.8 - 4310.9) was higher than that measured in DBS (2416.0 ng/mL, 95% CI: 2230.6 - 2556.1) but half-life was equivalent [101.9 hours (97.1-106.7) when measured in plasma versus 103.6 hours (96.7-110.4) in DBS]. These values are similar to those obtained from plasma in the literature [171,172,174], although this study was conducted with healthy volunteers. As a result, because the difference between DBS and plasma PK profiles is due to higher apparent concentrations in plasma but are eliminated at the same rate, AUC obtained from plasma measurement was higher. The DBS/plasma AUC ratio of 0.57 was very close to the blood/plasma ratio of 0.56 obtained using linear regression and Bland-Altman approach (CHAPTER IVa), indicating that our model fitted well our data. This ratio is expected since the apparent difference in PK parameters between DBS and plasma measurements is due to the predominant distribution of LF in the plasma. Erythrocytes occupy 41% (mean haematocrit value for healthy volunteers) of the volume in the blood compartment explaining why LF concentrations in DBS were about 0.6 times lower than those measured in plasma. This also implies that LF elimination rate is not affected by this phenomenon.

Table 4-1.b: Summary of LF pharmacokinetic parameters measured in DBS and in plasma

Dose	Measured in DBS			Measured in Plasma			AUC DBS:Plasma ratio
	C_{max} (ng/mL)	Half-life (hours)	AUC (mg*h/L)	C_{max} (ng/mL)	Half-life (hours)	AUC (mg*h/L)	
Single dose of LF 480 mg	2416.0 (2230.6 - 2556.1)	103.6 (96.7-110.4)	65.6 (55.6 - 75.7)	4111.2 (3858.8 - 4310.9)	101.9 (97.1-106.7)	114.5 (99.5-129.4)	0.57 (0.55-0.59)

Figure 4.2.b illustrates well these parameters, with a higher Cmax for LF measured in plasma and parallel terminal concentrations on a log-scale, indicating the similar half-life of LF obtained using the two sampling methods. This figure also demonstrates the long terminal half-life of LF due to the elimination of the drug from the peripheral compartment. Due to the highest sensitivity of the measurements in plasma (resulting in a lower limit of detection), the concentrations of LF will be detectable for a longer period in plasma than in DBS.

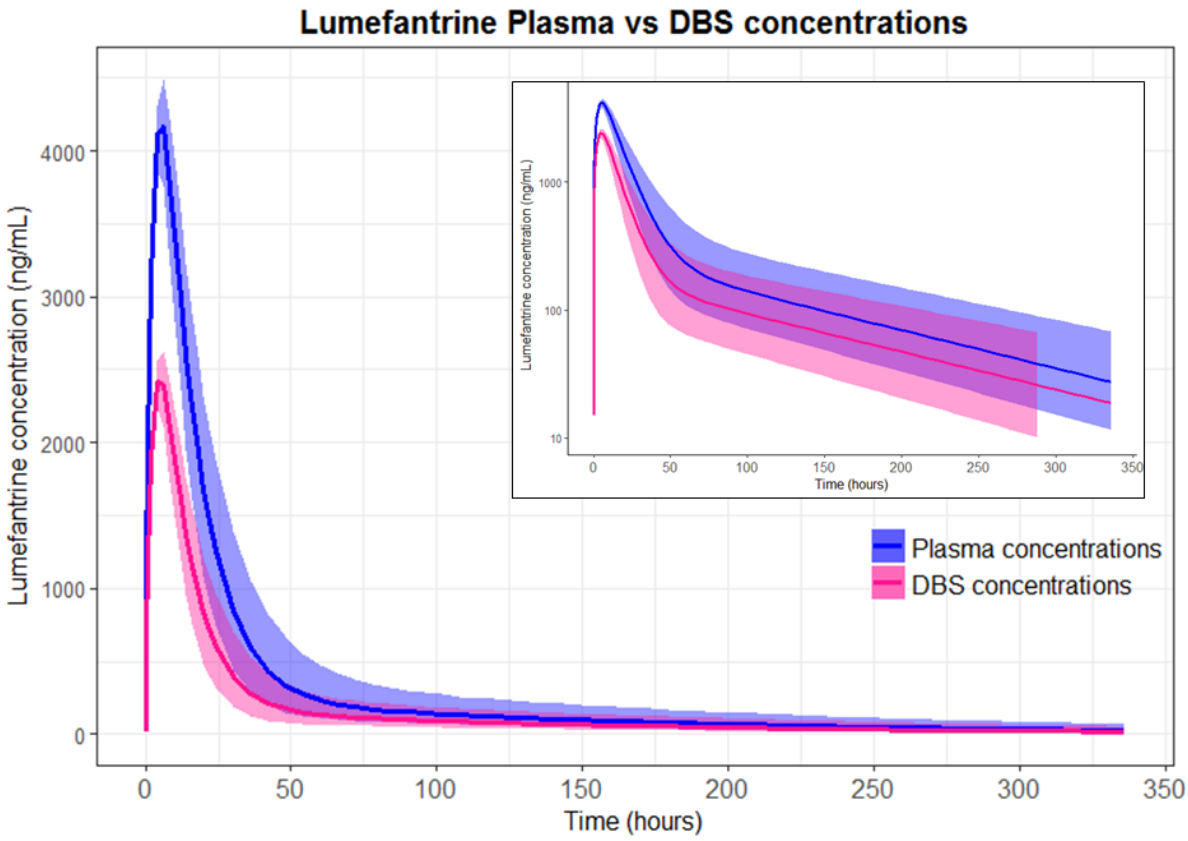

Figure 4-2.b: Estimated pharmacokinetic profiles of LF in DBS and in plasma after administration of a single adult dose of artemether-lumefantrine (480 mg of LF). The solid lines represent the median population prediction and the transparent margin represent the 95% prediction interval. The insert represent the same profiles on a log-scale.

In order to assess the day on which LF levels in DBS would become undetectable after a full artemether-lumefantrine course, predicted LF concentrations were simulated for 1000 individuals using the parameters obtained with the two-compartment model over 1080 hours (45 days). The median time point on which LF became undetectable for the 1000 individuals was 624 hours (26 days) and ranged from 360 hours (15 days) to 1056 hours (44 days). These estimations show the important inter-individual variability in LF concentrations [168] and that residual levels of LF can persist in the blood for a considerable amount of days therefore offering favorable conditions for the development of parasite resistance. At 720 hours (30 days), 23% of the individuals had concentrations above the analytical method's limit of quantification (LOQ) of 20 ng/mL (CHAPTER IVa). This is a valuable information as it indicates that a one-month record is a valid estimate to compare history of drug intake and drug measurements in epidemiological studies (although usually a two-weeks recall period is used for history of fever).

4.4. B) Conclusion

The current work validates the use of DBS to measure LF concentrations in field trials conditions. This study shows the feasibility of PK models and therapeutic drug monitoring using DBS. This is of importance given the fact that these fields have a wide potential to be explored, especially for infectious and parasitic diseases in developing countries. A refined PK analysis will be performed at a later stage to describe LF distribution in blood compartments using plasma proteins binding, hematocrit, erythrocyte and whole blood concentrations, aimed at improving our understanding of LF distribution in the blood compartments.

5. CHAPTER V: APPROPRIATENESS OF MALARIA DIAGNOSIS AND TREATMENT

5. Appropriateness of malaria diagnosis and treatment for fever episodes according to patient history and antimalarial blood measurement: a cross-sectional survey from Tanzania

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5.1. Abstract

Background

Monitoring the impact of case management strategies at large scale is essential to evaluate the public health benefit they confer. The use of methodologies relying on objective and standardized endpoints such as drug levels in the blood should be encouraged. We evaluated population drug use, diagnosis and treatment appropriateness in case of fever according to patient history and antimalarials blood concentration.

Methods

A cross-sectional survey took place between May and August 2015 in three regions of Tanzania with different levels of malaria endemicity. Interviews were conducted and blood samples were collected by dried blood spots through household surveys for further antimalarial measurements. Appropriate testing when individuals attended care was defined as a patient with history of fever being tested for malaria and appropriate treatment as i) having antimalarial in the blood if the test result was positive ii) having antimalarial in the blood if the person was not tested, and iii) no antimalarial in the blood when the test result was negative.

Results

Amongst 6391 participants included in the antimalarial analysis, 20.8% (1330/6391) had antimalarial drug detected in the blood. Only 28.0% (372/1330) of the individuals with antimalarials in their blood reported the use of antimalarials within the previous month. Amongst all participants, 16.0% (1021/6391) reported having had a fever in the previous two weeks and 37.5% of them (383/1021) had detectable levels of antimalarials in the blood. Of the individuals who sought care in health facilities, 69.4% (172/248) were tested and 52.0% (129/248) appropriately treated. When other providers were sought, 6% (23/382) of the persons were appropriately tested and 44.2% (169/382) appropriately treated. Overall, the proportion of individuals treated was larger than that being tested [47.3% (298/630) treated, 31.0% (195/630) tested].

Conclusion

Our study showed high prevalence of circulating antimalarial drug in the sampled population. Efforts should be made to increase mRDT use at all levels of health care and improve compliance to test result in order to target febrile patients that are sick with malaria and reduce drug pressure. Objective measurements collected at community level represent a

reliable tool to evaluate overall impact of case management strategies on population drug pressure.

5.2. Introduction

The implementation of artemisinin-based combination therapies (ACTs) and malaria rapid diagnostic tests (mRDTs) has been a cornerstone in the management of fever cases. These tools are essential components of the current global malaria control strategy [1,8,175]. In Tanzania, a good coverage of preventive and curative interventions, including the distribution of insecticide-treated nets and the adoption of ACTs as first-line therapy, has led to a decrease in the number of malaria cases of more than 75% between 2000 and 2015 [6]. Considerable efforts have been made to expand access to effective antimalarials in the public and private sector. In 2014, Tanzania has reported that sufficient ACTs had been distributed across the country to treat all patients attending public health facilities [20]. Malaria rapid diagnostic tests (mRDTs) have also been deployed to reach half of the population so far, and health workers have been trained in using them [6,103]. Recent surveys have reported that availability of malaria testing was 83% in the public sector where more than 70% of the suspected cases were tested [6,176]. This was not the case in the private sector where there was only 16% of testing available [176]. Diagnosis availability and compliance to diagnosis results are major factors to reach rational use of treatments. Although the Tanzania National Malaria Control Programme (NMCP) case management policies, recommended by the World Health Organization (WHO) requires parasitological confirmation of malaria prior to treatment for patients of all ages, there are concerns that many patients with malaria do not receive ACTs while others suffering from non-malaria fever do. This indicates that treatments are not always targeted to those in need [1,103,175]. While under-treatment needs to be addressed with improved access to drugs, over-treatment due to non-availability of malaria tests or lack of expertise of clinicians in the management of non-malaria fevers has becomes a concern increasingly important, especially with global decrease in proportion of febrile illnesses due to malaria [24,32]. Incorrect malaria prescriptions result in wastage of medication, delays in obtaining effective treatment for the true cause of illness, important drug pressure in the population [19] and hence emergence of parasite resistance to drugs [11].

Monitoring case management strategies and evaluating their impact are important activities to ensure that they confer the foreseen individual and public health benefit they are supposed to. To that end, interviews targeting caretakers of small children in community based cross-sectional surveys as well as data collection in health facilities or in the retail sector have been used to estimate levels of access to good quality drugs and the impact of mRDTs use on drug prescription [20,41,42,103,177]. These studies inherently suffer from potential biases, such as recall bias and inaccurate reporting due to fear to be judged or fear of not being appropriately cared for [47]. They are also more likely to detect a positive effect since they are usually

conducted in places where intense training and supervision have been undertaken, or are biased due to the Hawthorne effect [48]. Two studies conducted in Tanzania and in Cambodia showed that self-reported history is not reliable in terms of actual drug use. Indeed, 75% of patients presenting in a health facility in Tanzania and 50% in Cambodia had detectable concentrations of antimalarials in the blood, although all stated that they did not take any drug in the previous month [49,50]. Besides recourse to public health facilities, febrile patients often seek care in the private sector, and especially so among drug retailers that are usually prohibited to sell and perform mRDT testing which is the case in Tanzania, except for registered accredited drug dispensing outlets (ADDOs) [19,20,32,178,179]. As a result, a considerable amount of patients are prescribed antimalarial treatments presumptively. For all these reasons, the overall impact of the implementation of mRDTs and ACTs is difficult to assess precisely. There is a need to apply more rigorous and reliable methodologies to evaluate the appropriateness of case management for fever episodes at large scale. The aim of this study was to use antimalarials blood levels as an objective and standardized endpoint to evaluate population drug use, to compare these results with self-reported history, and to assess diagnosis and treatment appropriateness in case of fever episode at population level.

5.3. Materials and methods

5.3.1.Study design

This cross-sectional survey included three types of surveys conducted concurrently in randomly selected wards (which is the smallest administrative area and includes five to seven villages): 1) household-based surveys, 2) drug outlet-based surveys and 3) exit interviews in health facility-based surveys.

5.3.2.Study areas and population

The surveys took place in 2015, after the rainy season in three regions of Tanzania: Mtwara, Mwanza and Mbeya with populations of 1 270 854, 2 772 509 and 2 707 410 respectively [104]. The IMPACT2 project [20], whose main objective was to assess the impact of the Affordable Medicines Facility-malaria (AMFm) initiative on the supply and demand of ACTs, served as a basis for the choice of the regions in this study. In this study performed in 2012, the level of malaria prevalence amongst all age groups was moderately high for Mtwara and Mwanza (17.4% and 16.1% respectively), and low for Mbeya (2.3%). Tanzania is an area of year-round malaria transmission, with a bimodal pattern, peaking after the rainy season. Each region includes urban and rural districts, although the populations are predominantly rural. Tanzania has four different administrative levels, the highest level being the region. Regions are divided into districts and these are sub-divided into divisions and further into wards. Fever case

management in the public sector is provided by a network of regional and district hospitals as well as health centres and dispensaries at lower administrative levels [18]. The private sector includes for profit and not-for-profit facilities (hospital and clinics) and a drug outlets network which is mainly constituted of regulated and non-regulated drug shops, while registered pharmacies are almost exclusively located in major urban areas [180,181].

5.3.3.Study sampling

One urban and two rural districts were selected in each study region. Three wards were randomly selected proportionally to their population size in each district. In each urban ward, four streets and in each rural ward, two villages as well as two sub villages in each village were randomly selected. After obtaining the list of the households within each village/street, 20 households were randomly sampled for the household surveys. In each sampled household, up to six participants were randomly selected from the complete list of the household members until a total of 60 individuals per sub village/street was reached, resulting in 240 individuals sampled per ward. All consenting individuals were eligible to participate. The exclusion criteria were individuals with a severe illness requiring immediate referral and those under three months of age.

5.3.4.Data collection procedures

Household surveys

Interviews were conducted with a questionnaire in Swahili, first with the head of household. The questions also included information on time to the closest health facility and closest pharmacy or drug retailers. Randomly selected members were then asked about demographic information, history of fever in the previous two weeks as well as history of antimalarial use in the previous months. Members who reported fever in the previous two weeks were asked about treatment seeking behaviour including place where they sought care, information on malaria diagnosis testing and drugs received and ingested. Data was collected using electronic tablets with the help of the Open Data Kit collection tool (ODK). Each visited household was mapped using Global Positioning System. In addition, blood spots were collected from finger prick onto filter paper to assess the presence of malaria antigens and for further drug concentrations measurements.

Outlet surveys

Drug-outlet surveys were conducted in all private and public outlets surrounding and serving the selected villages. These included small district hospitals, public and private health centres, dispensaries, pharmacies, registered ADDOs and non-registered drug retailers, general stores

and kiosks. Following verbal consent of the most senior staff present at the moment of the survey, details about antimalarials stocks and diagnostic tools (mRDTs and microscope) available at the time of the visit were recorded.

Exit interviews of patients in health facilities

In each ward, the main health facilities (which included district hospitals, health centres and/or dispensaries) serving the surveyed villages were selected for the exit interviews. After completion of their consultation with health facility staff and after visiting the health facility pharmacy for possible treatment procurement, patients were interviewed and information were collected on demographics, administration of malaria diagnostic testing (microscopy or mRDTs), test result and drug obtention. In addition, mRDT was performed on site by our field investigators.

5.3.5.Laboratory procedures

Capillary blood samples were taken by fingerprick on all subjects interviewed during the household surveys. One drop of blood was immediately used for mRDTs analysis (ParaHIT-*f*test, Span diagnostic Ltd, Surat, India, detecting HRP-2 antigens) and four drops were applied on filter paper cards (FTA DMPK-B cards, Whatman, GE Healthcare). These were able to dry at room temperature for at least two hours before being placed in a specific bag with desiccant and stored in a -10°C freezer at the end of the day and finally transferred to a -80°C freezer within one month. Concentrations of seven antimalarials and two metabolites, namely amodiaquine, N-desethyl-amodiaquine, lumefantrine, desbutyl-lumefantrine, mefloquine, chloroquine, quinine, sulfadoxine and pyrimethamine, were determined in the dried blood spots (DBS) samples by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) [27, CHAPTER IV]. The LC-MS/MS platform enables to detect residual blood levels if the drug was taken up to four weeks prior to the analysis (given the long half-lives of the measured antimalarials). Due the very short half-life of artemisinin compounds, and because they are rapidly degraded due to haemolysis with the current collection procedure, their analysis were not performed in these DBS samples.

5.3.6.Definitions

Fever was defined as any illness with fever reported in the two weeks prior to the survey. Malaria infection was defined as a positive mRDT result on the day of the survey. Antimalarials identified during the outlet surveys were classified according to their active ingredients and drug formulation. For data analysis, outlets were considered to have mRDTs and antimalarials in stock if the study team observed at least one non-expired test or at least one complete non-expired treatment of any antimalarial for any age/weight group. Individuals were

considered having antimalarials in the blood if at least one of the nine antimalarials/metabolites were measured in their corresponding DBS sample, at a concentration equal or higher than the lower limit of quantification. The latter is the minimal concentration that confidently provides a bias and coefficient of variation within ±20% [148]. For our analysis, dispensaries, public, private or mission health centres and small district hospitals were classified as "health facilities". Pharmacies, registered (ADDOs) and non-registered drug retailers, general stores and kiosks were grouped as "non-health facility antimalarial providers". Traditional practitioner, neighbours, friends or even home (if medication available) were classified as "other places". Appropriate diagnosis was defined as a patient with history of fever being tested for malaria (by mRDT or microscopy) and appropriate treatment as having antimalarial in the blood or in agreement with mRDT result. Treatment was also considered as being appropriate if a febrile individual was not tested and had antimalarials detected in the blood, as per WHO guidelines when diagnostic testing is not possible [175].

5.3.7.Data management and statistical analysis

Data was stored on the ODK Aggregate data repository at the end of each survey day. During each interview, key data such as demographic information and mRDT results were also collected on paper forms. These data were cross-checked twice with electronic data at the end of each day. R (version 3.4.0) was used for data cleaning and management and to produce summary statistics as well as graphics using the ggplot2 package. P-values were calculated using Pearson X^2 statistics with significance defined as $p < 0.05$.

5.4. Results

The household survey included a total of 6485 participants. On average, 120 individuals were interviewed in each village. The outlet survey included 2 hospitals, 19 health centres, 39 dispensaries, 78 ADDOs, 57 drug stores, 9 pharmacies and 4 general stores or kiosks.

5.4.1.Population characteristics in the household surveys

In the household survey, 4503/6485 (69.4%) participants were sampled in the two regions with moderately high malaria endemicity [2141/6485 (33.0%) in Mtwara and 2362/6485 (36.4%) in Mwanza] and 1982/6485 (30.6%) in the region with low endemicity (Mbeya) (Table 5.1). The majority of the participants lived in rural districts (4280/6485, 66.0%). The sample included 3573 (56.0%) females with a median age of 20 years (age range from three months to 95 years) and 2802 (44.0%) males with a median age of 14 years (age range from three months to 100 years). On the day of the survey, 1039/6485 (16.0%) participants reported a history of fever in

the last two weeks prior the survey and 1136/6485 (17.5%) were tested positive for malaria by mRDT.

Table 5-1: Population characteristics of participants in household surveys and febrile outpatients exiting health facilities

	Participants in household surveys (N=6485)			Febrile outpatients, health facility exit interviews (N=226)		
	N	%	95% CI	N	%	95% CI
Total	**6485**	**100.0**	**-**	**226**	**100.0**	**-**
Sex						
Male	2846	43.9	(42.9-45.0)	99	43.8	(38.4-49.2)
Female	3623	55.9	(55.0-57.0)	127	56.2	(50.8-61.6)
Missing	16	0.2	-	0	0.0	-
Age						
0-4 years	1153	18.1	(17.3-18.9)	148	65.5	(60.3-70.7)
5-9 years	1014	15.9	(15.1-16.6)	48	21.2	(16.8-25.7)
10-14 years	775	12.1	(11.5-12.8)	9	4.0	(1.8-6.1)
15-24 years	990	15.5	(14.8-16.3)	6	2.7	(0.9-4.4)
25-44 years	1437	22.5	(21.7-23.4)	10	4.4	(2.2-6.7)
45-59 years	559	8.8	(8.2-9.3)	3	1.3	(0.1-2.6)
60-100 years	454	7.1	(6.6-7.6)	2	0.9	(-0.1-1.9)
Missing	103	1.6	-	0	0.0	-
Area						
Urban	2205	34.0	(33.0-35.0)	50	22.1	(17.6-26.7)
Rural	4280	66.0	(65.0-67.0)	176	77.9	(73.3-82.4)
Missing	0	0.0		0	0.0	
Region						
Mwanza	2362	36.4	(35.4-37.4)	45	19.9	(15.5-24.3)
Mbeya	1982	30.6	(29.6-31.5)	37	16.4	(12.3-20.4)
Mtwara	2141	33.0	(32.1-34.0)	144	63.7	(58.5-69.0)
Missing	0	0.0	-	0	0.0	-
Had a fever in the previous 2 weeks*						
Yes	1039	16.0	(15.3-16.8)	100	-	-
No	5440	83.9	(83.1-84.6)	0	-	-
Don't know	6	0.1	-	0	-	-
RDT result						
Positive	1136	17.5	(16.7-18.3)	106	47.1	(41.6-52.6)
Negative	5346	82.5	(81.7-83.3)	119	52.9	(47.4-58.4)
Missing	3	0.0	-	1	0.4	-
Took any antimalarial drugs in the previous 4 weeks*						
Yes	810	12.5	(11.8-13.2)	NA**		
No	5664	87.3	(86.7-88.0)	NA		
Don't know	11	0.2	-	NA		

*based on self-report , ** Not applicable

5.4.2. Prevalence of *Plasmodium falciparum* and antimalarials in the blood of the surveyed population

Out of the 6485 participants, 94 were excluded from the antimalarial analysis because their blood samples were not found or were mislabelled. Thus, 6391 participants remained with a valid antimalarial measurement.

Mtwara was the region with the highest malaria prevalence [25.9%, 95% Confidence interval (CI): 24.4-27.5], followed by Mwanza (21.1%, 95%CI: 19.7-22.5) and Mbeya (4.1%, 95%CI: 3.4-4.9). The presence of antimalarials was detected in the blood of 20.8% (95%CI: 20.0-21.6, 1330/6391) of individuals in total. The proportion of individuals with residual levels of antimalarials in the blood was 19.3% (95%CI: 17.9-20.7) in Mtwara, 28.0% (95%CI: 26.4-29.5) in Mwanza and 14.1% (95%CI: 12.8-15.3) in Mbeya (Figure 5.1).

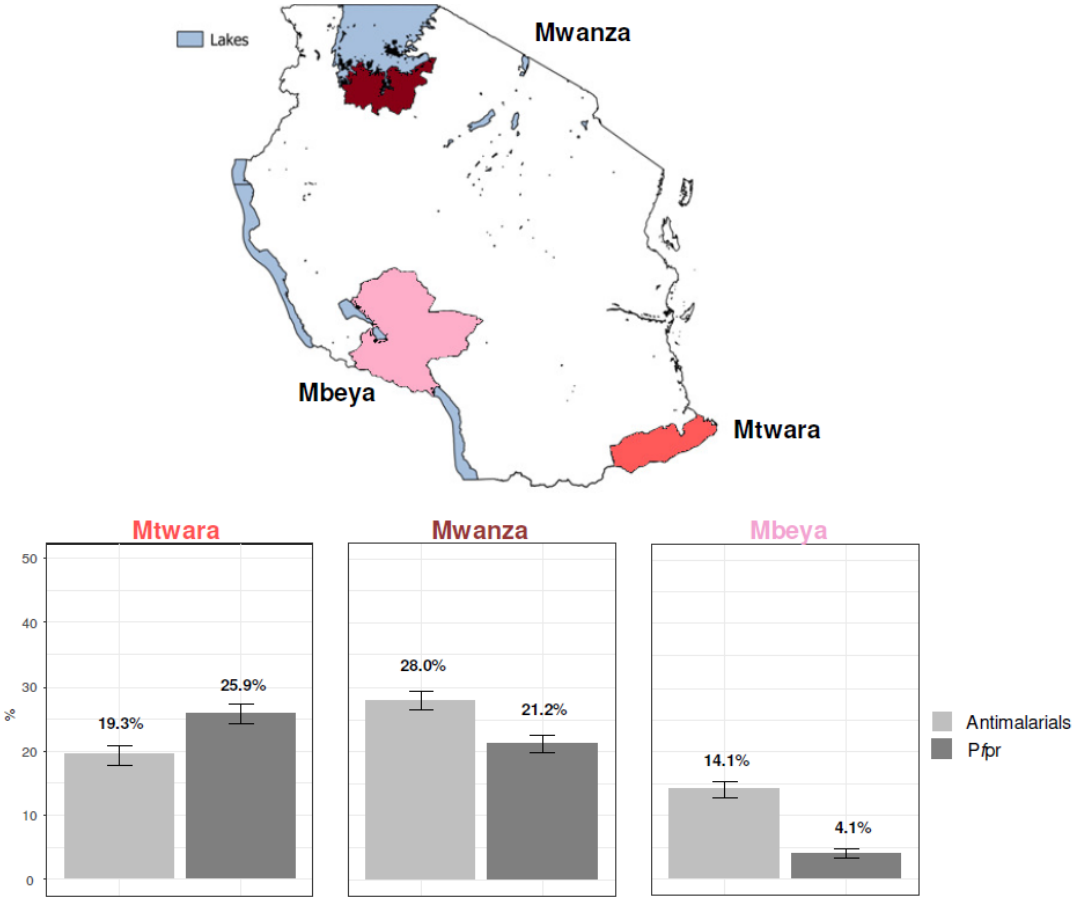

Figure 5-1: Proportions of individuals with residual antimalarials in their blood and individuals with *Plasmodium falciparum*. The presence of antimalarials in the blood was measured using dried blood spots samples and parasite prevalence using mRDT. These proportions were obtained from household surveys in three regions of Tanzania.

5.4.3.Reliability of medical history

Out of the 1330 individuals with antimalarials in their blood, only 28.0% (372/1330) reported the use of antimalarials within the previous month, irrespective of fever status in the previous two weeks, as represented in Figure 5.2A. There was only a 21% (372/1768) overlap between the individuals reporting antimalarial use and those having detectable concentrations of antimalarials in their blood.

Amongst the 6391 participants with a valid antimalarial measurement, 16.0% (1021/6391) reported having had a fever in the previous two weeks. About 37.5% (383/1021) of them had detectable levels of antiamalarials in the blood (Figure 5.2B). The overlap between the individuals reporting a fever and those having detectable concentrations of antimalarials in their blood was 20% (383/1968). Participants who did not report any fever in the previous two weeks accounted for the majority of the individuals who had residual antimalarial levels detected in their blood [71.2% (947/1330)].

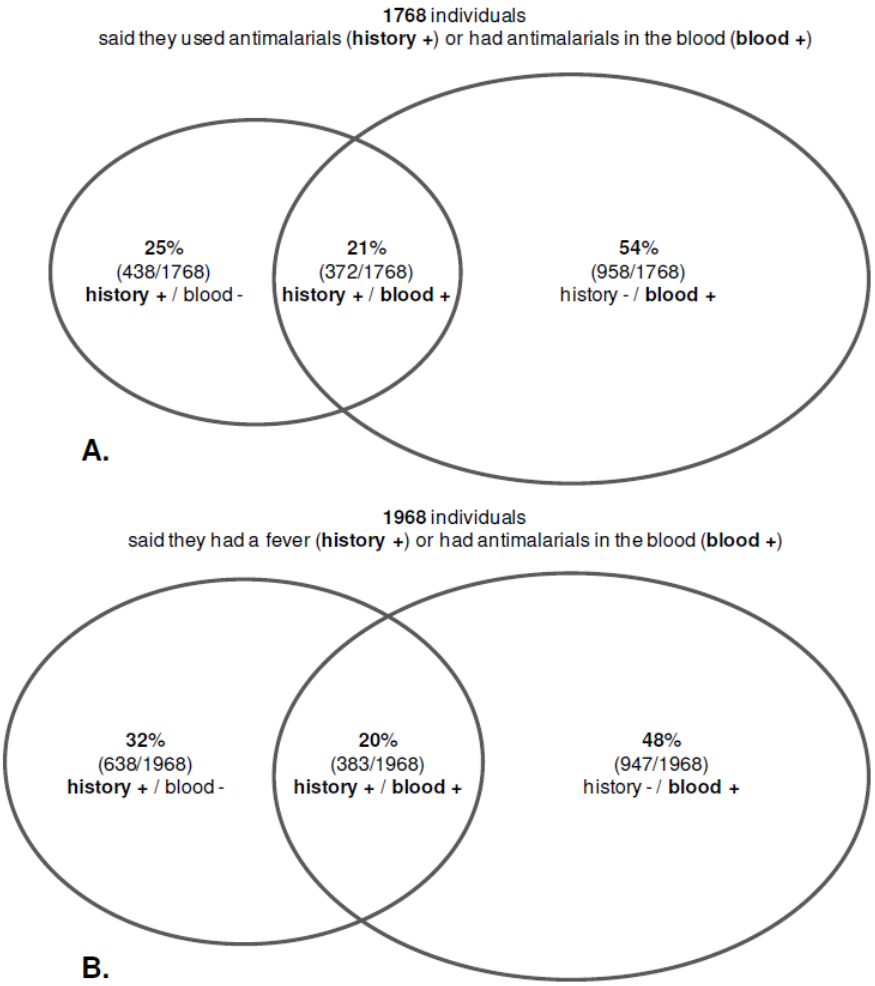

Figure 5-2: Overlap between self-reported history of antimalarial use (A) or fever (B) and antimalarials in the blood. Overlap between: (A) individuals reporting antimalarial use in the previous month or (B) individuals reporting fever in the previous two weeks, and individuals with detectable concentrations of antimalarial drugs in their blood (dried blood spots samples) in the household surveys.

5.4.4.Seeking care behaviour in case of fever

Figure 5.3 presents the proportion of febrile individuals who sought care and those amongst them who had antimalarials in their blood, according to the type of care providers they visited. Amongst the 37.5% (383/1021) of febrile individuals who had antimalarials in the blood, the proportion who sought care in health facilities was lower than in non-health facility antimalarial providers [11.3% (115/1021) vs 16.6% (170/1021)]. 8.3% (84/1021) said they did not seek care and 1.3% (13/1021) reported seeking care by a friend or by a traditional healer or took a drug from their home.

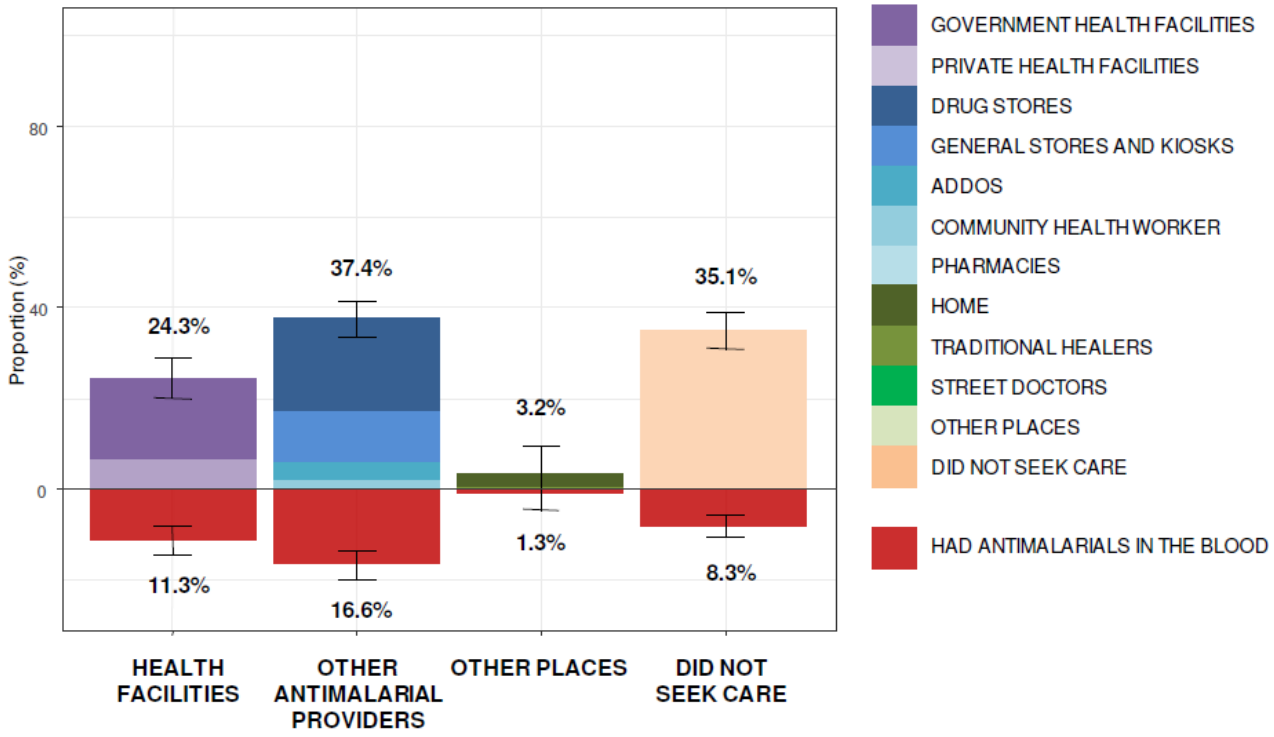

Figure 5-3: Prevalence of individuals with antimalarials in their blood according to health seeking behaviour. The top of the chart is based on the self-reported history of health-seeking behaviour of individuals who had a febrile episode in the previous two weeks. The bottom of the chart presents the corresponding prevalence of individuals with antimalarial drug in their blood.

5.4.5. Appropriateness of malaria diagnosis (according to medical history) and treatment (based on antimalarials in the blood) in health facilities and non-health facility antimalarial providers

As shown in Figure 5.4, proportion of participants with fever who reported they had been tested for malaria at the place they sought care was 31.0% (195/630), with a large statistically significant difference between health facilities and non-health facility antimalarial providers [69.4% (172/248) versus 6.0% (23/382) respectively; p<0.001)]. The overall proportion of people being appropriately treated was 47.3% (298/630). This proportion was 52.0% (129/248) in health facilities vs 44.2% (169/382) for non-health facility antimlarial providers (p=0.04). Only half of the individuals who were tested positive by mRDT in the health facilities had antimalarials detected in their blood [52.9% (74/140)]. This was close to those who were not tested [44% (33/73) in health facilities and 42.9% (154/359) in non-health facility antiamalarial providers]. One third of the individuals tested negative in the health facilities had detectable levels of antimalarials in their blood [36.4% (8/22)]. Overall, the proportion of individuals treated was larger than that being tested [45.2% (285/630) treated, 31.0% (195/630) tested].

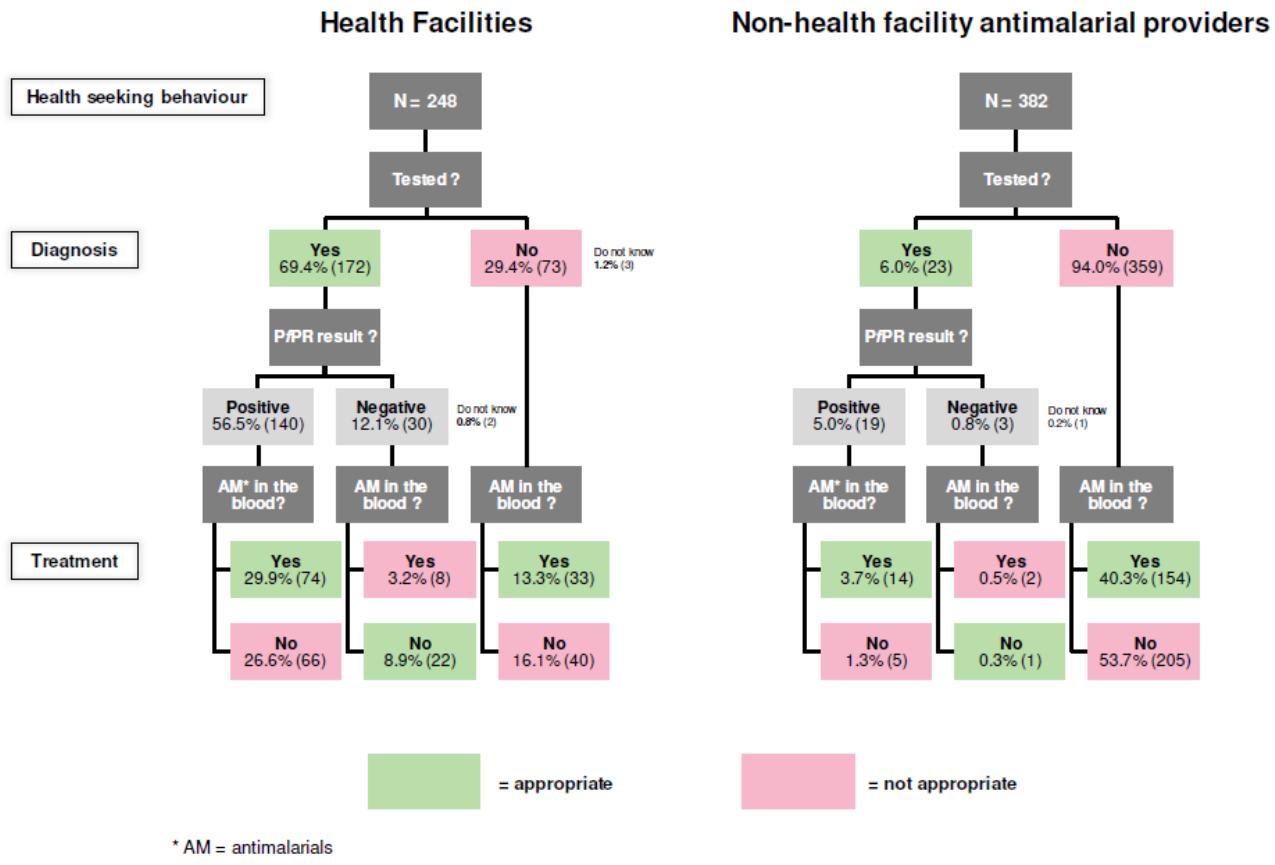

Figure 5-4: Proportion of febrile individuals appropriately diagnosed and treated for malaria. Appropriate diagnosis was defined as a patient with history of fever being tested for malaria (by mRDT or microscopy) and appropriate treatment as having antimalarials in the blood if the mRDT result was positive or if the person had not been tested. The left side of the figure reports individuals who sought care in health facilities and the right side of the figure those who sought care in non-health facility antimalarial providers. The upper part of the organigram is built upon self-reported history and the bottom part on results of antimalarials measured in blood samples collected during the household survey, which constitute more objective information.

5.4.6. Appropriateness of malaria treatments assessed by medical history and antimalarials in the blood in household surveys

When assessed based on participant's medical history recorded in household surveys, the proportion of individuals who mentioned being appropriately treated according to diagnostic test result or who stated being treated presumptively was significantly higher than when it was assessed on the basis of presence or absence of antimalarials in the blood, as shown in Figure 5.5 (65.3% *versus* 52.0%, $p<0.01$ in health facilities and 66.0% *versus* 44.3%, $p<0.001$ for non-health facility antimalarial providers).

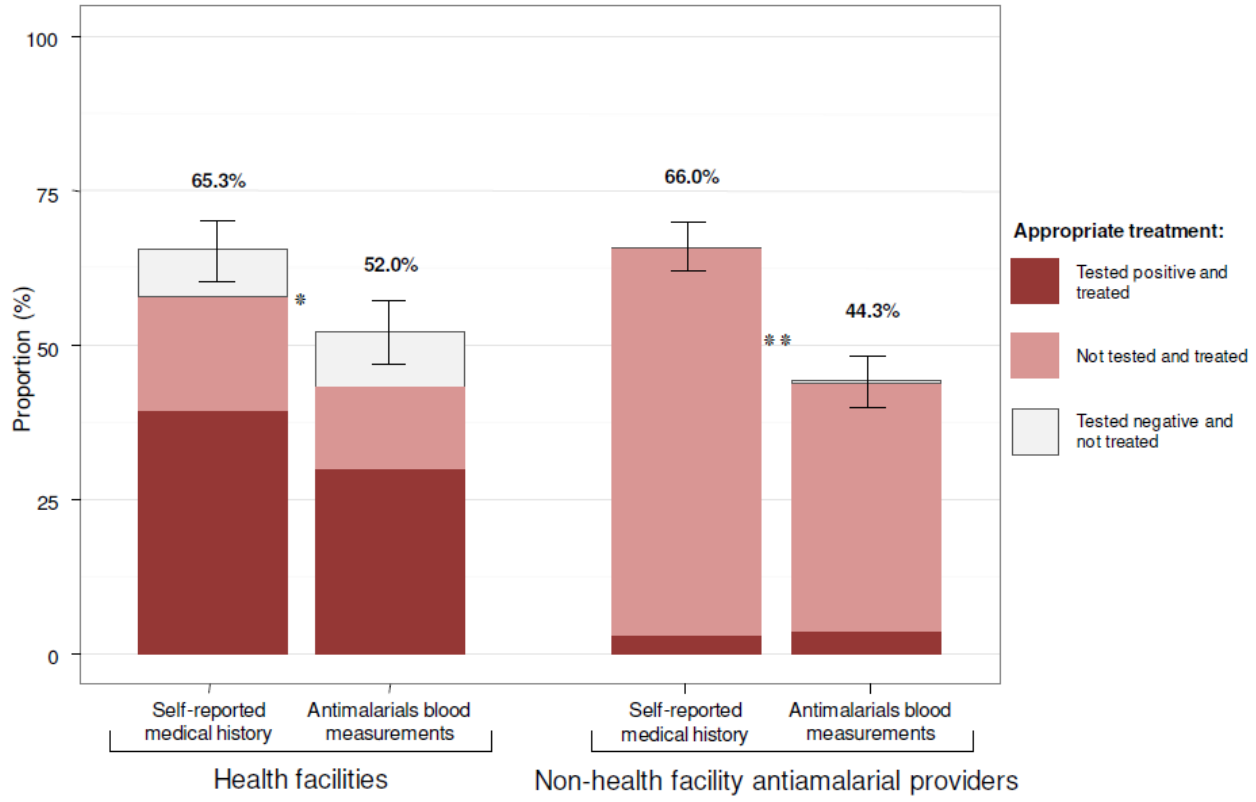

Figure 5-5: Comparison between appropriateness of treatment assessed according to self-reported medical history and antimalarials blood measurements. Proportions of febrile individuals interviewed in household surveys appropriately treated for malaria when they sought care in health facilities (left side of the figure) and non-health facility antimalarial providers (right side of the figure) assessed according to self-reported medical history and antimalarials blood measurements. There is a significant difference (*: $p<0.01$, **: $p<0.001$) between the proportion of febrile patients appropriately treated for malaria assessed according to self-reported medical history and antimalarials blood measurements.

5.4.7.Availability of antimalarial treatments and mRDTs in each region

Mbeya was the only region in which all health care providers (health facilities and non-health facility antimalarial providers) had antimalarials in stock (Table 5.2). The availability of malaria blood testing (by mRDT or microscopy) was the highest in Mbeya in all types of care providers (88.9% in health facilities and 7.9% in non-health facility antimalarial providers). 68.8% of the health facilities in Mtwara and 80.8% in Mwanza had malaria diagnosis tools available. Very few non-health facility antimalarial providers had mRDTs in stock in these two regions (0.0% in Mtwara and 1.1% in Mwanza). In both types of care providers and in each region, the commodities to treat were higher than the potential to test for malaria.

Table 5-2: Proportion of screened outlets with antimalarials and malaria diagnosis tools available in stock. This table reports stocks available on the day of the survey. Health facilities include hospitals, public and private health facilities and dispensaries. Non-health facility antimalarial providers include pharmacies, drug stores, ADDOs and general stores. Malaria diagnosis tools include mRDTs and microscopy.

	Mbeya				**Mtwara**				**Mwanza**			
	Health facilities (N=18)		**Non-HF AM* providers (N=38)**		**Health facilities (N=16)**		**Non-HF AM providers (N=21)**		**Health facilities (N=26)**		**Non-HF AM providers (N=89)**	
	N	% (95%CI)	N	% (95%CI)	N	% (95%CI)	N	% (95%CI)	N	% (95%CI)	N	% (95%CI)
% of outlets with antimalarials in stock	18	100.0	38	100.0	16	100.0	20	95.2 (87.6-102.9)	26	100.0	81	91.0 (86.0-96.0)
% of outlets with mRDTs in stock or microscopy	16	88.9 (76.7-101.1)	3	7.9 (0.7-15.1)	11	68.8 (49.7-87.8)	0	0.0	21	80.8 (68.1-935.)	1	1.1 (-0.71-2.96)

Non-HF AM* =non-health facility antimalarial.

5.4.8. Exit interviews

A total of 456 outpatients were interviewed in 37 nearby health facilities (3 hospitals, 17 health centres and 17 dispensaries) but for the present analysis, only the 226 febrile patients were considered (Table 5.1). The proportion of interviewees that reported they had been tested at the health facility was 65.9% (149/226), a proportion that is close to that obtained through household surveys [69.4% (172/248)]. 65.0% (147/226) had received appropriate treatment, a proportion that is higher than that in household surveys, although this difference was not statistically significant (p=0.16). During the interviews, 47.1% (106/226) febrile participants were tested positive by mRDT by our field investigators, a proportion which is lower than that reported through household surveys [81.4% (140/172), p<0.01].

5.5. Discussion

To our knowledge, this is the first study investigating the presence of antimalarials in the blood of the general population. The measurement of nine antimalarials provided a reliable endpoint and allowed a comprehensive assessment of drug use and current malaria case management landscape in the studied communities.

This study showed that close to one fifth (20.8%) of individuals in the community had residual antimalarials in their blood, even in Mbeya, a region of low endemicity of malaria. The absence of relationship between the level of transmission and the drug-prescribing behaviour has also

been observed in another study in Tanzania [182]. The high prevalence of individuals with antimalarials in the blood in Mwanza and Mbeya in comparison to the prevalence of *Plasmodium falciparum* does not seem to be related to low testing available since these two regions had higher proportions of health care facilities with malaria diagnostic tools in stock. However, having diagnostic tools available does not guarantee their usage, or that clinicians are compliant to tests results. The testing habits might be lower in these two regions, and especially so in Mbeya where the probability of a fever being malaria is much lower than in Mwanza or Mtwara because of the higher altitude [18]. More access to drugs might be one explanation. Indeed our team was able to visit 115 nearby outlets in Mwanza, 56 in Mbeya but only 37 in Mtwara for roughly the same number of individuals in the vicinity. Mwanza was the region with the highest proportion of individuals with antimalarials in the blood, and also the highest prevalence of fever (34.0% in Mwanza vs 14.6% in Mtwara and 9.3% in Mbeya). More febrile episodes in Mwanza could be due to a higher prevalence of other causes of fever (e.g. arboviruses) which might be wrongly allocated to malaria [183]. Alternatively, the population of Mwanza might have a lower level of malaria immunity than that of Mtwara, with a proportion of Plasmodium infections that progress to clinical manifestations being higher in this setting of lower endemicity, and hence higher prevalence of individuals treated with antimalarials [184]. Our results confirm this hypothesis with 35% of the individuals tested positive for malaria in Mwanza reporting having had a fever in the previous two weeks, against only 23% in Mtwara.

Whatever the reasons are for the high prevalence of people with antimalarials in the blood, access to drugs does not seem to be a major issue in these communities, but rather appropriate case management to target those febrile patients that are sick with malaria. The important drug pressure across these three regions is worrying because the occurrence of a low drug level in blood induces strong selective pressure on parasites and causes the emergence of drug resistance [12,39].

When using an objective endpoint such as the concentration of antimalarials in the blood, it appears that self-reporting of drug intake is unreliable, with only 28% concordance for individuals having detectable levels of antimalarials in their blood reporting the use of such treatments within the previous month, and 54% pretending having taken any drug actually having no residual antimalarials in their blood. Poor agreement between history taking and antimalarial concentration measurement has already been shown in two previous small-scale studies that were conducted among Tanzanian and Cambodian patients attending a health facility [49,50], and in a cross-sectional survey in Uganda [185].

Amongst the individuals who had detectable levels of antimalarials in their blood, 71% said they did not have fever in the previous two weeks and 6% said they had fever but did not seek care. Again, these results show that history taking is very unreliable and that all previous studies that described health seeking behaviour and drug consumption [3,42,177] should be taken with caution, as they leave aside a considerable part of the population using drugs. Although it has been shown that history validity can be improved [186], new technology platforms such as LC-MS/MS performed on DBS samples allow to move from subjective to objective and reproducible data. There is obviously a question of feasibility (ideally the DBS should be stored frozen) and cost (50 dollars for measurement of nine different antimalarials by LC-MS/MS in one DBS sample) to conduct large-scale surveys, but certainly such objective assessment could be used as a validation method for other more practical tools.

Another main finding of this study is the poor diagnosis and treatment practices in case of febrile episode. Overall, only one third of the febrile individuals being tested for malaria at the place they sought care and about half being appropriately treated according to their diagnostic test result, or presumptively if no diagnostic tool was available. Presumptive treatment is thus still common, and far from the goal set by the WHO of systematic testing of suspected malaria cases and treatment upon result [175]. The low testing rate is mainly due to the high proportion of individuals seeking care outside the health facilities where patients are usually not tested. Allowing drug retailers to perform malaria testing might be one way of reducing the numbers of antimalarials sells and consumption. In Tanzania, an ongoing research in which dispensers from ADDOs in intervention districts are trained to perform RDTs and treating with first-line treatment has already shown encouraging results with an increase from 0 to 65% of suspected malaria patients visiting a shop being tested [19].

The proportion of febrile individuals appropriately tested for malaria was much higher in health facilities than in non-health facility antimalarial providers (70% vs 6.0%), but this difference was not much reflected in a better targeting of treatment to individuals with malaria. Indeed, the proportion of patients appropriately treated was only eight percentage point higher in health facilities than in non-health facility antimalarial providers [52.0% (129/248) vs 44.2% (169/382), p=0.04]. This can be partly explained by the fact that, according to antimalarial drugs detected in the blood of the interviewees, only half of the patients tested positive were treated. The same proportion of antimalarial drugs was detected in the blood of patients who were not tested.

In the literature, a decrease by up to three-quarters in ACT prescription has been observed after mRDTs implementation, between 2006 and 2008 [26,187]. A recent systematic review including 14 studies showed that the overall compliance to positive and negative mRDT results

was 97% and 78% respectively and that lower levels of health care workers complied better that the more professional counterparts [188]. Our results are less encouraging since the fever cases were not always tested and appropriately treated. Studies rolled out in places where training has been done are more likely to detect a positive effect and a change observed at a given time point might not be sustainable. Besides, our findings add to previous household surveys conducted in the same regions three years before which showed no significant change in the proportion of febrile individuals obtaining an antimalarial at the population level [20,103]. This was explained by a reduction in the use of health facilities. Indeed, ADDOs are now recognized to be the principal source of medicines in Tanzania [189].

Our results call for interventions to reinforce the whole system for a public health impact. Health facilities priorities should focus on improving systematic testing and treating all positive cases. As for non-health facility antimalarial providers, our findings support the suggestions already drawn from two recent trials that introducing mRDTs into regulated private sector settings can improve malaria testing and reduce over-treatment [19,190]. Testing and treating should also be encouraged at low level of health care, e.g. at the level of community health workers, as it has been proven to improve well-targeted ACT treatments in randomised cross-over trials in Africa [191,192].

In addition, care should be taken when assessing appropriateness of treatment in the population. Our study observed a higher proportion of appropriate treatment when assessed by self-reported medical history than by antimalarials measured in the blood and there are several potential reasons to explain such discrepancies: in case of a positive test result, the treatment received may have not been an antimalarial or patient might have poorly adhered to their treatments [178,193,194]. In the case of a negative result, patients could have obtained a treatment from the informal sector and not reporting it.

Exit interviews confirmed the findings of the household surveys. Only 58% of the patients tested positive by our field investigators prior leaving the health facilities had received treatment against malaria during their consultation. The most likely scenario is poor compliance by health professionals to an mRDT positive result and no presumptive treatment prescribed to fever cases not tested. In contrast, a rather small proportion of the patients tested negative received a treatment (11%), which indicates a good compliance to negative results. Such findings imply that if more febrile patients would be tested, the amount of unnecessary treatment distributed would decrease.

Our study has several limitations. The history of fever recall was based on a two-week period in order to be able to compare with previous surveys in the same areas, but residual

antimalarial drugs can eventually be detected in the blood for up to four weeks. This could explain why a proportion (71%) of individuals who did not report a fever in the previous two weeks had detectable levels of antimalarials in the blood. However, as already mentioned before, history of drug intake was made on a four weeks period record in order to make the comparison between self-reported use and blood levels of antimalarials possible.

The evaluation of diagnosis appropriateness from household surveys was based on self-reported medical history only, and thus subjected to report bias. This limitation is nevertheless inherent to all studies of this kind, except for those that rely on direct observation of the consultation, which are subject to the Hawthorne effect though.

Finally, our study did measure diagnosis and treatment practices in different settings (urban or rural) and different transmission areas, but only at one point in time.

5.6. Conclusion

Despite recent efforts that have been made to improve access to diagnostic tools and to reduce antimalarials overuse, there is still a considerable antimalarial drugs pressure at the population level. Improving rational use of drugs is necessary to prevent the development of resistance. The present findings indicate that the goal of the WHO guidelines of systematic diagnostic testing and treatment upon result is far from being reached, and that antimalarial treatments are not targeted to the individuals in need. As resources are invested into the development and implementation of new diagnostic tools and effective treatments, it is of paramount importance to make sure that those tools are used to their full potential, and properly enforced. New health care interventions should not only be evaluated for their impact at the level of health facilities, but also at the level of the community. However, household surveys that collect information on health-seeking behaviour or practices through self-history are subject to important biases. Evaluation or monitoring tools that rely on objective measurements such as drug concentration in the blood should be favoured, if feasible.

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Ethical approval

This epidemiological study received approval from the Swiss Ethics Committees on research involving humans and from two responsible local authorities (Institutional review board of the Ifakara Research Institute and the National Institute for Medical Research in Tanzania). Interviews were conducted and capillary blood samples were obtained after receiving written informed consent in Kiswahili by the participants or their responsible caretaker.

6. CHAPTER VI: PREDICTORS OF RESIDUAL ANTIMALARIALS IN THE BLOOD

6. Predictors of residual antimalarial drugs in the blood in community surveys in Tanzania

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6.1. Abstract

Background

Understanding pattern of antimalarials use at large scale helps ensuring appropriate use of treatments and preventing the emergence and spread of resistant parasites. We estimated the proportion of individuals in community surveys with residual antimalarials in their blood and identified the factors associated with the presence of lumefantrine and/or desbutyl-lumefantrine (LF/DLF) or sulfadoxine-pyrimethamine (SP).

Methods

A cross-sectional survey was conducted in 2015 in three regions of Tanzania with different levels of malaria endemicity. Interviews were conducted and blood samples were collected through household surveys for further antimalarial measurements using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). In addition, diagnosis and treatment availability were investigated through outlet surveys. Multilevel mixed effects logistic regression models were used to estimate odds ratios for having LF/DLF or SP in the blood.

Results

Amongst 6391 participants included in the analysis, 12.4% (792/6391) had LF/DLF and 8.0% (510/6391) SP in the blood. A multivariate analysis identified factors associated with higher odds of detecting LF/DLF in the blood; these include fever in the previous two weeks (OR=2.6, $p<0.001$), living in a district with higher malaria prevalence (OR=1.5, $p<0.001$) and living in a ward in which all visited Drug Stores had artemisinin-based combination therapies in stocks (OR=2.7, $p=0.020$). Participants in older age groups were less likely to have LF/DLF in the blood (OR=0.9, $p<0.001$). Odds of having SP in the blood were higher for pregnant women (OR=4.6, $p<0.001$), when living in Mwanza (OR=3.9, $p<0.001$ compared to Mbeya), for individuals that reported an episode of fever in the previous two weeks (OR=1.7, $p<0.001$) and for participants in older age groups (OR=1.2, $p<0.001$).

Conclusion

The most significant predictors identified were expected. History of fever in the past two weeks and young age were significant predictors of residual LF/DLF in the blood, which is encouraging. Antimalarial drug pressure was high in the population and hence the use of recommended first-line drugs in combination with malaria Rapid Diagnostics Tests should be promoted to ensure appropriateness of treatment.

6.2. Introduction

Artemisinin-based combination therapies (ACTs) are the most potent weapon in treating *falciparum* malaria [12], representing the second highest impact intervention on malaria control between 2000 and 2015 [195]. In response to the development of parasite resistance to conventional antimalarials such as chloroquine, the World Health Organisation (WHO) recommended ACTs as first-line therapy for uncomplicated malaria in 2006 [175] when the Ministry of Health and Social Welfare of Tanzania had already recommended artemether-lumefantrine (Alu) as standard therapy in 2005 [17]. The use of antimalarials as combination therapy is yet clearly of benefit for the individuals [35], but artemisinin itself is rapidly eliminated while the "partner drugs" persist in the bloodstream as monotherapy for weeks due to their long half-lives [36]. Despite its poor efficacy and a high proportion of resistant parasites in Tanzania [196,197], sulfadoxine-pyrimethamine (SP) is still widely used as self-medication outside of intermittent preventive treatment during pregnancy (IPTp) [198,199]. Both sulfadoxine and pyrimethamine have long half-lives. The extensive and often inadequate use of these long-acting compounds constitute a critical factor contributing to drug pressure [39], which in turn is a potent force selecting resistant parasites [34,40]. Thus, correct use of antimalarials is now one of the five goals and recommendations of WHO Global plan for artemisinin resistance containment [12]. Understanding the pattern of antimalarial drugs usage will help designing specific and targeted interventions aimed at reducing drug pressure and monitoring the implementation of diagnostic and treatment policies.

Previous studies have explored the effect of environmental and behavioral factors on population drug use. Most of them relied on questionnaires [196,198,200,201] although there was no indication that information collected through self-reporting was reliable. With such methodology, uptake of antimalarials was reported to be low [40,47]. On the other hand, studies conducted during the chloroquine or SP eras [34,40,202,203] which used biochemical markers in addition to questionnaires showed high prevalence of individuals with detectable drug in urine or blood, although the tests used had a rather poor sensitivity. Two recent studies using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), a sensitive analytical method, showed that 52% of the patients upon admission to a health facility in Cambodia [50] and 74% in Tanzania [49,50] had detectable antimalarials in their blood, although all of them stated that they had not taken any drug in the previous four weeks. These observations indicate frequent recourse to self-medication and poor reliability of self-reporting when assessing drug use. Our study first aimed at evaluating the actual state of population drug pressure by estimating the proportion of individuals in community surveys

with residual antimalarials in their blood, and then at identifying the factors associated with the presence of lumefantrine (LF) or SP in the blood.

6.3. Methods

A cross-sectional investigation including household-based and drug outlet-based surveys was conducted concurrently. Detailed study setting, population and sampling have been described elsewhere (CHAPTER V) and can be summarized as follows.

6.3.1.Study setting and population

Surveys were conducted in three regions of Tanzania with different levels of malaria endemicity, i.e moderately high for Mtwara and Mwanza (17.4% and 16.1% respectively) and low for Mbeya (2.3%) [20]. Our study took place from May to August 2015 following the rainy season. In this country where the population is predominantly rural, over 95% of inhabitants of mainland Tanzania are at risk of malaria [1]. Alu is free of charge in public health facilities (HFs) for under five children, pregnant women, elderly and those who cannot afford to pay [20]. Although no-longer indicated for malaria case management, SP is still widely available both in the private and public sector, according to recent outlet surveys [176].

6.3.2.Study sampling and data collection

Tanzania has four different administrative levels: regions, divisions, districts and wards (which include five to seven villages). In each region, one urban and two rural districts were selected. Three wards per district, four streets or sub villages per ward, 20 household per sub village/street and up to six individuals per household were randomly selected. The questionnaire in Swahili was divided into 3 parts: i) household-related questions on indoor residual spraying (IRS), time to the closest HF and closest drug retailer; ii) demographic information, use of bed nets, pregnancy and history of fever in the previous two weeks; iii) information on health seeking behaviour. Capillary blood was collected from all participants for malaria Rapid Diagnostics Tests (mRDTs) analysis (ParaHIT-*f* test, Span diagnostic Ltd, Surat, India, detecting HRP-2 antigens) and applied on filter paper cards (FTA DMPK-B cards, Whatman, GE Healthcare) for further analysis of seven antimalarials and two metabolites by liquid chromatography coupled to tandem mass-spectrometry (LC-MS/MS) in the dried blood spots (DBS) samples [131,204]. The drug outlet surveys included all HFs (small district hospitals, public and private health centres, dispensaries) and drug stores [pharmacies, registered accredited drug dispensing outlets (ADDOs) and non-registered drug retailers, general stores and kiosks] surrounding the selected villages. Diagnostic tools (mRDTs and microscope) and antimalarials in stocks at the time of the visit were recorded.

6.3.3.Data management and statistical analysis

The Open Data Kit collection tool (ODK) programmed on electronic tablets was used to collect the data and record Global Positioning System coordinates of each household and drug outlet visited. Data was stored on the ODK Aggregate data repository at the end of each survey day. R (version 3.4.0) was used for data cleaning and management and to produce logistic regressions as well as forest plots using the lme4 and sjPlot packages.

6.3.4.Variable definitions

Individuals were considered having an antimalarial in the blood if the concentrations measured in their corresponding DBS sample were equal or higher than the lower limit of quantification (LOQ) (CHAPTER IV). Age was categorized as presented in Table 6.1, based on the age distribution of the sampled population. Mean malaria endemicity was classified according to the most recent epidemiological profile of mainland Tanzania from the last Malaria Indicator Survey [18]. The following variables were used as binary variables: history of fever (in the two weeks prior to the survey), mRDT result (performed on the day of the survey), bednet use (the previous night), IRS (the previous year), pregnancy (at the time of the survey) and living in an urban or rural district. Participants were asked to estimate the time to reach the closest HF or drug store (DS) using the mode of transport they would normally use. Outlets were considered to have mRDTs and antimalarials in stock if, at least one non-expired test and respectively one complete non-expired treatment of any antimalarial for any age/weight group was observed.

6.3.5.Multilevel mixed effects logistic regression analysis

The presence of lumefantrine and/or desbutyl-lumefantrine (LF/DLF) and the presence of sulfadoxine and/or pyrimethamine (SP) were analyzed as outcome variables. A bivariate analysis was first performed to select variables based on the p-value of the log-likelihood ratio tests, with a cut-off of 0.2 [205]. Multilevel mixed effects logistic regression models were then performed by a backward step-by-step procedure. Independent variables and their interactions were retained when significance level was ≤0.05 and if they showed a model fit improvement [reduction in the Akaike Information Criteria (AIC) value of the model]. According to sampling design, regions, district and wards were included as nested random effects.

6.4. Results

In total, 6485 participants were included in the household surveys but 94 were excluded of the present analysis because their DBS sample was not found or mislabelled. On average, 237 individuals were interviewed in each ward, ranging from 166 to 380. The outlet surveys included 2 hospitals, 19 health centres, 39 dispensaries, 78 ADDOs, 57 DSs, 9 pharmacies and 4 general stores or kiosks.

6.4.1.Site and study participants description

Females were 55.9% (3573/6391) while males were 43.8% (2802/6391). The median age was 17 years and ranged from three months, one of the inclusion criteria, to 100 years. Overall, 4421 participants were sampled in regions of high endemicity (2304 in Mwanza and 2117 in Mtwara) and 1970 in Mbeya, the region of low endemicity. Majority of the participants lived in rural districts [65.8% (4206/6391)]. Summary statistics for all assessed variables are shown in Table 6.1.

Table 6-1: Sample characteristics of included individuals and strength of association between variables and presence of LF/DLF (A) and presence of SP (B) in the blood. In the bivariate analysis, p-values are based on log-likelihood ratio tests. Variables with more than two categories were considered as ordinal, except for the regions. Net use was defined as use in the previous night and $PfPR_{2-10}$ as malaria endemicity.

A)

	Total participants	**Participants with LF/DLF in the blood**					
	N (%)	N (%)	95% CI	Bivariate analysis		Multivariate analysis	
				ORs	*p*-value	ORs	*p*-value
Total	**6391** (100.0)	**792** (12.4)	11.7-13.1	-	-	-	-
Sex							
Male	2802 (43.8)	350 (12.5)	11.5-13.5	0.97	0.673	-	-
Female	3573 (55.9)	441 (12.3)	11.4-13.2				
Missing	16 (0.3)	-	-				
Age							
0-4 years	1135 (18.1)	157 (13.8)	12.1-15.5	0.92	<0.001	0.92	<0.001
5-9 years	996(15.8)	170 (17.1)	15.1-19.0				
10-14 years	765 (12.2)	111 (14.5)	12.4-16.6				
15-24 years	973 (15.5)	83 (8.5)	7.1-10.0				
25-44 years	1422 (22.6)	129 (9.1)	7.8-10.3				
45-59 years	552 (8.8)	79 (14.3)	11.9-16.8				
60-100 years	445 (7.1)	49 (11.0)	8.6-13.5				
Missing	103 (1.6)	-	-				
Pregnant							
Yes	104 (1.6)	10 (9.7)	4.9-14.5	0.71	0.297	-	-
No	1569 (24.6)	135 (8.7)	7.5-9.9				
Not applicable	4718 (73.8)	-	-				
Had a fever in the previous 2 weeks							
Yes	1021 (16.0)	247 (24.2)	22.0-26.4	2.70	<0.001	2.62	<0.001
No	5364 (83.9)	544 (10.1)	9.5-10.8				
Missing	6 (0.1)	-	-				
mRDT result							
Positive	1117 (17.5)	222 (19.9)	17.9-21.8	1.25	0.023	1.14	0.183
Negative	5272 (82.5)	570 (10.8)	10.1-11.5				
Missing	2 (0.0)	-	-				
Net use							
Yes	4180 (65.4)	555 (13.2)	12.4-14.1	1.36	<0.001	1.30	0.004
No	2211 (34.6)	238 (10.8)	9.7-11.8				
Missing	0 (0.0)	-	-				
IRS in the previous year							
Yes	1073 (16.8)	147 (13.7)	12.0-15.4	0.99	0.802	-	-
No	5316 (83.2)	645 (12.1)	11.4-12.9				
Missing	2 (0.0)	-	-				
Area							
Urban	2185 (34.2)	172 (7.9)	6.9-8.8	1.59	0.359	-	-
Rural	4206 (65.8)	620 (14.7)	13.8-15.7				
Missing	0 (0.0)	-	-				
Region							
Mbeya (reference)	1970 (30.8)	151 (7.7)	6.7-8.6	-	-	-	-
Mwanza	2304 (36.1)	338 (14.7)	13.5-15.9	2.15	0.137	-	-
Mtwara	2117 (33.1)	303 (14.3)	13.1-15.6	2.49	0.075	-	-
Missing	0 (0.0)	-	-				
$PfPR_{2-10}$ of the ward							
0 < 1%	1421 (22.2)	244 (17.2)	15.5-18.8	0.97	0.655	-	-
1 - < 5%	0 (0.0)	-	-				

5 - < 10%	1453 (22.7)	202 (13.9)	12.4-15.4				
10 - < 50%	2062 (32.3)	183 (8.9)	7.8-9.9				
> 50%	1455 (22.8)	163 (11.2)	9.8-12.6				
Missing	0 (0.0)	-	-				
$PfPR_{2-10}$ of the district							
0 < 1%	1244 (19.5)	48 (3.9)	3.0-4.8	1.53	0.002	1.52	<0.001
1 - < 5%	0 (0.0)	-	-				
5 - < 10%	1470 (23.0)	138 (9.4)	8.1-10.6				
10 - < 50%	2259 (35.3)	343 (15.2)	13.9-16.4				
> 50%	1418 (22.2)	263 (18.6)	16.8-20.2				
Missing	0 (0.0)	-	-				
Time to the closest HF*							
<15 min	2134 (33.4)	276 (12.9)	11.7-14.1	0.85	0.011	0.84	0.007
15 min to 1h	3483 (54.5)	430 (12.3)	11.4-13.3				
1h to 2h	564 (8.8)	57 (10.1)	8.0-12.2				
> 2h	167 (2.6)	26 (15.6)	10.9-20.2				
Don't know	43 (0.7)	-	-				
Time to the closest DS**							
<15 min	2911 (45.5)	331 (10.7)	9.7-11.6	1.05	0.385	-	-
15 min to 1h	2599 (40.7)	354 (13.6)	12.5-14.7				
1h to 2h	461 (7.2)	60 (13.0)	10.4-15.6				
> 2h	63 (1.0)	9 (14.3)	7.0-21.5				
Don't know	357 (5.6)	-	-				
ACTs in stocks in all the visited HF of the ward							
Yes	6391 (100.0)	792 (12.4)	11.7-13.1	-	-	-	-
No	0 (0.0)	-	-				
Missing	0 (0.0)	-	-				
AM* in stocks in all the visited HF of the ward**							
Yes	6391 (100.0)	792 (12.4)	11.7-13.1	-	-	-	-
No	0 (0.0)	-	-				
Missing	0 (0.0)	-	-				
mRDTs in stocks in all the visited HF of the ward							
Yes	4986 (78.0)	534 (10.7)	10.0-11.4	0.68	0.139	-	-
No	1405 (22.0)	258 (18.4)	16.7-20.1				
Missing	0 (0.0)	-	-				
ACTs in stocks in all the visited DS of the ward							
Yes	5528 (86.5)	667 (12.1)	11.3-12.8	2.88	0.008	2.69	0.020
No	399 (6.2)	16 (4.0)	2.4-5.6				
Missing	464 (7.3)	-	-				
AM in stocks in all the visited DS of the ward							
Yes	5694 (8.9)	669 (11.7)	11.0-12.4	2.53	0.039	-	-
No	233 (3.6)	14 (6.0)	3.4-8.6				
Missing	464 (7.3)	-	-				
mRDTs in stocks in all the visited DS of the ward							
Yes	713 (11.2)	56 (7.9)	6.2-9.5	0.73	0.126	-	-
No	5217 (81.6)	627 (12.0)	11.3-12.8				
Missing	464 (7.3)	-	-				

*HF = Health Facility , **DS = Drug Store, ***AM = antimalarial drug

B)

	Total participants	**Participants with SP in the blood**					
	N (%)	N (%)	95% CI	Bivariate analysis		Multivariate analysis	
				ORs	*p*-value	ORs	*p*-value
Total	**6391** (100.0)	**510** (8.0)	7.4-8.5	-	-		
Sex							
Male	2802 (43.8)	200 (7.1)	6.3-7.9	0.97	0.036	-	-
Female	3573 (55.9)	310 (8.7)	7.9-9.4				
Missing	16 (0.3)	-	-				
Age							
0-4 years	1135 (18.1)	67 (5.9)	4.7-7.1	1.21	<0.001	1.18	<0.001
5-9 years	996(15.8)	43 (4.3)	3.3-5.4				
10-14 years	765 (12.2)	41 (5.4)	4.0-6.7				
15-24 years	973 (15.5)	83 (8.5)	7.1-10.0				
25-44 years	1422 (22.6)	168 (11.8)	10.4-13.2				
45-59 years	552 (8.8)	62 (11.2)	9.0-13.4				
60-100 years	445 (7.1)	39 (8.8)	6.6-11.0				
Missing	103 (1.6)	-	-				
Pregnant							
Yes	104 (1.6)	28 (27.2)	20.0-34.4	4.91	<0.001	4.60	<0.001
No	1569 (24.6)	166 (10.7)	9.4-12.0				
Not applicable	4718 (73.8)	-	-				
Had a fever in the previous 2 weeks							
Yes	1021 (16.0)	127 (12.4)	10.7-14.1	1.58	<0.001	1.71	<0.001
No	5364 (83.9)	383 (7.1)	6.6-7.7				
Missing	6 (0.1)	-	-				
mRDT result							
Positive	1117 (17.5)	26 (2.3)	1.6-3.1	0.26	<0.001	0.28	<0.001
Negative	5272 (82.5)	484 (9.2)	8.5-9.8				
Missing	2 (0.0)	-	-				
Net use							
Yes	4180 (65.4)	356 (8.5)	7.8-9.2	1.07	0.541	-	-
No	2211 (34.6)	154 (7.0)	6.1-7.9				
Missing	0 (0.0)	-	-				
IRS in the previous year							
Yes	1073 (16.8)	82 (7.6)	6.3-9.0	0.44	<0.001	0.46	<0.001
No	5316 (83.2)	428 (8.0)	7.4-8.7				
Missing	2 (0.0)	-	-				
Area							
Urban	2185 (34.2)	224 (10.2)	9.2-11.3	0.57	0.145	-	-
Rural	4206 (65.8)	286 (6.8)	6.2-7.4				
Missing	0 (0.0)	-	-				
Region							
Mbeya	1970 (30.8)	134 (6.8)	5.9-7.7				
Mwanza	2304 (36.1)	281 (12.2)	11.1-13.3	1.59	0.241	3.92	<0.001
Mtwara	2117 (33.1)	95 (4.5)	3.7-5.2	0.56	0.156	1.02	0.942
Missing	0 (0.0)	-	-				
$PfPR_{2-10}$ of the ward							
0 < 1%	1421 (22.2)	131 (9.2)	8.0-10.5	0.89	0.102	-	-
1 - < 5%	0 (0.0)	-	-				
5 - < 10%	1453 (22.7)	107 (7.4)	6.2-8.5				
10 - < 50%	2062 (32.3)	169 (8.2)	7.2-9.2				
> 50%	1455 (22.8)	103 (7.1)	6.0-8.2				
Missing	0 (0.0)	-	-				

$PfPR_{2-10}$ of the district							
0 < 1%	1244 (19.5)	96 (7.7)	6.5-9.0	0.73	0.044	0.77	0.010
1 - < 5%	0 (0.0)	-	-				
5 - < 10%	1470 (23.0)	175 (11.9)	10.5-13.3				
10 - < 50%	2259 (35.3)	191 (8.5)	7.5-9.4				
> 50%	1418 (22.2)	48 (3.4)	2.6-4.2				
Missing	0 (0.0)	-	-		-		
Time to the closest HF*							
<15 min	2134 (33.4)	194 (9.1)	8.1-10.1	0.83	0.003	0.85	0.031
15 min to 1h	3483 (54.5)	279 (8.0)	7.2-8.8				
1h to 2h	564 (8.8)	27 (4.8)	3.3-6.3				
> 2h	167 (2.6)	8 (4.8)	2.1-7.5				
Don't know	43 (0.7)	-	-				
Time to the closest DS**							
<15 min	2911 (45.5)	264 (9.1)	8.2-10.0	0.89	0.105	-	-
15 min to 1h	2599 (40.7)	200 (7.7)	6.8-8.6				
1h to 2h	461 (7.2)	36 (7.8)	5.7-9.9				
> 2h	63 (1.0)	3 (4.8)	0.3-9.2				
Don't know	357 (5.6)	-	-				
ACTs in stocks in all the visited HF* of the ward							
Yes	6391 (100.0)	510 (8.0)	7.4-8.5	-	-	-	-
No	0 (0.0)	-	-				
Missing	0 (0.0)	-	-				
AM* in stocks in all the visited HF of the ward**							
Yes	6391 (100.0)	510 (8.0)	7.4-8.5	-	-	-	-
No	0 (0.0)	-	-				
Missing	0 (0.0)	-	-				
mRDTs in stocks in all the visited HF* of the ward							
Yes	4986 (78.0)	454 (9.1)	8.4-9.8	1.48	0.238	-	-
No	1405 (22.0)	56 (4.0)	3.1-4.8				
Missing	0 (0.0)	-	-				
ACTs in stocks in all the visited DS of the ward							
Yes	5528 (86.5)	467 (8.4)	7.8-9.1	1.27	0.839	-	-
No	399 (6.2)	17 (4.3)	2.6-5.9				
Missing	464 (7.3)	-	-				
AM in stocks in all the visited DS of the ward**							
Yes	5694 (8.9)	479 (8.4)	7.8-9.0	1.39	0.875	-	-
No	233 (3.6)	5 (2.1)	0.6-3.7				
Missing	464 (7.3)	-	-				
mRDTs in stocks in all the visited DS of the ward**							
Yes	713 (11.2)	96 (13.5)	11.4-15.6	1.73	0.223	-	-
No	5217 (81.6)	388 (7.4)	6.8-8.0				
Missing	464 (7.3)	-	-				

*HF = Health Facility , **DS = Drug Store, ***AM = antimalarial drug

6.4.2.Prevalence of antimalarials in the blood of the surveyed population

Antimalarial drugs were present in the blood of 20.8% (1330/6391) (95%CI: 20.0-21.6) of individuals in total: 53.4% (710/1330) had lumefantrine (LF), 14.2% (189/1330) desbutyl-lumefantrine (DLF), 20.5% (272/1330) sulfadoxine (SD), 26.5% (352/1330) pyrimethamine (PM), 1.9% (26/1330) quinine (Q), 0.6% (8/1330) mefloquine (MF), 0.2% (3/1330) chloroquine (CQ), 0.1% (2/1330) amodiaquine (AQ) and 6.7% (86/1330) N-desethyl-amodiaquine (DAQ). Additionally, 59.5% (792/1330) of individuals had LF and/or DLF (107 individuals had both and 685 had one or the other) and 38.3% (510/1330) SP. When considering parent drug and metabolite or combined treatment as one drug (LF/DLF, AQ/DAQ and SP), 6.6% (88) of the 1330 participants with residual drug concentrations in the blood had more than one antimalarial in the blood: 84 individuals had two different drugs, 2 had three drugs and 2 had five drugs. The proportions of participants with each type of antimalarial in their blood per region are detailed in Figure 6.1. In Mtwara, the proportion of individuals with LF/DLF in their blood [74.1% (303/409)] was significantly higher than in Mwanza [50.9% (338/664), $p<0.001$] and Mbeya [54.5% (151/277), $p<0.001$]. Inversely, the proportion of individuals with SP in their blood was significantly higher in Mwanza [42.3% (281/664), $p<0.001$] and Mbeya [48.4% (134/277), $p<0.001$] than in Mtwara [23.2% (95/409)].

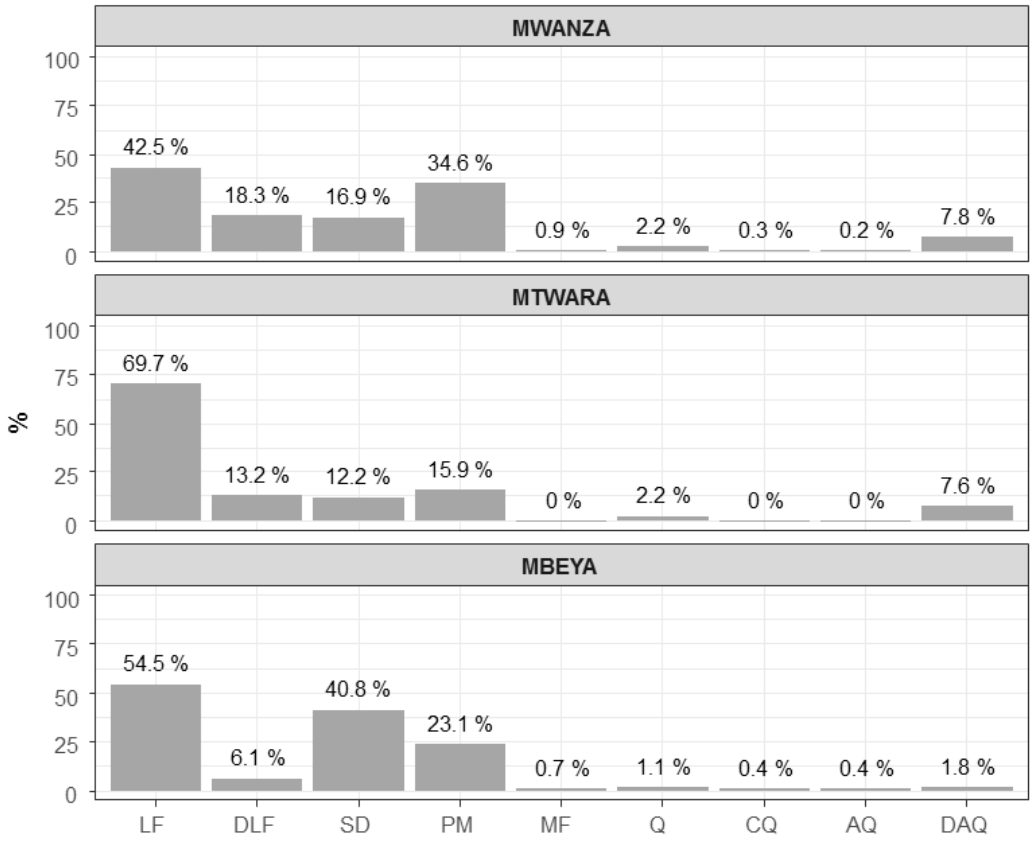

Figure 6-1: Percentage of participants with residual antimalarial concentrations in each study region. LF, lumefantrine; DLF, desbutyl-lumefantrine; SD, sulfadoxine; PM, pyrimethamine; MF, mefloquine; Q, quinine; CQ, chloroquine; AQ, amodiaquine; DAQ, N-desethyl-amodiaquine.

6.4.3.Variables associated with the presence of antimalarials

The full list of variables and interaction terms investigated in the multivariate logistic regression model to assess their association with LF/DLF or SP in the blood are summarized in Table 6.2, while the results of the bivariate and multivariate analysis can be found in Table 6.1. In the multivariate analysis, before including interaction terms (Figure 6.2), an increasing age (OR=0.9, p<0.001) and living further away from a HF (OR=0.8, p=0.007) were associated with lower odds of detecting LF/DLF. Having had a fever in the previous two weeks (OR=2.6, p<0.001), living in a district with higher malaria prevalence (OR=1.5, p<0.001), sleeping under a bed net (OR=1.3, p=0.004) and living in a ward in which all visited DSs had ACTs in stocks (OR=2.7, p=0.020) were associated with higher odds of having DLF/LF in the blood. A statistically significant interaction between age and mRDT result (p<0.001) (Table 6.3) was noted and showed that increasing age was associated with lower odds of having LF/DLF in the blood only for the individuals tested positive by mRDT. A significant interaction between district parasite prevalence and fever (p<0.001) also showed that the likelihood of having

LF/DLF in the blood according to fever was higher at low endemicity than at high endemicity. More details on interactions are provided in Table 6.3.

Table 6-2: Multivariate logistic regression models used in the present analysis.

	LF/DLF Model	SP Model
Outcome	Presence of lumefantrine and/or desbutyl-lumefantrine in the blood	Presence of sulfadoxine and/or pyrimethamine in the blood
Regions	All	All
Number of observations	6279	6278
Variables		
Included	Age; fever in the previous 2 weeks; mRDT result; net use; PfPr of the district; time to the closest HF; ACTs in stocks in DS	Age , pregnant, fever in the previous 2 weeks, mRDT result, IRS in the previous year, region, time to the closest HF
Excluded	Sex; pregnant; IRS in the previous year; area; region; PfPr of the ward; time to the closest DS; ACTs, AM, mRDTs in stocks in HF; AM, mRDTs in stocks in DS	Sex; net use; area; PfPr of the district and of the ward; time to the closest DS; ACTs, AM, mRDTs in stocks in HF; AM, ACTs, mRDTs in stocks in DS
Nested random effects	Regions/districts/wards	Regions/districts/wards
Interaction terms included	Age*mRDT result; PfPr of the district* fever in the previous 2 weeks	Region* pregnant; Region*time to the closest HF

Being pregnant (OR=4.6, p<0.001), living in Mwanza (OR=3.9, p<0.001 compared to Mbeya), having had a fever in the previous two weeks (OR=1.7, p<0.001) and increasing age (OR=1.2, p<0.001) were associated with higher odds having SP in the blood. Being tested positive by mRDT (OR=0.3, p<0.001), living in a house that had been sprayed by IRS in the previous year (OR=0.46, p<0.001), living in a district with higher malaria prevalence (OR=0.8, p=0.010) and living further from a HF (OR=0.8, p=0.031) were also associated with lower odds of having SP in the blood. Considering the interaction between region and pregnancy (p=0.002) showed that the likelihood of having SP in the blood when pregnant was much higher in Mbeya and Mtwara than in Mwanza (Table 6.3). The significant interaction between region and time to the closest HF (p=0.002) revealed that individuals living further from a HF were less likely to have SP in their blood in Mwanza only.

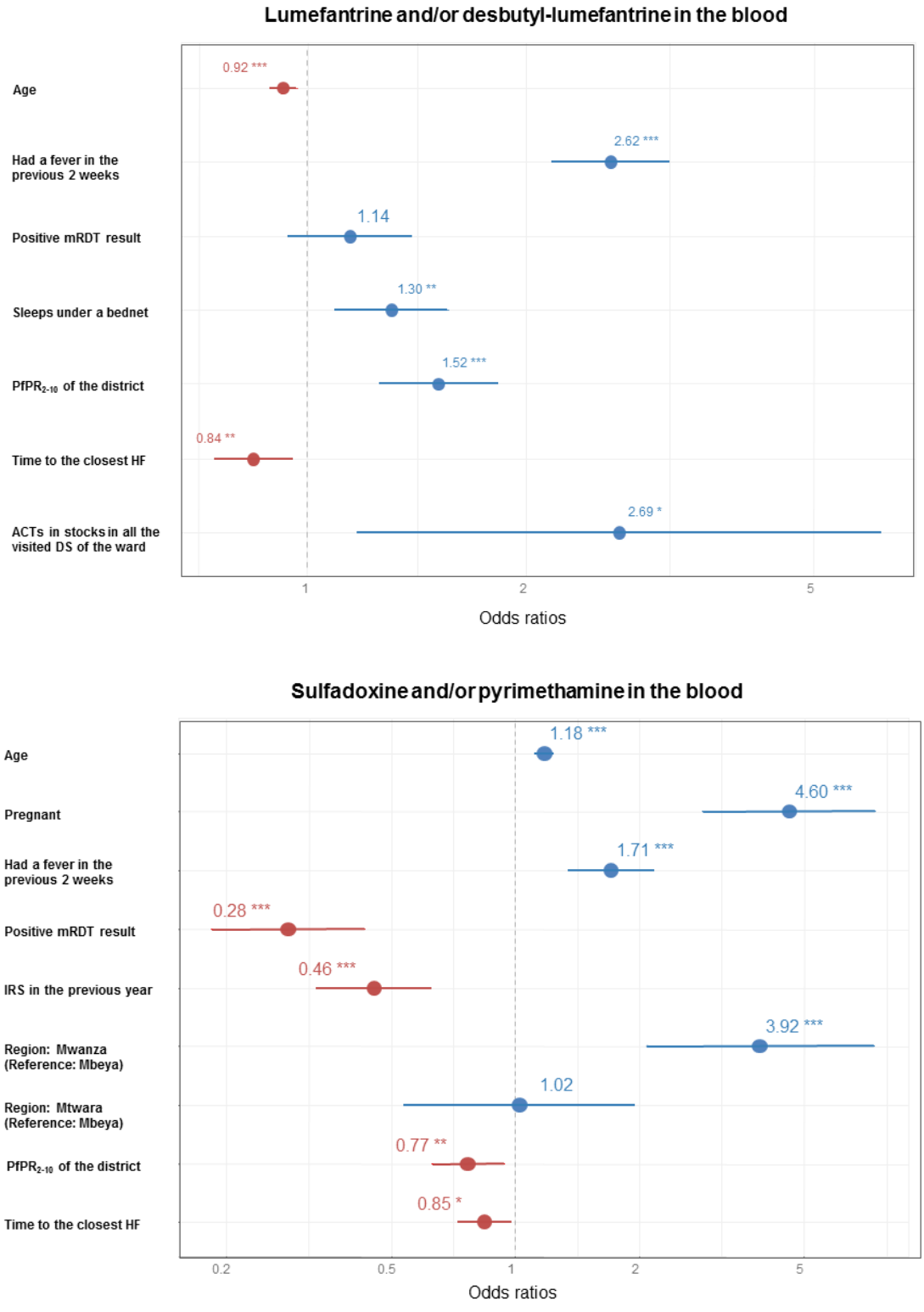

Figure 6-2: Multivariate analysis of the determinants for the presence of LF/DLF (A) and SP (B) in the blood of the surveyed individuals. Lines width corresponds to 95% CI bounds and the number above each line correspond to the association's OR. * indicate the strength of the significance (levels of significance: *= p<0.05, ** = p<0.01, *** = p<0.001) obtained from the multivariate analysis.

Table 6-3: Odds ratios of having LF/DLF or SP in the blood estimated by multivariate logistic regression including interaction terms. These tables only present the variables included in the final adjusted models.

Participants with LF/DLF in the blood		
	Multivariate analysis	
	ORs	*p*-value
Variables		
Age	0.99	0.560
Had a fever in the previous 2 weeks	17.29	<0.001
mRDT result	3.65	<0.001
Net use	1.31	0.003
$PfPR_{2-10}$ of the district	2.01	<0.001
Time to the closest HF	0.84	0.006
ACTs in stocks in all the visited DS of the ward	2.52	0.029
Interactions terms		
Age*mRDT result	0.68	<0.001
$PfPR_{2-10}$ of the district*fever in the previous 2 weeks	0.75	<0.001
Participants with SP in the blood		
	Multivariate analysis	
	ORs	*p*-value
Variables		
Age	1.18	<0.001
Pregnant	12.61	<0.001
Had a fever in the previous 2 weeks	1.70	<0.001
mRDT result	0.29	<0.001
IRS in the previous year	2.17	<0.001
Region		
Mwanza	11.30	<0.001
Mtwara	1.68	0.323
$PfPR_{2-10}$ of the district	0.77	0.013
Time to the closest HF	1.20	0.211
Interactions terms		
Mwanza*Pregnant	0.16	0.003
Mtwara*Pregnant	0.74	0.656
Mwanza*Time to the closest HF	0.57	0.002
Mtwara*Time to the closest HF	0.76	0.223

Table explanation: when including a statistically significant interaction between age and mRDT result (p<0.001), odds of having LF/DLF in the blood decreased with increasing age, but only for the individuals tested positive (OR=0.67 for those tested positive and OR=0.99 for those tested negative). Participants with a positive mRDT result were more likely to have LF/DLF in the blood (OR=3.6, p<0.001), but ORs decreased when age increased (OR=2.4). After including an interaction between district parasite prevalence and fever (p<0.001), the OR of having LF/DLF in the blood according to fever were higher at low endemicity (OR=8.0) than at high endemicity (OR=6.0). Participants were more likely to have LF/DLF in the blood when living in a district of higher endemicity, especially if they did not have a fever (OR=1.6 for those who did not have a fever and OR=1.2 for those who had a fever).

An interaction between region and pregnancy (p=0.002) showed that pregnant women were more likely to have SP in their blood, especially if they lived in Mbeya and Mtwara region (OR=12.7 in Mbeya, OR=9.5 in Mtwara and OR=1.8 in Mwanza). Odds of having SP in the blood were higher when living in Mwanza, but to a lesser extent for pregnant women (OR=1.8 for pregnant women and OR=11.2 for other participants). An interaction between region and time to the closest HF (p=0.002) revealed that odds of having SP in the blood significantly decreased when living further from a HF, but only for those living in Mwanza (OR=0.7). This association was not significant when living in Mbeya (OR=1.2, p=0.211) or Mtwara (OR=0.9, p=0.223). Furthermore, participants living in Mwanza were more likely to have SP in the blood, especially when living close to a HF (OR=11.2 when living close to a HF and OR=6.4 with increasing distance).

6.5. Discussion

Knowing how antimalarial medication circulates in the population and what the most important drivers of antimalarial consumption are may help to improve treatment appropriateness and prevent the emergence and spread of resistant parasites. To our knowledge, this is the first study assessing drug consumption by means of antimalarial blood concentrations including such a large sample size [34,40,202].

Overall, 20.8% of the study participants had antimalarials in the blood. LF/DLF composed more than half (59.5%) of the drugs detected in our sampled population. Most of the factors significantly associated with the presence of LF/DLF in the blood were expected, including history of fever, which had the highest ORs. Indeed, when fever occurs, antimalarials and antibiotics are commonly used as presumptive treatment [34,47], with or without parasitological confirmation. However, odds of having LF/DLF in the blood according to fever were modulated by district parasite prevalence and were found to be higher at low endemicity. This could be explained by the fact that districts with highest malaria endemicity are also the most rural. Shortage of drugs and long waiting time at HFs due to disease burden, long distance to HFs and inability to pay for health care are factors discouraging people to seek treatment in case of fever and which might not apply in urban districts where inhabitants are wealthier and access to care is less of a concern [199,206,207]. The association between younger age and the presence of LF/DF in the blood adds to previous studies reporting that small children are more likely to be taken to the HF and being treated with ACTs [34,201,202,206,207]. The interaction between age and mRDT result revealed that odds of having LF/DLF in the blood in case of positive mRDT decreased when age increased. This supports previous observations that children are better cared than older people, which is encouraging since children are the most vulnerable population [208]. The effect of the distance to HF and drug stocks in DSs on the presence of LF/DLF confirms that access to treatment (including distance to health care provider and treatment availability) leads to more drug consumption, and hence drug pressure [34,198,209]. In our data, distance to DS did not play a role on pattern of drug use, probably because all households were located within a short radius of DSs. Such leveling effect has previously been reported in the literature [198]. We could not detect an effect of drugs availability in HFs on antimalarial use since all HFs visited during the survey had ACTs in stock. However, the significant effect of stocks of ACTs in DSs shows the importance of these providers, as often reported in the literature ([189], CHAPTER V).

SP accounted for 38.3% of all antimalarials detected although it has been shown to have low therapeutic value in Tanzania [17,210], and that SP super-resistant haplotypes are widespread

throughout the country [197]. SP had been officially abandoned as first line treatment in 2006 in Tanzania [17] but is still present in HFs for IPTp [17]. Amongst all individuals, 8.0% (510/6391) had SP in the blood. According to 2015-16 DHS surveys [3], 8.6% of women aged between 15-49 years are pregnant on average. Considering that women aged between 15-49 years represent around 25.0% of the total population [104], one would expect around 2.0% of the participants to have SP in their blood, much lower than the proportion measured in our study. As mentioned before, this large discrepancy goes in line with previous studies reporting the common use of SP as self-medication [196,199,211]. Measuring SP in the blood provides interesting insight on how these treatments are used in different regions of the country and by different population groups. For example, in the univariate analysis, female were more likely to have SP in the blood. However, this association was no longer significant when adjusting for pregnancy in the multivariate analysis as previously reported in the literature [34,40,49,201]. Indeed, it was pregnancy that was a strong predictor of the presence of SP in the blood, which reflects adherence to IPTp programs [17]. This association was nevertheless mitigated in Mwanza, the reason possibly being that in Mbeya and Mtwara the use of SP is more restricted to IPTp while in Mwanza, treating malaria with SP is more common practice. This hypothesis is reinforced by the fact that Mwanza was the region with the highest proportion of individuals with SP in the blood, and the only region for which distance to HF had significant influence on the presence of SP in the blood. Age was also significantly associated with the presence of SP in the blood but contrary to DLF/LF, residual levels of SP were more likely to occur in older people, who have more often recourse to self-medication [212]. These results tend to show that compliance to standard malaria treatment recommendations is lower in Mwanza and in adults.

Almost all antimalarials detected in the studied population were part of the recommended treatments in Tanzania, which is encouraging. Beside LF/DLF and SP, which were the most prevalent, 123 individuals (9.3% of all antimalarials detected) were found with quinine, mefloquine, amodiaquine or its metabolite N-desethyl-amodiaquine (which were more prevalent due to amodiaquine's short half-life of 3.9h when used in ACTs [213]). Quinine is the drug of choice for treatment of severe malaria and second line drug in case of treatment failure with Alu. Amodiaquine and mefloquine can both be found Tanzania in combination with artesunate as a viable option for treating uncomplicated malaria [17]. Three individuals had chloroquine in their blood although it had been banned from the therapeutic armamentarium in 2001.

Previous studies conducted in Tanzania reported that around 2.0% of the fever in low and 8-20% in highly endemic settings ([214,215][25] Boillat et al, unpublished) are due to malaria. In

our study, 1021 individuals reported a fever in the previous two weeks (Table 6.1), and 630 of them said they sought care because of their fever (CHAPTER V). Considering that, on average, 10.0% of fevers are due to malaria according to literature, 63 individuals should have received an antimalarial. This number can be doubled because LF can be detected in DBS samples for up to one month (unpublished data) and history of fever recall was based on a two-week period. These estimations should have resulted in a prevalence of 19.7% (1260/6391) of the total surveyed population (providing all participants who sought care in the previous month were treated) having LF detected in their blood if none had a diagnostic test done and 2.0% (126/6391) if all had been tested and treated upon result. Together with the 2.0% of the population constituted by pregnant women receiving SP through IPTp, we would expect 4.0% of the participants to have an antimalarial in the blood, which is far from what we measured (20.8%). This highlights the urgent need to implement interventions aimed at decreasing drug pressure, notably encourage the use of mRDTs to target treatments to individuals with malaria only.

Our study's main limitation resides in the fact that we did not collect all possible information on potential factors influencing drug use. Socio-economic determinants, knowledge and attitude on malaria management might have been of interest, although differences in wealth might not have been much different, at least in rural areas.

6.6. Conclusion

Understanding pattern of antimalarial drug use has implication in malaria control (access to care as well as diagnostic and treatment policies implementation) and surveillance (parasite resistance). The most significant predictors identified were encouraging since they implied that people took a drug when they had a fever and that children were treated with standard first line treatment, i.e. artemether-lumefantrine. However, the long-standing belief that access to treatment should be increased might need to be revisited [216], as proven by the number of people having drugs in their blood and the good availability of drugs in the health facilities and drug stores. Efforts need rather to be focused on the appropriate use of these drugs, namely to target antimalarial treatment to those who have malaria, with the appropriate dosage and correct adherence, and to recommend the use first-line efficacious drugs in adults [217]. This study has shown that drug pressure was highest in districts of high transmission and in most accessible locations. This observation calls for interventions to be targeted to such areas since they constitute favorable conditions for development and spread of resistant strains.

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7. CHAPTER VII: RESIDUAL ANTIMALARIALS AND RESISTANCE

7. Do residual antimalarials in the blood of individuals sampled in the community predict the presence of resistant parasites? A cross-sectional survey in Tanzania

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7.1. Abstract

Background

Artemisinin-based combination therapies treatment failures have already been observed due to the presence of partner-drug resistance. We investigated whether the presence of residual lumefantrine (LF) or sulfadoxine-pyrimethamine (SP) blood levels in individuals sampled in the community predicted the presence of *P. falciparum* harbouring molecular markers of resistance.

Methods

Community-based cross-sectional surveys took place in three regions of Tanzania with different levels of malaria endemicity. Dried blood spot samples were collected for analysis of antimalarials and specific mutations known to be associated with LF or SP resistance.

Results

Individuals with high LF blood concentrations were more likely to have wild-type parasites (OR=4.80, 95%CI: 1.57-14.70) and a recent episode of fever (OR=2.76, 95% CI: 1.42-5.34) while those with low LF concentration were more likely to have parasites harbouring the *pfmdr1* N86/184F haplotype (OR=4.80, 95%CI: 1.57-14.70) and less likely to have fever (OR=0.36, 95% CI: 0.19-0.70). Low SP concentrations were associated with higher probability of carrying parasites harboring the *pfdhfr/pfdhps* quintuple or sextuple mutations compared to higher concentrations (OR=19.63, 95%CI: 1.44-268.25).

Conclusion

Our data provide evidence that low LF and SP concentrations in the blood of individuals surveyed in the community was associated with the presence of parasites habouring drug resistance markers.

7.2. Introduction

Global malaria control and elimination highly depend on the sustained efficacy of artemisinin-based combination therapies (ACTs) [10]. Due to its short half-life, artemisinin is rapidly cleared from the bloodstream. The partner drug (with longer half-life) persists as a vulnerable monotherapy for weeks after treatment, particularly at sub-therapeutic concentrations, selecting for newly acquired tolerant parasites [36,96]. This constitutes the main weakness of current ACTs and treatment failures have already occurred in the absence of artemisinin resistance, mainly due to partner drug resistance [218,219].

In Tanzania, sulfadoxine-pyrimethamine (SP) was introduced as first-line treatment for uncomplicated *falciparum* malaria in 2001 and replaced in 2006 by artemether-lumefantrine (ALu) due to widespread resistance and high rate of treatment failure [17,101,220,221]. In patients, parasites harboring multiple copies or the N86/184F haplotype of the *pfmdr1* gene are selected by ALu treatment and can withstand high lumefantrine (LF) concentrations [37,96,97,222,223]. In 2010-11 in Tanzania, the prevalence of the *pfmdr1* haplotype claimed to be associated with LF tolerance ranged from 17 to 26% [223]. Regarding SP, the combination of the *pfdhfr* triple mutations (51I/59R/108N) with the *pfdhps* double mutations (437G/540E) confers high-level resistance [197,221]. Recently, these super-resistant haplotypes have been reported to be widespread throughout Tanzania [197].

The influence of LF concentrations on the selection of tolerant parasites has been observed in clinical trials settings [37,97,224,225]. However, there is little direct evidence that drug pressure in the community leads to the emergence of parasite resistance. Previous studies [39,69] showed that in areas of low transmission, drug pressure was the critical factor for parasite resistance, which was not the case in high transmission places. These results illustrate the complex interplay between parasite prevalence, immunity and drug pressure and call for more research using sensitive and reliable methods for drug pressure assessment and integrating various determinants. The aim of our study was to investigate whether the presence of residual LF or SP in the blood of individuals sampled in the community predicted the presence of *Plasmodium falciparum* harbouring molecular markers of resistance.

7.3. Methods

7.3.1.Study area and population

A community-based cross-sectional survey was conducted in three regions of Tanzania from May to August 2015, following the rainy season. The regions represented heterogeneous malaria endemicity, i.e moderately high for Mtwara and Mwanza (17.4% and 16.1%

respectively) and low for Mbeya (2.3%) [20]. Over 95% of inhabitants of mainland Tanzania are at risk of malaria and the population is predominantly rural [1]. Detailed study setting, population and sampling have been described elsewhere (CHAPTER V).

7.3.2.Study sampling and data collection.

Tanzania has four different administrative levels: regions, divisions, districts and wards. In each region, one urban and two rural districts were selected. Random selection was then carried out as follow: three wards per district, four streets or sub villages per ward, 20 household per sub village/street and up to six individuals per household. All consenting individuals were eligible to participate with the exception of those under three months of age or with a severe illness requiring immediate referral. Interviews were conducted in Swahili and capillary blood was collected from all participants. The Open Data Kit collection tool (ODK) programmed on electronic tablets was used to collect the data.

7.3.3.Laboratory procedures

One drop of capillary blood was used for mRDTs analysis (ParaHIT-*f* test, Span diagnostic Ltd, Surat, India, detecting HRP-2 antigens) and four applied on filter paper cards (FTA DMPK-B cards, Whatman, GE Healthcare). Concentrations of seven antimalarials and two metabolites [amodiaquine (AQ), N-desethyl-amodiaquine (DAQ), lumefantrine (LF), desbutyl-lumefantrine (DLF), mefloquine (MF), chloroquine (CQ), quinine (Q), sulfadoxine (SD) and pyrimethamine (PM)] were further measured in the dried blood spot (DBS) samples using a validated liquid-chromatography coupled to tandem mass-sprectrometry (LC-MS/MS) methodology ([131], CHAPTER IV). Artemisinin compounds were not analysed due their very short half-life and their rapid degradation when using the current sampling procedure. The *P.falciparum* DNA was extracted from the same DBS samples and amplified by polymerase chain reaction assay (PCR). Sequencing was then used for the assessment of Single Nucleotide Polymorphisms (SNPs) related to parasites drugs resistances. The SNPs analysed were the *pfmdr1* mutations N86Y and Y184F, *pfcrt* mutations C72S, M74I, N75E and K76T/I/N, *pfdhfr* mutations A16V/S, N51I, C59R, S108N/T and I164L and *pfdhps* mutations S436A/F, A437G, K540E, A581G and A613T/S. The number of copy of the *pfmdr1* gene was also assessed by real-time PCR.

7.3.4.Data analysis

R (version 3.4.0) was used for data analysis to produce summary statistics, logistic regressions and plots using the lme4 and ggplot2 packages.

Drug concentrations were categorised as presented in Table 7.1. A meta-analysis showed that LF plasma concentration >200 ng/ml at day 7 were associated with >98% cure rates and that

lower concentrations constituted favourable conditions for the development of resistance [38]. Taking into account the ratio between LF DBS and plasma concentrations (CHAPTER IV) of approximately 0.5, a cut-off concentration of 100 ng/ml was chosen in our analysis to distinguish between low and high concentrations. Since there is no accurate estimate of in-vivo minimal inhibitory concentration of sulfadoxine (SD) and pyrimethamine (PM) [226], the median concentration measured in our study sample was thus chosen as cut-off concentration (700 ng/ml for SD and 10 ng/ml for PM). Individuals were considered to have an antimalarial in the blood if the concentration measured in their corresponding DBS sample was equal or higher than the lower limit of quantification (LOQ) of the analytical method (CHAPTER IV).

For molecular markers, individuals carrying mixed infections (wild and mutant types) were analysed as harbouring the polymorphism associated with ALu or SP tolerance. Parasites with more than 1.5 copies of the *pfmdr1* gene were considered as having multiple copies [227] (with no distinction between 2 copies and more, as it has been described not to influence the parasites' susceptibility to LF [225]).

Age was categorized based on the age distribution of our sampled population. Mean malaria endemicity of the district was classified according to the categories used in the most recent epidemiological profile of mainland Tanzania [18]. Variables with more than two categories were considered as ordinal, except for the drug concentrations and the regions.

Odd Ratios (OR) for the association between molecular markers of LF and SP tolerance/resistance in the blood of individuals and the presence of lumefantrine and/or desbutyl-lumefantrine (LF/DLF) and SP in the blood, or the presence of low drug concentrations were first estimated using bivariate analysis. Then, ORs for the association between molecular markers of LF and SP tolerance/resistance and the presence of drug in the blood at low concentrations were estimated by multivariate mixed effect logistic regression. ORs were adjusted for all variables that had an influence on the presence of molecular markers of resistance [previously selected by bivariate analysis with a significance level <0.20 and if they showed a model fit improvement (reduction in the Akaike Information Criteria value of the model)]. Regions, district and wards were included as nested random effects.

Table 7-1: Characteristics of individuals carrying parasites in which A) *pfmdr1* gene and B) *pfdhfr* and *pfdhps* were genotyped and strength of association between variables and presence of mutations assessed by bivariate and multivariate analysis. Variables with more than two categories were considered as ordinal, except for the drug concentrations and the regions. $PfPR_{2-10}$ was defined as malaria endemicity.

A)

	Total participants	**Participants carrying parasites harboring the *pfmdr1* N86/184F mutant haplotype**					
	N (%)	N (%)	95% CI	Bivariate analysis		Multivariate analysis	
				ORs	95% CI	ORs	95% CI
Total	**683** (100.0)	**444** (65.0)	11.7-13.1	-	-	-	-
LF blood concentration categories							
>100 ng/ml	20 (2.9)	6 (30.0)	13.1-46.8	Reference		Reference	
20-100 ng/ml	52 (7.6)	35 (67.3)	56.6-78.0	4.52	1.46-14.02*	4.27	1.35-13.48*
<Limit of quantification[a)]	611 (89.5)	403 (65.9)	62.8-69.1	4.22	1.58-11.29**	4.38	1.58-12.10**
Missing	0 (0.0)	0 (0.0)	-				
Age							
0-4 years	109 (16.0)	67 (61.5)	53.8-69.1	0.93	0.84-1.03*	0.89	0.80-0.99*
5-9 years	155 (22.7)	106 (67.9)	61.8-74.1				
10-14 years	152 (22.3)	110 (72.4)	66.4-78.3				
15-24 years	106 (15.5)	68 (64.1)	56.5-71.8				
25-44 years	104 (15.2)	62 (59.6)	51.7-67.5				
45-59 years	37 (5.4)	21 (56.8)	43.4-70.1				
60-100 years	19 (2.8)	10 (52.6)	33.8-71.5				
Missing	1 (0.1)	0 (0.0)	-				
Had a fever in the previous 2 weeks							
Yes	188 (27.6)	106 (56.1)	50.1-62.0	0.57	0.40-0.82**	0.58	0.40-0.84**
No	497 (72.4)	338 (68.4)	65.0-71.9				
Missing	0 (0.0)	0 (0.0)	-				
IRS in the previous year							
Yes	143 (21.0)	82 (57.3)	50.5-64.1	0.68	0.45-1.03	0.68	0.45-1.03
No	539 (79.0)	362 (67.0)	63.7-70.4				
Missing	0 (0.0)	0 (0.0)	-				
$PfPR_{2-10}$ of the district							
0 < 1%	5 (0.7)	3 (60.0)	24.0-96.0	1.21	0.93-1.58	1.18	0.91-1.52
1 - < 5%	0 (0.0)	0 (0.0)	-				
5 - < 10%	56 (8.2)	31 (55.4)	44.4-66.3				
10 - < 50%	288 (42.2)	186 (64.6)	59.9-69.2				
> 50%	334 (48.9)	224 (67.1)	62.8-71.3				
Missing	0 (0.0)	0 (0.0)	-				

a) Limit of quantification of LF = 20 ng/ml
Significance: * =p<0.05; **=p<0.01

B)

	Total participants	Participants carrying parasites harboring the *pfdhfr/pfdhps* quintuple mutation					
	N (%)	N (%)	95% CI	Bivariate analysis		Multivariate analysis	
				ORs	95% CI	ORs	95% CI
Total	605 (100.0)	452 (74.7)	71.8-77.6	-	-	-	-
SP blood concentration categories							
>700 ng/ml of SD and/or 10 ng/ml of PM	7 (1.2)	3 (42.9)	12.1-73.6	Reference		Reference	
20-700 ng/ml of SD and/or 2-10 ng/ml of PM	13 (2.1)	12 (92.3)	80.1-104.5	19.63	1.44-268.25*	19.86	1.44-273.97*
<Limit of quantification of SD and PM[a)]	583 (96.4)	436 (74.8)	71.8-77.7	4.67	0.94-23.33	4.75	0.95-23.81
Missing	2 (0.3)	0 (0.0)					
Region							
Mwanza (reference)	303 (50.1)	262 (86.5)	83.2-89.7				
Mtwara	259 (42.8)	155 (59.8)	54.8-64.9	0.22	0.14-0.33	0.22	0.14-0.32***
Mbeya	43 (7.1)	35 (83.4)	76.3-94.4	0.89	0.35-2.24	0.90	0.35-2.29
Missing	0 (0.0)	0 (0.0)	-				

a) Limits of quantification = 20 ng/ml for SD and 2 ng/ml for PM
Significance: * = p<0.05; ** = p<0.01, ***=p<0.001

7.4. Results

7.4.1.Concentrations of antimalarials measured in the surveyed population

Antimalarials were present in the blood of 20.8% (1330/6391) (95%CI: 20.0-21.6) of participants. The concentration range detected in the 1330 individuals were 6–2165 ng/ml (median=24) for DAQ, 20–8202 ng/ml (median=133) for Q, 20–82046 ng/ml (median=693) for SD, 2–458 ng/ml (median=10) for PM, 4–214 ng/ml (median=15) for MF, 20–436 ng/ml (median=30) for DLF and 20–4459 ng/ml (median=43) for LF (Figure 7.1; only 2 and 3 individuals had AQ and CQ in the blood, respectively, these results are thus not shown).

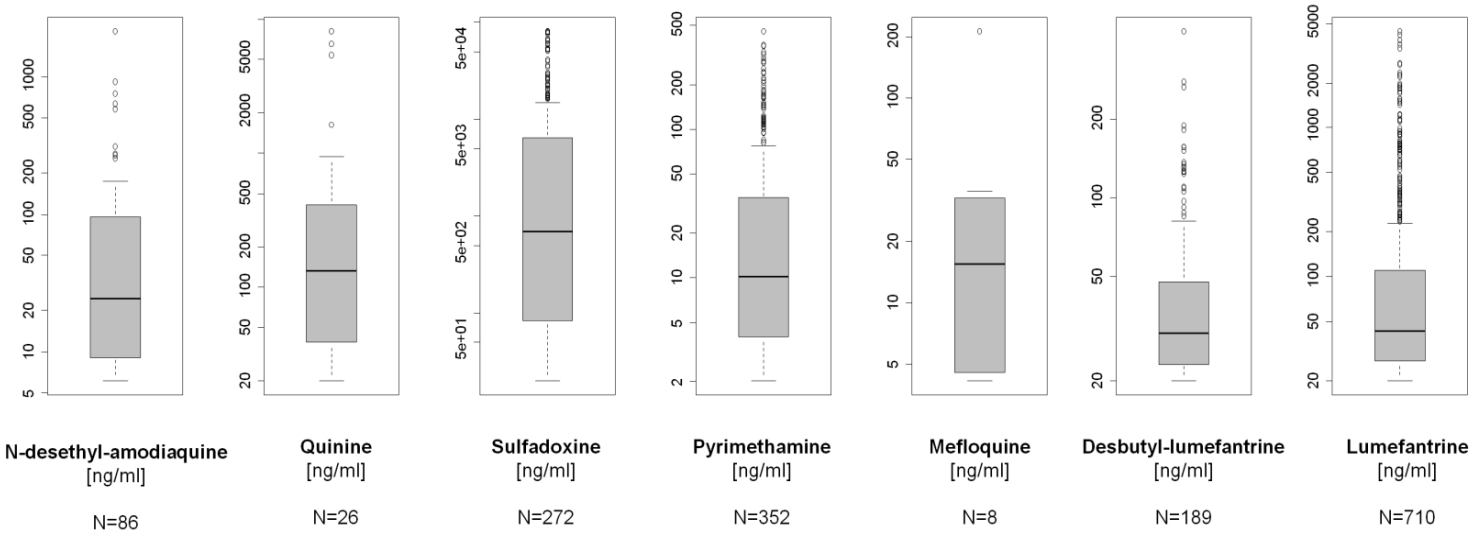

Figure 7-1: Concentrations distributions of each antimalarial, detected in 1330 DBS samples collected in the household survey. The number of samples, median concentration, interquartile ranges, lowers and upper adjacent values and outlier values are shown for each antimalarial. Two and three individuals had detectable levels of amodiaquine and chloroquine respectively, hence these drugs are not represented on this figure.

7.4.2. Relation of prevalence of antimalarials to prevalence of molecular markers of resistance

Amongst the 6485 participants in the household surveys, 17.5% (1136) were tested positive for malaria. The molecular markers analysis was performed in a sub-sample of 683 positive individuals randomly selected from the three regions. Amongst those, 682, 589, 614 and 616 were successfully genotyped for SNPs on the *pfmdr1*, *pfcrt*, *pfdhfr* and *pfdhps* genes, respectively and 635 for the copy number of the *pfmdr1* gene.

The N86/184F haplotype (formed by a mutation on the Y184F codon) and multiple copies of the *pfmdr1* gene were harbored by 65.1% (444/682) and 8.8% (56/635) of genotyped samples, respectively. LF/DLF was detected in 12.5% (85/682) of the samples for which the *pfmdr1* 86/184 haplotype was assessed and in 12.4% (79/635) of the samples analyzed for *pfmdr1* the copy number. Amongst the samples harboring the N86/184F haplotype and multiple copies of the gene, 10.8% (48/444) and 7.1% (4/56) had detectable levels of LF/DLF, respectively (Table 7.2). Having LF in the blood was not associated with higher odds of carrying a parasite with the mutant N86/184F haplotype (OR=0.64, 95%CI: 0.40-1.01) or an increased copy number of the *pfmdr1* gene (OR=0.52, 95%CI: 0.18-1.47). The wild type *pfcrt* 76K allele was present in 85.4% (502/588) of the genotyped isolates. Since this wild type allele is ubiquitous in Tanzania [228], it was excluded from the present analysis.

Table 7-2: Distribution of *pfmdr1* single nucleotide polymorphisms (N86Y, Y184F) and *pfmdr1* multiple copies according to the presence of lumefantrine and/or desbutyl-lumefantrine (LF/DLF) in the blood of the individuals carrying the parasites. P-values were measured using Pearson Chi-square test. Bold underline indicates the amino acid changes.

	***Pfmdr1* polymorphisms**						***Pfmdr1* copy number**			
	N86/Y184 (Wild type)	N86/184**F** (Mutant)	OR	95% CI (p-value)	86**Y**/Y184 (Mutant)	86**Y**/184**F** (Mutant)	N copy=1 (Wild type)	N copy>1 (Mutant)	OR	95% CI (p-value)
	N (%)	N (%)			N (%)	N (%)	N (%)	N (%)		
Total	**232 (34.0)**	**444 (65.1)**			**5 (0.7)**	**1 (0.2)**	**556 (87.6)**	**79 (12.4)**		
LF/DLF in the blood										
Yes	37 (15.9)	48 (10.8)	0.64	0.40-1.01 (0.073)	0 (0.0)	0 (0.0)	52 (9.4)	4 (5.1)	0.52	0.18-1.47 (0.216)
No	195 (84.1)	396 (89.2)			5 (100.0)	1 (100.0)	504 (90.6)	75 (94.9)		

The *pfdhfr/pfphps* quintuple or sextuple mutations (formed by the triple *pfdhfr* 51I/59R/108N mutations combined with *pfdhps* 437G/540E double or 437G/540E/581G triple mutations) harbored by 74.5% (451/605) of genotyped samples. SP was detected in 3.3% (20/605) of the samples genotyped for the *pfdhfr*/*pfdhps* haplotype and in 3.3% (15/452) of those harboring the quintuple or sextuple mutations. Having SP in the blood was not significantly associated with carrying a parasite with these mutations (OR=1.28, 95%CI: 0.42-3.92) (Table 7.3).

Table 7-3: Distribution of *Pfdhfr* (A16V/S, N51I, C59R, S108N/T, I164L) and *pfdhps* (S436A/F, A437G, K540E, A581G and A613T/S) single nucleotide polymorphisms according to the presence of sulfadoxine/pyrimethamine (SP) in the blood of the individuals carrying the parasites. P-values were measured using Pearson Chi-square test. Bold underline indicates the amino acid changes.

	***Pfdhfr* polymorphisms**		***Pfdhps* polymorphisms**		***Pfdhfr* and *Pfdhps* polymorphisms**			
	Other[a]	51**I**/59**R**/108**N** triple mutation[b]	Other[c]	437**G**/540**E** double or 437**G**/540**E**/581**G** triple mutations[d]	Other	Quintuple or sextuple mutations[e]	OR	95% CI (p-value)
	N (%)	N (%)	N (%)	N (%)	N (%)			
Total	**87 (14.2)**	**527 (85.8)**	**87 (14.1)**	**529 (85.9)**	**154 (25.5)**	**451 (74.5)**		
SP in the blood								
Yes	3 (3.4)	18 (3.4)	2 (2.3)	18 (3.4)	4 (2.6)	15 (3.3)	1.28	0.42-3.92 (0.664)
No	84 (96.6)	509 (96.6)	85 (97.7)	511 (96.6)	149 (96.7)	436 (96.7)		

a. including single (A**I**CSI, ANC**N**I) and double mutations (A**IR**SI, A**I**C**N**I, AN**RN**I)

b. A**IRN**I

c. including single (**A**AKAA, S**G**KAA, SA**E**AA) and double mutations (**F**A**E**AA)

d. S**GE**AA, S**GEG**A, **FGEG**A, **AGE**AA, **FGE**AA, S**GE**A**S** or **FGE**A**S**

e. *pfdhfr* A**IRN**I + *pfdhps* S**GE**AA, S**GEG**A, **FGEG**A, **AGE**AA, **FGE**AA, S**GE**A**S** or **FGE**A**S**

7.4.3.Relation of *pfmdr1*, *pfdhfr* and *pfdhps* mutations to LF and SP concentration

LF concentrations measured in the genotyped samples harboring the *pfmdr1* mutant N86/184F haplotype were lower (median = 33.7 ng/ml) than those measured in samples harboring wild type (WT) haplotype (median=69.9) (Figure 7.2). Median LF concentrations did not differ significantly (p=0.074, measured using Mann-Whitney rank sum test) between both haplotypes but as shown in Figure 7.2 and 7.3, the distribution's density at low concentrations was higher for the N86/184F haplotype than for the wild type. The association between *pfmdr1* multiple copies and LF levels was not considered because only four samples harbored multiple copies of the *pfmdr1* gene together with detectable LF levels. By bivariate analysis, having LF in the blood at concentrations <100 ng/ml was associated with higher probability of carrying a parasite with the *pfmdr1* N86/184F haplotype compared to higher concentrations (OR=4.52, 95%CI: 1.46-14.02). Similar results (OR= 4.27, 95%CI: 1.35-13.48) were obtained when calculated by multivariate analysis after adjusting for other significant predictors (age, a febrile episode in the previous 2 weeks, IRS in the previous year and district malaria prevalence) (Table 7.1). As illustrated in Figure 7.3, individuals with high LF blood concentrations (>100 ng/ml) were more likely to have WT parasites (OR=4.80, 95%CI: 1.57-14.70) and a recent episode of fever (OR=2.76, 95% CI: 1.42-5.34) while those with low LF

concentration were more likely to have mutant parasites (OR=4.80, 95%CI: 1.57-14.70) and less likely to have fever (OR=0.36, 95% CI: 0.19-0.70).

Concentrations of PM measured in the genotyped samples harboring the quintuple or sextuple mutations were significantly lower than those measured in samples harboring other SNPs (median = 131.0 ng/ml and 3.7 ng/ml respectively, p=0.034). Amongst the 10 genotyped samples with detectable SD concentrations, only one did not harbor quintuple mutations. By bivariate analysis, having SP in the blood at concentrations <700 ng/ml of SD and/or <10 ng/ml of PM was associated with higher probability of carrying parasites harboring the *pfdhfr/pfdhps* quintuple or sextuple mutations compared to higher concentrations (OR=19.63, 95%CI: 1.44-268.25). Similar results (OR=19.86, 95%CI: 1.44-273.97) were obtained by multivariate analysis after adjusting for the regions (the only other significant predictor) (Table 7.1). No significant interactions were found in the final multivariate models.

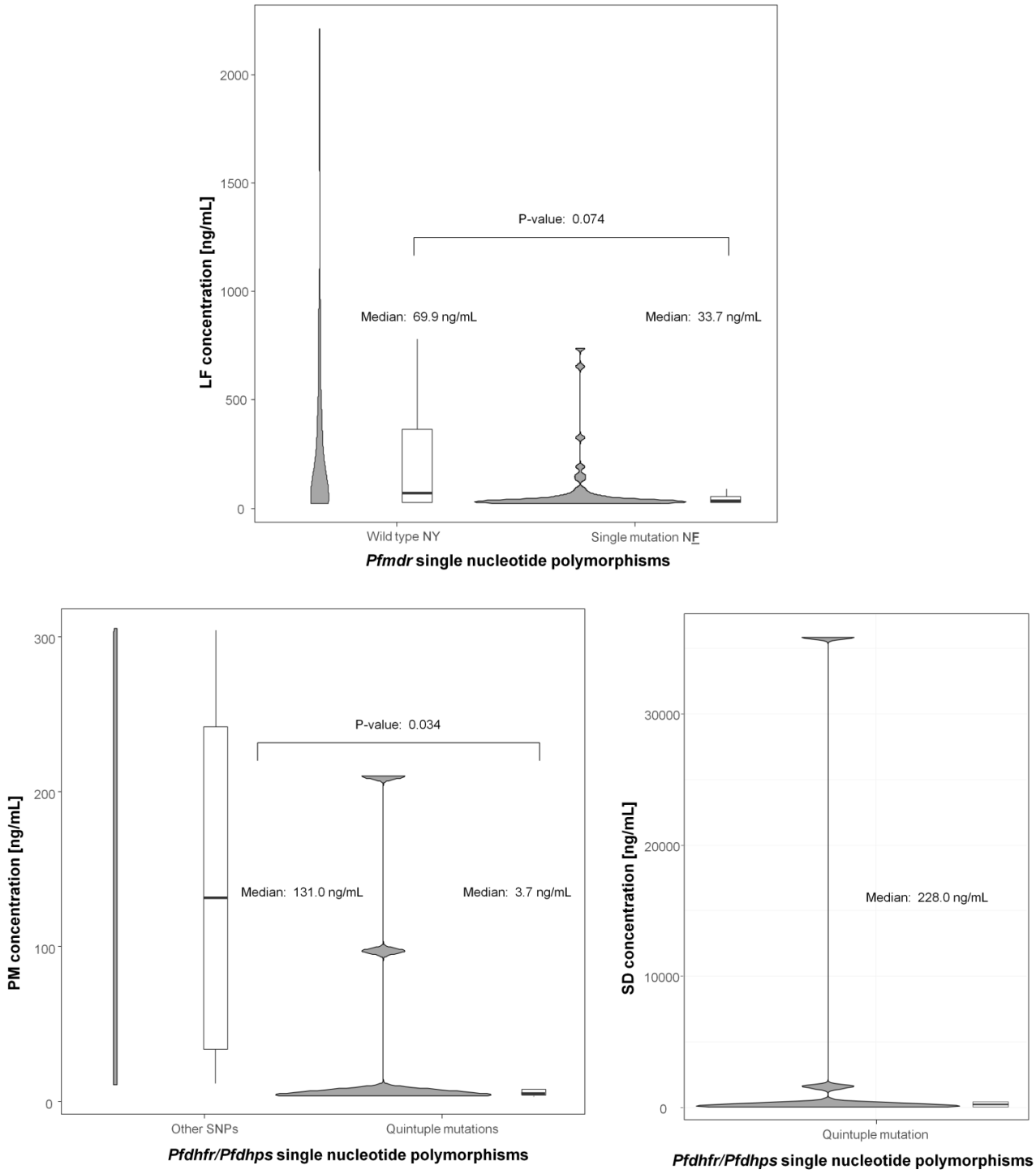

Figure 7-2: Concentrations distributions of lumefantrine (LF), pyrimethamine (PM) and sulfadoxine (SD) measured in DBS samples of individuals carrying *Plasmodium falciparum* harbouring different single nucleotide polymorphisms (SNPs) of the *pfmdr1* , *pfdhfr* and *pfdhps* genes, respectively. The box plots represent the median concentration, interquartile ranges, lower and upper adjacent values (excluding outlier values). The violin plots represent the total distribution of the data (including outliers), as well as the distribution's density, using the width of the plots. Mann-Whitney rank sum test was used to compare median drug concentrations between SNPs.

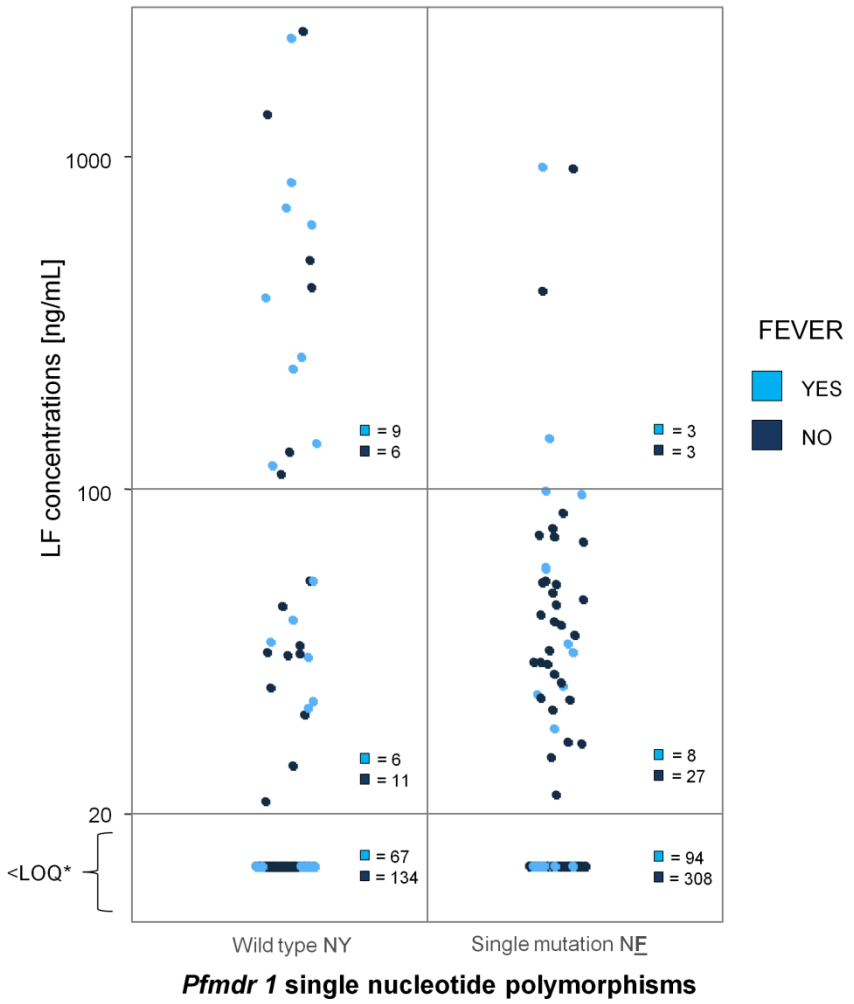

Figure 7-3: Concentrations distributions of lumefantrine (LF) measured in DBS samples of individuals carrying *Plasmodium falciparum* harbouring different SNPs of the *pfmdr1* gene, in relationship with fever. Individuals with LF concentrations >100 ng/ml were more likely to report a febrile episode in the previous two weeks (OR=2.76, p=0.003). *LOQ = limit of quantification of LF (20 ng/ml).

7.5. Discussion

The present survey provides the first data on level of parasite resistance when in relation to residual concentrations of LF in individuals sampled in the community.

ALu had officially replaced SP in 2006 in Tanzania. An increase from 8% to 33% in the occurrence of the *pfmdr1* N86/184F combination from 2006 to 2010 has been reported, excluding mixed infections [221,229]. In the present study, this proportion was 44% (184/415). A study conducted between 2006 and 2011 in Tanzania reported that all samples had one copy of the *pfmdr1* gene [222]. In our study, this figure was 12%. This increase reflects probably the intense use of ALu as first-line treatment in the last nine years and possibly the resulting drug pressure on the parasites in this country.

Unexpectedly, the *pfmdr1* N86/184F haplotype was not more frequent in individuals with residual LF/DLF levels in their blood. A few studies conducted more than a decade ago investigated the association between CQ and SP blood levels and the presence of parasites harboring resistance markers in individuals sampled in the community [79,80,83,230,231]. They showed an association between the presence of drugs and the occurrence of the pfcrt 76T mutation, but not with the *pfmdr1* 86Y mutation, although it was known to be selected by CQ treatments [232]. It is important to stress that the dynamic of resistance is not expected to be the same for monotherapies such as CQ and ACTs due to the presence of the artemisinin component during the first three days of treatment. Furthermore, these studies focused on children and pregnant women populations and do not allow to do a straightforward comparison with our data. In the present survey, individuals harboring LF concentrations above 100 ng/ml were less likely to carry parasites including the *pfmdr1* mutant N86/184F haplotype than those with low or no detectable LF levels. The tolerant parasites selection might result from two events: i) they are present during the primary infection and are selected by the treatment, ii) newly acquired tolerant parasites emerge from the liver and are exposed to sub-therapeutic drug levels from a previous treatment [35,39]. The latter is the main cause of resistance selection because the chances of partner drug mutation emerging from primary infections are low due to the artemisinin [233]. The selection of tolerant parasites is less likely to occur soon after ACT intake due to the presence of the two drugs at high concentrations. The problem arises if parasites exposure to treatment occurs later, when only the partner drug remains in the blood stream at sub-therapeutic level [12,226]. At this point, less susceptible parasites have a survival advantage which leads to their selection and spread of resistance [39]. This window of selection is one of the three key drivers of resistance [35,39]. Our findings are in line with a previous study suggesting that *pfmdr1* N86 selection operates mainly during the elimination phase of LF, precisely 20-30 days after treatment initiation [96].

By multivariate analysis, having had a fever in the previous two weeks and being of an older age were significantly associated with decreased risk of carrying a LF tolerant parasite. Individuals with fever were less likely to carry tolerant parasites because they were treated more recently (as witnessed by their high LF concentrations). The detected WT parasites are most likely those responsible for the primary infection recently treated and not killed yet. Moreover, WT parasites were exposed to a broader range of concentrations due to the higher absolute variability of the concentrations in the early phase of the treatment. Malaria disease severity is directly correlated with parasite density [234]. Tolerant parasites in non-febrile individuals might thus be low densities infections resulting from a previous treatment. The prevalence of resistance to LF being a function of age has already been observed suggesting that acquired immunity played a critical role in the clearance of mutant parasites [226,230,235].

According to previous studies, resistances are more likely to occur in high transmission areas [69,236], because people living in such areas are more likely to be infected at a time when residual drug levels are present in their blood [12,226] and because the resistant parasites are more likely to be transmitted. In our study, this tendency was observed for LF but was not significant. O'Meara et al. mathematical model showed that immunity (linked to age and transmission intensity), treatment rate, drug decay kinetics and presumptive treatment rate are important factors in the spread of resistance, findings also supported by our data [235]. Their model concluded that the spread of partial resistance was driven by drug treatment of uninfected, non-immune individuals.

Many individuals with no LF detectable in their blood carried parasites with the mutant *pfmdr1* N86/184F haplotype. Previous study showed a strong selection of N86/184F persisting for about two months after a single treatment with ALu [237] and that *P.falciparum* can persist at low densities in a host for months after treatment [238]. It is thus conceivable that the tolerant parasites selected by previous treatment or by exposure to sub-therapeutic levels of LF in case of re-infection would remain in the bloodstream for a considerable period, even when drug levels have become undetectable. Since individuals were considered to have an antimalarial in their blood if a concentration equal or higher than the analytical method's LOQ was measured, one could also argue that parasites were in presence of low LF levels which could not be detected but could still have an effect on tolerance selection. Furthermore PCR positivity can persist several weeks after treatment even if parasites are dead [239].

Our findings have several implications. First based on the literature, it is unclear whether ALu therapies can reliably eradicate parasites carrying the N86/184F mutation [240]. The present study is reassuring on that matter since no mutations were found at high concentrations, implying that the artemether component or the synergies between the two drugs clear the mutant parasites effectively.

Then, the median concentrations measured in our sampled population for each drug were very low and are typically expected to be encountered from two (for LF and PM) to six (for SD) weeks after treatment intake [170,171,241–251]. Such concentrations are favorable conditions for tolerance/resistance selection. Studies reported that parasites with the N86/186F haplotype were able to withstand estimated LF concentrations two-fold higher than the N86/Y186 haplotype [97] and that they could re-infect at higher concentrations than WT [37], implying that they can spread more easily [240], with increased risk of recrudescence after ALu treatment [97]. According to authors, some parasites carrying the *pfmdr1* N86/184F haplotype were able to grow at estimated LF blood concentrations of 500nM (=264 ng/ml). The latter concentration is higher than 87% (620/710) of those measured in our study sample. In our

analysis, 73% (521/710) of the concentrations measured in our study sample were below the 100 ng/ml threshold.

In 2010-2011, the prevalence of *pfdhfr/pfdhps* quintuple mutations in Tanzania was on average 77% [252]. In our study, this figure was 75% showing that continuous use of SP maintains the selection of resistance (although not recommended anymore outside of IPTp programs [17]. Similarly to LF, low SP concentrations were associated with carrying resistant parasites. This association was not very strong due to the fact that only 20 individuals had parasites and SP in their blood and that the quintuple mutation in Tanzanian parasite population has been high for many years [252]. Furthermore, there was a significantly lower proportion of SP resistant strains in Mtwara compared to Mwanza and Mbeya, already noticed in 2010-2011 [252], which cannot be explained by drug use nor transmission intensity, because as previously reported (CHAPTER V), Mtwara was not the region with the lowest proportion of individuals with antimalarials in their blood nor the lowest malaria endemicity. Kavishe *et al.* [197] speculated that factors related to population movement and cross-border spread of resistance could contribute to these differences.

Our study has several limitations. First, the final sample size was rather small due to the fact that only few people had LF together with parasites harboring multiple copies of *pfmdr1* in their blood or SP together with parasites. Then, it was impossible due to study design to know if the parasites were remaining from a previously treated infection or if they originated from a new infection. This information would have helped interpreting the role of LF levels on tolerance selection. Finally, due to their high prevalence, mixed infections were excluded from the analysis. We postulated that individuals carrying a mixed infection were carrying the polymorphism associated with ALu tolerance. This assumption is debatable but the same magnitude of the effect was observed when analyzing mixed infections as a third distinct group.

7.6. Conclusion

We demonstrated that the presence of low LF concentrations in the blood of individuals surveyed in the community was associated with the presence of parasites habouring markers of drug resistance. This proves *in vivo* the direct correlation between residual antimicrobial drug and occurrence of resistance. These results highlight the fact that the selection of resistant parasites is expected to remain as long as the population is treated by artemisinins combined with long half-life parent compounds. In this context, the argument that long half-life antimalarials confer protective efficacy a few days after treatment should be weighed against the increased risk of mutant parasites selection. The latter is of particular concern

because the prevalence of resistant parasites was already high in our survey and seemed to be increasing since ALu introduction, in 2006 [6]. Although the latest data still show good treatment efficacy of ALu in Tanzania [253], a better targeted use of antimalarials in favor of parasitologically confirmed malaria cases only should be strongly promoted [235]. Our data support the current view that combinations should ideally include drugs with close half-lives [240]. Lately, the possibility of using triple combination therapy to prevent resistance has been investigated with encouraging results [254].

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8. CHAPTER VIII: GENERAL DISCUSSION

The main purpose of this thesis was to evaluate the usefulness of measuring concentrations of antimalarial drugs in the blood to assess drug pressure in the general population and to understand how levels of residual antimalarial drugs in community surveys interrelate with malaria prevalence, access to care, diagnosis and treatment practices as well as drug resistance. We drew on previous evidence that the use of drugs cannot be accurately reflected by self-report [49,50], suggesting that methodologies relying on objective and standardized endpoints should be used, or at least added, to monitor interventions aimed at improving access and rational use of drugs in Tanzania [26,27].

First, it was important to measure antimalarials using sensitive analytical methodology adapted to field conditions. For this purpose, we developed a LC-MS/MS assay for the simultaneous analysis of the main antimalarial drugs used in combination with artemisinin derivatives in Tanzania in dried blood spots (DBS) samples (CHAPTER IV). The proposed assay was found to be suitable for the analysis of residual antimalarials in DBS samples collected at large scale thanks to the high sensitivity achieved, the convenience of a single extraction procedure and a good stability of the compounds in the DBS samples.

The developed assay was applied within the frame of a PK study including 16 healthy volunteers who received a single adult dose of ALu. LF concentrations in plasma and in DBS were highly correlated at all time points (R=0.97) (CHAPTER IVa) and lumefantrine (LF) half-life calculated from plasma and DBS measurements was similar (101.9 hours in plasma *versus* 103.6 hours in DBS) (CHAPTER IVb). This confirmed that LF concentrations determined in DBS could be used as surrogate to plasma concentrations.

The analytical method was then used for measuring residual antimalarial drugs concentrations in 6485 DBS samples collected in a cross-sectional survey rolled-out in three regions of Tanzania with different levels of malaria endemicity (CHAPTER V). This study showed that amongst 6391 participants included in the antimalarial analysis, close to one fifth (20.8%) had antimalarials detected in their blood. There was no relationship between the level of malaria transmission and the proportion of drug circulating in the blood of the population at regional level. According to antimalarials measurement, self-reporting of drug intake appeared to be unreliable and there was room for improvement with regard to diagnosis and treatment practices in case of febrile episode. Indeed, 31% of the individuals with fevers seeking care were appropriately tested and 50% appropriately treated for malaria.

Understanding patterns of antimalarial drugs use might help to improve treatment appropriateness and decreasing drug pressure. This was addressed in CHAPTER VI where we used a multivariate analysis to determine the factors associated with the presence of

lumefantrine and/or desbutyl-lumefantrine (LF/DLF) or sulfadoxine-pyrimethamine (SP) in the blood, which were the antimalarials identified as most frequently used in our surveyed population.

Finally, the main consequence of drug pressure is the emergence of parasite resistance. In this context, we investigated in CHAPTER VII whether the presence of residual LF or SP in the blood of individuals sampled in the community predicted the presence of *Plasmodium falciparum* harbouring molecular markers of resistance. We found that having LF or SP in the blood was not associated with higher odds of carrying a parasite harboring mutations known to reduce parasites susceptibility. However, by multivariate analysis, having LF or SP in the blood at low concentrations was significantly associated with higher probability of carrying a mutant parasite.

All these aspects provide a unique perspective on drug pressure and its relation to diagnosis and treatment environment and drug resistance in settings with different levels of malaria endemicity. In this section we will discuss questions, challenges and limitations that have arisen throughout the project. We will present the implications of our research findings and how they can be further implemented by addressing the following points:

- Determination of antimalarials concentrations in DBS
- Drug measurements: gold standard to evaluate new diagnosis and treatment strategies and their impact on drug pressure?
- Challenges in improving malaria case management at population level
- Residual antimalarials: implications for parasites resistance and case management

Table 8-1: Contribution of each chapter of the thesis to innovation, validation and application

Chapter title	Related objectives	Innovation	Validation	Application
IVa) LC-MS/MS method for the simultaneous analysis of 7 antimalarials and two active metabolites in dried blood spots for applications in field trials: analytical and clinical validation **IVb)** Lumefantrine pharmacokinetic using plasma and dried blood spots: a comparison in healthy volunteers	To develop and validate a LC-MS/MS assay for the simultaneous analysis in dried blood spots (DBS) of the main antimalarial drugs used in combination with artemisinin derivatives in Tanzania.	Development of a methodology for antimalarial drugs blood measurement using a new sampling approach.	First validation of DBS sampling versus plasma sampling of an antimalarial using population pharmacokinetics approach.	The methodology could be used to assess DBS drug concentrations in the frame of an epidemiological cross-sectional study and an efficacy study.
V) Appropriateness of malaria diagnosis and treatment of fever episodes according to patient history and antimalarial blood measurement: a cross-sectional survey from Tanzania	To measure blood levels of residual antimalarial drugs (primarily LF and SP) in representative samples of people in malaria endemic areas and to compare these results with self-reported history of drug use. To use antimalarials blood measurements in community surveys to assess diagnosis and treatment appropriateness in case of fever episode at population level.	New approach to assess appropriateness of case managements instead of self-reported history.	-	Drug measurements could complement or replace traditional survey methods to assess drug use and case managements. Results could be used by health authorities to develop interventions aimed at improving diagnosis and treatment appropriateness.
VI) Predictors of residual antimalarial drugs in the blood in community surveys in Tanzania	To use antimalarials blood measurements in community surveys to evaluate the actual state of population drug pressure and to identify the factors associated with the presence of LF or SP in the blood.	New approach to assess drug pressure at population level and to determine factors associated with the use of LF and SP. First report exploring factors associated with LF in the blood.	-	Results could be used by health authorities to plan targeted interventions aimed at decreasing antimalarial drugs pressure at population level. The methodology could be used as a new approach to assess treatment policies implementation.
VII) Do residual antimalarials in the blood of individuals sampled in the community predict the presence of resistant parasites? A cross-sectional survey in Tanzania	To investigate whether the presence of residual LF or SP in the blood of individuals sampled in the community predicts the presence of *Plasmodium falciparum* harbouring molecular markers of resistance.	First results of the effect of LF blood concentrations in the blood of individuals surveyed in the community on the selection of *P.falciparum* harbouring resistance markers.	Provides in vivo proofs of the direct correlation between residual antimicrobial drug and occurrence of resistance, already observed in clinical trials settings.	These results could be used by health authorities to develop interventions aimed at promoting better targeted use of antimalarials. Antimalarial drugs combinations should include drugs with close half-lives.

8.1. Determination of antimalarials concentrations in DBS

The building block of this thesis was to develop an analytical assay meeting the specific needs required by field epidemiological studies (CHAPTER IVa). Our study design necessitated that the analytical method could offer the convenience of a single extraction procedure and a single analysis of several drugs from different classes, in order to be applied to a large number of samples. The one step extraction and multiplex analysis also resulted in decreasing analytical costs.

The assay was sensitive enough to reach lower concentrations than in previously published methods [113] [2 ng/ml (sulfadoxine) and 20 ng/ml (chloroquine, quinine, pyrimethamine, mefloquine, LF and DLF)], which was necessary due to the fact that the concentrations measured in the samples were mainly low residual concentrations. Most of the concentrations detected in the DBS samples collected in Tanzania were close to the method's limit of quantification (LOQ). A higher sensitivity of the method could have enabled to better discriminate individuals with very low residual concentrations from those without any drug in the blood, providing a more accurate picture of population drug use. But in order to achieve higher sensitivity, a last generation liquid-chromatography coupled to tandem mass-spectrometry (LC-MS/MS) platform and a complex sample preparation would have been needed, resulting on an increase in time and costs. An analytical method needs to be chosen according to its intended use and in our case, the gain in sensitivity needed to be balanced against the increase in costs and analytical time. In order to assess drug pressure before and after intervention at larger scale, a simple and cheap method is adequate. In contrast, to assess drug pressure in a specific population or in the frame of pharmacokinetics studies, a more tedious and expensive but sensitive method should be chosen.

The stability of antimalarials in DBS samples is a critical element. A benefit of the DBS sampling technology is the supposed analytes stabilisation after the blood spots have become dry [93]. In our case, the stability provided by the solid and dried form was sufficient to store the samples at room temperature for 24 hours, enough time to ship the samples to a laboratory or a health facility (HF) where they could be stored between -10 and -20°C.

We noticed during the analytical method's development the important influence of the haematocrit and volume of blood spotted on the results accuracy. While the latter can be easily addressed in field conditions by using glass capillaries of defined volume coated with heparin, the haematocrit effect is more difficult to handle. Recently, new devices have been developed to elude haematocrit and blood volume issues [255–258] but again, the relating

costs are important and some studies have reported an overestimate of analytes concentrations [256–259].

The sensitive and rapid analytical developed (to be used in field conditions/remote settings) can detect LF concentrations for up to one month after treatment intake (CHAPTER IVb). The comparison between pharmacokinetic (PK) parameters of LF obtained from plasma and DBS measurements showed that DBS can be used as a confident surrogate to venous sampling (CHAPTER IVb). Our methodology could support an accurate interpretation of clinical trial outcome and could improve our understanding on the parasites phenotype associated with decreased susceptibility and its selection after treatment intake. However, regarding the other antimalarials, our study showed that a correlation between DBS and plasma is necessary due the fact that drugs distribution is not even in the different blood compartments and to the lack of PK/PD studies based on whole blood sampling (CHAPTER IVa).

8.2. Drug measurements: gold standard to evaluate new diagnosis and treatment strategies and their impact on drug pressure?

8.2.1. Comparison between traditional surveys and drug measurements

Table 8.2 gathers the type of information obtained, the methods used and the limitation of "traditional surveys" (such as household surveys using questionnaires, HF and private medicines retails outlets surveys) and surveys using drug measurements.

Measuring drug in the blood of the population allows moving from subjective to objective and reproductive data. Using drug measurements is reliable and reproducible, but this methodology lacks capturing information such as treatment seeking behavior and diagnosis. If it can identify accurately the type of antimalarials used in the population, the amount and exact timing of drug intake cannot be assessed. LF's inter-individual variability in terms of absorption and half-life [168] brings variability on the number of days up to which LF can be detected in the blood (CHAPTER IVb).

The limitations relating to the DBS sampling technology have been discussed before (CHAPTER VIII, section 8.1) and are few, thanks to the possibility of using capillary tubes to standardize the volume of capillary blood sampled and by the good stability of the drugs in DBS for 24 hours at room-temperature. The main question with regard to drug measurements at population level is whether it is easily implementable and affordable. The LC-MS/MS platform is expensive (approximately 50 dollars for measurement of nine different antimalarials in one DBS sample) and requires technicians specifically trained for the machine's maintenance.

An alternative could be to use liquid chromatography coupled to UV detection (LC-UV) which requires less-skilled technologist and lower start-up costs [260]. The main drawbacks of LC-UV is the loss of sensitivity and selectivity [261] which implies the need of an extensive sample clean-up and to restrict to a lower number of drugs analyzed simultaneously. If drug measurements are used to evaluate drug pressure before and after an intervention with the aim of assessing the impact of the intervention's implementation, a less sensitive methodology can be used. This would decrease the power of the study but would still be able to catch the magnitude of the change in drug pressure. Moreover, some University laboratories in sub-Saharan Africa (including Tanzania) are already well-equipped with facilities such as liquid or gas chromatography coupled to UV detection to perform biomedical analysis [262]. The use of already existing platforms could push forward local capacities and give opportunities to train local laboratory technicians and data analysts.

Traditional survey methods such as HF and drug outlet surveys relying on exit interviews, recording of drug stocks and prescription rates have the advantages of providing information on the availability and quality of malaria rapid diagnostic tests (mRDTs) and drugs at different levels of the private and public health sector. Information collected can serve as baseline indicator in countries to scale up diagnostic tools and treatments availability [263]. To some extent, they also inform on diagnosis and prescription habits, guidelines compliance, knowledge and ability of health workers. They reflect the potential capacity of the health system.

Table 8-2: Comparison between traditional surveys and surveys using drug blood measurements

References	Survey design	Data collection methodology	Information collected	Limitations
Traditional surveys				
[32,263–269]	- Health facility surveys (private and public sector) - Private medicine retails outlet surveys	- Audit sheets (filled by direct observation or report from providers) - Patients exit interviews - Mystery shopper - Providers register books	**Diagnostic tools:** - Stocks (number, brand, manufacturer) - Distribution (sold or used in the previous 7 days) - % of patients tested - Microscopy services available **Antimalarials:** - Prices, availability, market share, brand - % of patients tested, compliance to test result - % of patients prescribed (AM and ATB) - Formulation, brand **Other:** - % of patients referred - Knowledge and ability to test and appropriately treat patients - Safety disposal	- No information on how mRDTs are being used - Providers often had a waiver to perform mRDTs under pilot study conditions - Providers report subjected to bias (stocks, pricing, sales) - Hawthorn effect - No certainty that patients ingested drugs - No information on the number of visits in private medicine outlets → difficult to assess the proportion of visitor receiving a drug - Do not include diagnosis-only service providers (private laboratories) - Recording errors - Time consuming for field workers - Cost burden - Intense training needed - Respondent fatigue
[3,20,42,270,271]	Household surveys	Questionnaires	- Treatment seeking behavior - % of people with fever obtaining an antimalarial or ACT (by treatment source) - Information on economic status, demographics, access to treatment, preferences	- Unreliability of self-reported data (mRDTs, antimalarials prescription, treatment seeking behavior, antimalarial consumption) - Large sample size needed - Respondent fatigue - Time consuming for field workers - Cost burden - Lack of harmonization for history of fever recall (previous 48h *versus* previous 2 weeks) - No certainty that patients ingested drugs
Drug measurements				
-	Household surveys	Dried blood spot samples	- % of people with antimalarials in the blood - Type of antimalarials used in the population - Treatment seeking behavior - % of people with fever obtaining an antimalarial or ACT (by treatment source)	- Unreliability of self-reported data (mRDTs, treatment seeking behavior) - Lack of harmonization for history of fever recall (previous 48h *versus* previous 2 weeks) - Cost burden - Logistical needs required by sampling method

However, regarding coverage of appropriate antimalarials treatment at the community level, such design pain tackling the actual component of drug use.

Demographic health surveys (DHS) and malaria indicator surveys (MIS) have been collecting information on intervention and malaria related outcomes in Tanzania since 2000 [272]. They have the advantage of informing on the final effect of an intervention at the community level, to help understand the context influencing treatment seeking behavior and drug use. They can also replace HF data relating to malaria prevalence in children under five and case management when they are not very reliable. Their main limitation is the unreliability of information ([40,69] and in CHAPTER V: only 28% concordance for individuals having detectable levels of antimalarials in their blood reporting the use of such treatments within the previous month). In fact, the need for developing more sensitive and specific methods to assess impact of interventions has already been expressed due to, for example, recall bias or omissions from the interviewees and gaps in disease identification due to non-specific symptoms [271].

More generally, traditional surveys provide data available at national level, but they result in high costs and human resources burden and no data on quality of care are collected [271]. Also, due to the length of the survey, questionnaires in community surveys have mainly focused on children under five [3], leaving aside the adult population which is not similar regarding health seeking behavior and case management (CHAPTER VI, [34,198]).

8.2.2.What method should be used?

Household surveys are necessary because what is happening in the HF does not reflect the way the whole population is managed. Indeed, it is of paramount importance to assess the association between the implementation of an intervention and public health gain, and to ensure interventions reach those in need [266]. As shown in Figure 8.1, under real conditions, there is a measureable "effectiveness decay" resulting from the effect of diverse elements of the health system (including challenges in financing, procurement, work force, supply chain and adherence, change in care seeking behavior) [273].

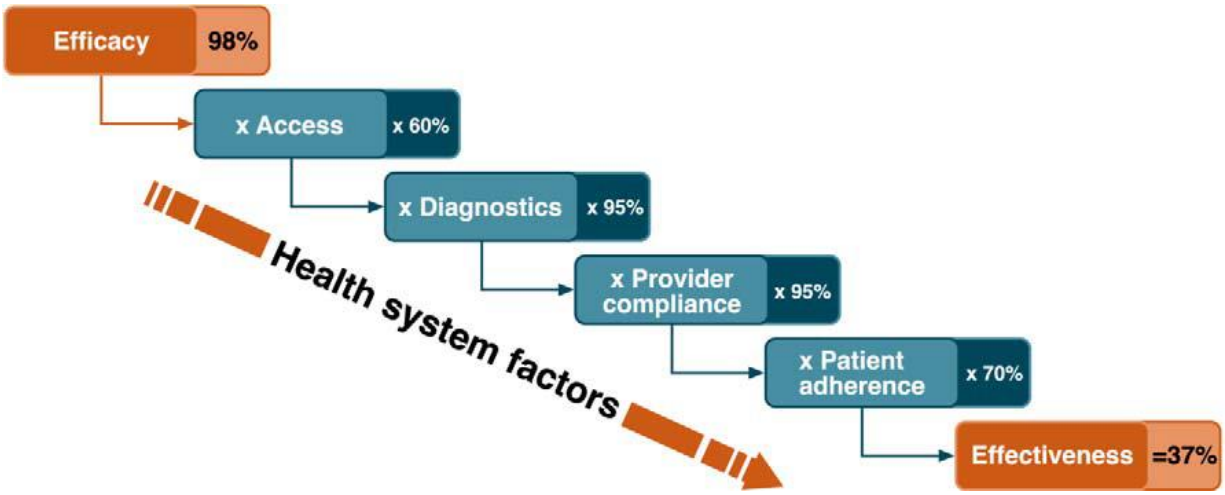

Figure 8-1: Systems effectiveness framework showing how intervention's efficacy (here ACTs) translates into effectiveness. Source: malERA Consultative Group on Health Systems and Operational Research [274].

Household surveys are one step further from outlet surveys and can measure the effectiveness of an intervention more than its efficacy [273,274]. If programs aim at improving rational use of drugs, then drug pressure and resistance should be the most valuable final outcomes, and should be measured in the community due to the potential influence of many actors within and outside the health system.

The data need furthermore to be informative, practical in terms of collection and interpretation [273]. The "minimal needed data" is a balance between collectable and informative. Our study was a proof of concept and showed that reliable and informative data can be collected. Drug measurements could furthermore be integrated into existing intervention packages (for example, along with malaria prevalence assessment by mRDTs where capillary blood is collected anyways).

Questionnaires are important to understand the context which has a major role in determining an implementation performance [266]. Blood measurement could be used to assess drug prevalence, concentrations (in relation to resistance) and the type of treatment while questionnaires can inform on the frequency of drug use and to describe the sociological and behavioral context in which drugs are used, whose importance is not to be disputed [40]. But thanks to the high quality of data provided by drug measurements, the sample size could be smaller. Moreover, there is a need to establish a tool for analyzing effectiveness decay and to understand and mitigate this effect [273]. Drug measurements, maybe performed on a sub-sample of individuals, combined with questionnaires and HF surveys could be a tool to assess and understand this effectiveness decay.

8.3. Challenges in improving malaria case management at population level

8.3.1.Importance of engaging the whole health system for a public health impact

The final aim of implementing new case management is to improve not only short-term health outcomes but also long-term outcomes at individual and population level through rational use of drugs.

As presented in CHAPTER V and VI, 20.8% of the surveyed population had antimalarials in the blood. This overall drug pressure reflects good drug coverage and access, even at community level, thanks to initiatives such as the Affordable Medicines Facility-malaria (AMFm) initiative that achieved access to affordable drugs in remote places [265]. Our study showed that fever was highly correlated to the use of antimalarials, meaning that there is still a strong belief that fevers are mainly due to malaria. As explained in CHAPTER VI, if all fever cases were treated with an antimalarial, this would lead to a proportion of 19.7% (close to the 20.8% mentioned above) of study participants having drugs in their blood, instead of the 2.0% corresponding to real febrile malaria cases [25,214,215]. These results are less encouraging than those of other studies [188] and showed that more than availability of diagnosis and treatment, the actual use and compliance of mRDTs is an issue. Indeed, all of the surveyed HF had antimalarials in stocks and 80% of them had mRDTs (CHAPTER V). However, there was no correlation between the availability of mRDTs in HF and the prevalence of individuals with antimalarials in the blood (CHAPTER VI). Availability or mRDTs alone is not sufficient to ensure the appropriate use of ACTs (either because mRDTs are not used or because of poor compliance to test results) [30,182].

Systematic testing and treatment upon result will not be achieved as long as countries do not take a "total health system" approach to address the challenge of effective malaria case management, as recommended by the Global Fund [275]. Indeed, the poor diagnosis and treatment practices in case of febrile episode were mainly due to the high proportion of individuals seeking care outside HF (CHAPTER V and VI). This calls for a better understanding of drug and diagnosis coverage and quality in all provider types, and strategies designed to tackle these challenges, while considering the relationship between sectors.

8.3.2.Target interventions: where and how?

The cost of ignoring private (registered and unregistered) sector is high for public health but the choice of the appropriate approach to engage this sector is open to debate [276]. Lately, pilot programs have aimed at encouraging private providers to improve the quality and coverage of their care [276] but evidence of the benefit of such interventions to date is very mitigated.

Can we reduce antimalarial consumption by allowing drug retailers to perform malaria testing? A recent review assessing the implementation of mRDTs in private drug stores showed that although mRDTs were made available in the private medicine outlets, this was not sufficient to ensure their appropriate use [270]. Indeed, most of these studies showed increase testing and good compliance but most of them were pilot studies implemented at small scale, at one point in time, and were given a waiver to perform mRDTs [270]. Studies with more intensive interventions and supervision produced better results but uncertainties remain regarding the consistency of the observed effect in the long term and when scaled up at national level for the following reasons: i) simple interventions such as mRDTs requires complex supporting interventions [267] which have been proved not to be very successful at HF level, i) the regulatory framework prohibiting private providers to sell mRDTs makes the scaling up difficult [268] iii) a complete and successful training and supervision of all private drug retailers in a country is ambitious given the fact that some of them are not even registered [23]. Other challenges are the lack of referral system from private retailers to public facilities and the many rationales for providers to provide antimalarials to patient with a negative test such as fear for the patients that the disease worsens without antimalarial, patients demand, time consuming, or incentive to make sale although the test is negative [270].

Although drugs are more expensive in drug stores than in HF [189], other reasons motivate people to seek care outside of HF, such as feeling quality of service is poor in HF or not wanting to wait, not wanting to pay for a consultation and drug stock outs [189]. These reasons were also frequently noticed during our fieldwork. Efforts should thus be invested in training more HF staff, frequently supervise them and encourage them to retain where they are needed [277]. Another reason for not seeking care in HF is their remoteness, especially in rural areas [278]. Training more community health workers in villages could address this issue and could not only improve well-targeted ACTs treatment at community level, but also create opportunities for improving management of non-malaria fever by referring patients to the HF, only when needed [191].

Another important matter regarding case management is that even when appropriately tested in HF, half of the people tested positive did not have any antimalarial in the blood (CHAPTER V). It might be due to non-compliance of HF to positive results or non-adherence of patients to treatment. The latter raises a concern regarding resistance if people do not take the full course of their treatment and should also be addressed [194].

8.4. Residual antimalarials: implications for parasites resistance and case management

Pfmdr1, *pfdhfr* and *pfdhps* mutant isolates were frequent in the surveyed regions (CHAPTER VII). The use of antimalarials seems to be a factor contributing to the selection of resistant parasites in the host, but not in the way it was expected. Indeed, the presence of LF in itself in the blood was not associated with the presence of parasites harboring markers of drug resistance, but the presence of low LF concentrations did. Our results are consistent with previous studies that suggested the "window of selection" as a key driver of resistance [39]. According to Hasting and Ward [240] parasites carrying the *pfmdr1* N86/184F mutation can infect a new host 17 days after treatment onset. The simulation performed using the PK model constructed in CHAPTER IVa showed that 50% of individuals had detectable residuals LF levels for up to 26 days, meaning that mutant parasites can be selected during a period of at least 9 days. In Tanzania, in a settings of similar malaria endemicity as our study regions (21.0-24.6%), the average EIR (=average number of infectious mosquito bites [279]) was 1.1-1.3 per month in 2011 [280]. It implies that a person taking ALu has a chance of approximately 35% to be infected during that 9 days period of selection (chances being even higher during the peak of transmission after the rainy season). This constitutes the drawbacks of using drugs with long half-life such as LF and SP. The problem with shorter half-life compound is that they need to be given more frequently and for a longer period, which could decrease patients' adherence [35].

Another matter is that the presence of *pfmdr1* N86/184F increases the chances of resistance in artemisinin to appear [240]. Indeed, parasites harboring the 86N allele are less sensitive to artemisinins *in vitro* [281,282] and infections with LF tolerant parasites imply that artemisinins act as monotherapy, hence contributing to selection pressure for artemisinin resistance [240].

Our findings suggest that ALu is not robust enough to obviate the selection of mutant parasites and that a single treatment is sufficient for this selection to happen. This raises concerns about the speed with which the mutations are selected but also has implications in terms of case management. Indeed, a study suggested that patients presenting in a HF with a partner drug detected in their blood were 4 times more likely to fail subsequent treatment with the same ACT [283] and that they should then be treated with an alternate ACT. These data are consistent with the great number of ACT failure rate observed, regardless of whether artemisinin resistance was present [284]. This is explained by the fact that the artemisinin component is responsible for the rapid reduction in parasites load [35] which alleviates the symptoms while the partner drug ensures the successful therapeutic outcome, killing the remaining parasites ($10\text{-}10^4$) [14]. In ACTs, artemisinin is protected by the partner drug. If

artemisinin resistant parasites are present, they will be killed by the partner drug which makes the selection of artemisinin resistant parasites through ACTs unlikely. Despite the protective effect of the partner drug, artemisinin resistance has already emerged in South East Asia, which was attributed to the use of artemisinin-based monotherapies, substandard and counterfeit treatments [12]. In the reverse situation, resistance to partner drug can be selected during the selection window, leading to treatment failure [219].

In 2002, Talisuna *et al*. [69] observed lower CQ use in high parasite prevalence sites and attributed it to the acquisition of partial immunity in high transmission areas, resulting in less clinical demonstration of the disease. In our study, the assumption that higher parasite prevalence leads to lower drug use through lower frequency of clinical malaria cases was valid for adults, who were more likely to use SP (CHAPTER VI). However, in children who are more prompt to clinical malaria, the opposite trend was observed: LF prevalence increased with malaria endemicity. Moreover, due to their poor immunity, they are more at risk of selecting resistance [226,230,235]. Special attention should thus be given to increase appropriate case management in children, not only for a positive clinical outcome, but also to control the spread of resistance. Indeed, in high transmission areas where resistance can spread faster, their immunity is not sufficient to stop infection to evolve in clinical malaria and to stop the selection of resistant parasites. Moreover, as shown in CHAPTER VI, drug pressure is higher in highly endemic districts and in the most accessible locations. Interventions aimed at improving case management should be prioritized in these areas because they constitute favorable conditions for development and spread of resistance.

9. CHAPTER IX: RECOMMENDATIONS AND CONCLUSION

9.1. Recommendations

9.1.1. Implications of the research findings into policy and actions

Tanzania mainland's goal was to reduce the average country malaria prevalence to less than 1% by 2020 [1]. However, this country particularly leans on case management and long lasting insecticidal nets (LLINs), which are both threatened by resistance. There is an urgent need to make rational use of drug a real and sustainable objective. Investments are put in researching for new diagnostic tools and drugs (20% of the annual malaria research and development funding [285]), while those existing are not used to their full potential.

Based on our findings, we can draw the following recommendations for health authorities and policy makers in Tanzania:

- Use drug measurements to assess impact of interventions and programs
- Strengthen drug policy outside health facilities
- Target interventions towards high risks areas
- Target children at high endemicity and adults at low endemicity
- Prioritize health workers training
- Use of local capacities
- Increase consumer awareness

- **Use drug measurements to assess impact of interventions and programs**

Measuring drugs in the frame of population-based and school-based surveys can, combined with questionnaires limited to a few questions:

- help understand the most important drivers of antimalarials consumption
- help targeting interventions in the locations where medicine use appears inappropriate [286]
- assess whether programs such as Intermittent Preventive Treatment of malaria in pregnancy (IPTp) are correctly implemented (when used in population-based surveys)
- assist in decision-making to implement specific interventions and be used as outcome indicator [287] to further assess the success and impact of the implementation
- combination of available technologies such as high performance platforms (LC-MS or LC-UV) with mapping and modeling [288] can enable the collection of the minimal needed data of good quality [285] and predict data where they do not exist.

- **Strengthen drug policy outside health facilities**

The high prevalence of lumefantrine (LF) levels (more particularly at sub-therapeutic levels) and of the relating mutant parasites in the studied population challenges the rationale of increasing access to highly effective treatment such as ALu through private sector, without suitable use of diagnostic tools. More restrictive rules should be introduced to drug vendors, such as forbidding the sale of ACTs without prescription or without positive mRDT result. This should be done along with increased mRDTs roll-out with proper training and supervision.

Then, the relatively high proportion of adults self-treated with SP calls for a special attention on reinforcing the use of first line treatments, in Mwanza particularly. The supply of sulfadoxine-pyrimethamine (SP) outside health-facilities (HFs) for specific programs such as IPTp and IPTi should be forbidden due to the poor efficacy of this treatment.

- **Target interventions towards high risks areas**

By measuring antimalarials in the blood, we identified that drug pressure was higher in districts of high transmission and in most accessible locations. Such places should thus be targeted first.

- **Target children at high endemicity and adults at low endemicity**

In high transmission areas, children represent the major infectious reservoir [289]. Improved case management should especially be targeted towards children, not only for a positive clinical outcome, but also to control the spread of resistance. In lower transmission settings, since adults are the major drivers of transmission [289], the use of recommended first-line efficacious drugs in adults should be promoted in the perspective of pre-elimination [3].

- **Prioritize health workers training**

The majority of febrile individuals seek care outside HF. Proximity of good quality health care should be improved by training dedicated community health workers (who do not have any incentive of selling more drugs, unlike drug shops). In HF, health workers should be better trained and their ability in performing mRDTs should be assessed on a regular basis. Clinicians should also understand the stake of treating according to the test results.

- **Use of local capacities**

Resources should be used in developing local capacities, in terms of human resources and technology platforms. Laboratory technicians could then be involved in surveillance activities ranging from parasite surveillance, resistance surveillance and drug assessment in the population. This could also be applied to other infectious diseases [277].

- **Increase consumer awareness**

Behavior change from the consumer side is also paramount in order to achieve rational use of antimalarials and other antimicrobials. Interventions that increase awareness of multiple causes of fevers are needed to support the new rationalization for malaria treatment represented by parasitological diagnosis [290]. For example, radio, sms platforms could be used or messages could be given to children at school [3].

In our study, many people tested positive did not have drugs in their blood. The importance of treatment adherence should be emphasized using logos on drug boxes or in drug shops and by asking patients to take the first dose under observation [194]. Poor adherence has an effect regarding clinical outcome, but also in the selection of mutant parasites as it leads to sub-therapeutical drug concentrations.

9.1.2.Recommendations for future research

- Choose the appropriate study design
- Use of more advanced mathematical and statistical methodologies
- Monitor drug pressure and resistance
- Use dried blood spot in a broader antimicrobial stewardship effort
- Improve knowledge on drug inhibitory concentration and distribution
- Search for new drug combinations

- **Choose the appropriate study design**

In order to assess the impact of an intervention in a comprehensive manner, its effect should be evaluated both at HF level and in the community to see whether results obtained from HF surveys correlate with measures obtained in the population. Moreover, surveys should be conducted at repeated points in time to assess the sustainability of the effect, and in heterogeneous malaria transmission settings and heterogeneous proximity or easiness of transports to the HF and drug stores.

- **Use of more advanced mathematical and statistical methodologies**

The use of stochastic individual-based models of malaria transmission could simulate the prevalence of fevers that are due to malaria and thus the number of individuals that should have antimalarial detected in the blood. The simulation models can be parameterized using the survey data such as malaria endemicity, demographics, measures of access to care, use of

diagnosis and treatment regimens and outputs from the simulations compared with field data. Furthermore, understanding the mechanism of drug resistance both at individual and population level is crucial in order to embrace the dynamic of resistance [69]. The population component needs to be tackled using more advanced statistical methodologies such as bayesian statistics and mapping and taking into account other important geographical or climate factors which could have an influence on resistance development and spread [291]. Knowledge of special characteristics and predictors of diseases has proven to be valuable tools to assist health authorities in adapting appropriate and targeted interventions [291,292]. This analysis has already been initiated in collaboration with Dr. Penelope Vounatsou, Head of the Bayesian Modeling and Analysis group at the Swiss TPH.

- **Monitor drug pressure and resistance**

Understanding, tracking and mapping resistance is essential to understand how it spreads. Drug pressure, because it is linked to resistance, could thus be measured alongside with antimalarial susceptibility testing [277].

In our study, we did not know whether the parasites detected in our samples had been selected during the previous treatment or if they were new infections. Genotyping of a highly polymorphic gene such as *pfmsp2* [96] could define the genetic diversity of the infection and help distinguish reinfections from primary infections and should be used to understand the role of residual drug levels on parasite resistance.

- **Use dried blood spot in a broader antimicrobial stewardship effort**

Decrease in antimalarial use often goes hand in hand with an increase in antibiotic prescription [182,293]. For this reason, DBS should be used to simultaneously measure antimalarials and antibiotics to better understand the dynamic of antimicrobial consumption. A LC-MS/MS method for the analysis of the 12 most frequently used antibiotics in Tanzania (namely amoxicillin, metronidazole, ceftriaxone, trimethoprim, sulfamethoxazole, cephalexin, ciprofloxacin, doxycycline, chloramphenicol, erythromycin, penicillin V and G) in DBS has been developed by a Master student in Pharmacy [294] in the frame of the present thesis. This method is planned to be used in a new project aiming at assessing antimicrobial drug pressure at the population level and evaluating the impact of antibiotic stewardship intervention on antimicrobial drug pressure [295]. The spatial distribution of antimalarial and antibiotic use in the population will then be mapped and used to re-inforce and better target antimicrobial stewardship interventions.

- **Improve knowledge on drug inhibitory concentration and distribution**

Few reliable and reproducible information on the minimum inhibitory concentrations (MIC) of the studied drugs are available in the literature. Furthermore, after correlating LF concentrations in DBS and plasma, we realized that LF distribution inside the red blood cells is minimal. More extensive research on the MIC and the distribution of the drugs inside the erythrocytes (where drugs inhibit the intra-erythrocyte development [87,88]) should be conducted to better assess parasites susceptibility to drugs.

- **Search for new drug combinations**

We confirmed the limitation of combining short half-life and long half-life compounds. Studies should examine possibilities of combining drugs with shorter and similar half-life as well as using triple combinations therapies to prevent resistance.

9.2. Conclusion

We developed a sensitive LC-MS/MS method for the simultaneous analysis in DBS of the major partner drugs used in ACT combinations. This methodology was suitable for field studies and enabled us to collect objective and reliable data to illustrate the complex interplay between parasite prevalence and resistance, individual factors such as age and health seeking behavior as well as diagnosis and treatment practices. We were able to identify the principal determinants of drug use, which could be used in the future in malaria control (to assess access to care as well as diagnostic and treatment policies implementation) and surveillance (along with parasite resistance surveillance for example). We also demonstrated that the presence of low LF concentrations in the blood of individuals surveyed in the community was associated with the presence of parasites harboring markers of drug resistance. Despite efforts made to improve access to diagnostic tools, there is still a high drug pressure in the population. A better targeted use of antimalarials in favor of parasitologically confirmed malaria cases only should be urged in order to stop the spread of resistance. To that aim, the WHO guidelines of systematic diagnosis and treating upon results is an essential guideline to be followed and there is a need to support the use of mRDTs use with proper training in the public and in the private sector.

This observational work was a first step in the evaluation of drug pressure in the community, using reliable tools to have a deeper understanding of the relationship between drug pressure and parasite resistance at population level. It is now time to integrate available advanced technologies and statistical tools such as Bayesian statistics and mapping to create good quality data and predict data when they are not existing. Modeling could also be used as a pro-active approach to predict the effect of interventions on drug pressure.

The amount of drug pressure in the population should seriously be considered as Tanzania wants to progress towards malaria pre-elimination. High prevalence of individuals with drug in the blood (such as during mass drug administration) is only recommended in settings approaching interruption of transmission [296], where the risk of re-introduction of infection is minimal [297]. In other transmission settings, the temporary prophylactic effect of long half-life compounds provides a significant selective pressure for the emergence of drug resistance, which threatens the sustainability of gains in malaria control and prospects for elimination [298]. Strategies to stop the expanding resistance to artemisinins and partner drugs are urgently needed [273] and this starts by avoiding parasite exposure to a single selective agent, which is the central principle of resistance management [298].

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11. APPENDICES

APPENDIX 1: Example of flowsheet used during the inclusion visit

ETUDE DBS-ALU – INVESTIGATION EN PCL

Date d'inclusion: ☐☐ / ☐☐ / 2016

PARTICIPANT

Initiales (prénom, nom) : ☐☐ Sexe : ☐ M ☐ F

Année de naissance : ☐☐☐☐

Code d'identification du sujet : ☐☐☐☐ – ☐☐

Date de signature du consentement : ☐☐ / ☐☐ / ☐☐☐☐

VISITE DE SCREENING		
CRITERES D'INCLUSION :	OUI	NON
▪ Sujet âgé entre 18 et 45 ans	☐	☐
▪ Si sujet féminin : utilise une méthode de contraception fiable. Si une méthode de contraception hormonale est utilisée (orale, transdermique ou systémique), la volontaire a été avisée d'utiliser une méthode non-hormonale additionnelle jusqu'aux prochaines menstruations	☐	☐
▪ Absence de traitement médicamenteux concomitant (contraception hormonale tolérée)	☐	☐
▪ Le sujet comprend les procédures et accepter de participer à l'étude et souhaite donner son consentement écrit	☐	☐
▪ Le sujet accepte de participer à minimum 5 visites après l'administration du médicament	☐	☐
A remplir par la suite :		
▪ ECG 12 dérivations sans anomalie significative	☐	☐
▪ BMI entre 18 et 29 kg/m^2	☐	☐

CRITERES D'EXCLUSION :

	OUI	NON
▪ Antécédents médicaux majeurs (hépatiques, immunologique, rénal, hématologique, gastro-intestinal, génital, système nerveux central, cardiovasculaire, pulmonaire ou rhumatismal)	□	□
▪ Maladie sévère récente ou séquelles qui pourraient exposer le sujet à de plus gros risques ou pourrait confondre les résultats de l'étude	□	□
▪ Prolongation congénitale de l'intervalle QT ou toute autre condition connue pour allonger l'intervalle QT ou causer une arythmie cardiaque (historique d'arythmie cardiaque symptomatique, bradycardie ou maladie cardiaque sévère)	□	□
▪ Femme enceinte ou allaitante	□	□
▪ Historique familial de prolongation congénitale de l'intervalle QT ou de mort subite	□	□
▪ Perturbations connues de l'équilibre électrolytique	□	□
▪ Troubles hépatiques connus, y compris ceux ne nécessitant pas de traitement (syndrome de Gilbert toléré si léger)	□	□
▪ Traitement durant les 3 mois précédents avec un médicament connu pour avoir un potentiel de toxicité bien définie pour un organe majeur	□	□
▪ Historique d'hypersensibilité à un des composants du médicament	□	□
▪ Historique d'hypersensibilité à n'importe quel médicament, si considéré comme sérieuse	□	□
▪ Historique d'abus d'alcool ou de drogues	□	□
▪ Consommation d'une grande quantité d'alcool ou de vin (>0.5 L vin/jour)	□	□
▪ Utilisation de médicaments durant la semaine précédant l'étude (ou basé sur la règle des 5 demi-vies plasmatiques) et jusqu'à 48 heures post-traitement	□	□
▪ Participation à un essai clinique dans les 3 mois précédents, à moins qu'aucun traitement n'ait été administré et qu'un volume faible de sang ait été prélevé	□	□
▪ Occupation qui pourrait interférer avec les visites ou les prises de sang durant l'étude	□	□
▪ Statut psychologique qui pourrait avoir un impact sur la capacité du sujet à fournir un consentement éclairé	□	□
▪ Toute caractéristique présente ou de l'historique du sujet qui, selon l'avis de l'investigateur, pourrait confondre les résultants de l'étude ou qui pourrait représenter un risque potentiel pour le sujet	□	□

A remplir par la suite :		
▪ Test de grossesse (hCG) positif	☐	☐
▪ Hypertension (>150/90mmHG au repos) ou épisodes hypotensifs récurrents considérés comme cliniquement significatifs	☐	☐

INFORMATIONS A TRANSMETTRE AU SUJET :

	OUI	NON
▪ Le sujet a été informé qu'il devrait s'abstenir de consommer du grapefruit (jus et fruit entier) ainsi que des boissons alcoolisées 24 heures avant et jusqu'à 48 heures après la prise de Riamet®	☐	☐
▪ Le sujet a été informé qu'il devrait s'abstenir de prendre un médicament hors de celui administré durant l'étude une semaine avant et jusqu'à 48 heures après la prise de Riamet® (à l'exception des contraceptifs oraux). Prise de paracétamol acceptée après permission de l'investigateur	☐	☐
▪ Le sujet a été informé qu'il devrait s'abstenir de participer à un autre essai impliquant la prise d'un traitement jusqu'à la fin de l'étude	☐	☐
▪ Si sujet féminin : la volontaire a été avisée d'utiliser une méthode non-hormonale jusqu'aux prochaines menstruations	☐	☐
▪ Le sujet a été informé des effets indésirables les plus fréquents survenant à la suite de la prise de Riamet® (céphalées, vertiges, nausées, vomissements, douleurs abdominales, toux, faiblesse, fatigue, arthralgies, myalgies, palpitations, troubles du sommeil)	☐	☐
▪ Le sujet a été informé du fait que des vertiges et fatigues pouvaient survenir à la suite de la prise de Riamet® et que ceux-ci pouvaient limiter son habilité à conduire un véhicule	☐	☐

AUTRES INFORMATIONS :

Utilisation récente d'antimalariques : ☐ OUI ☐ NON

Si **OUI**, nom du traitement (spécialité ou principe actif) :

Quand le traitement a-t-il été pris ? ☐☐ (jours/semaines/mois)

INFORMATIONS SUR LE PARTICIPANT :

Taille : |_|_|_| cm Poids : |_|_|_| kg

BMI : |_|_| kg/m^2 Température : |_|_| °C tympanique

Fréquence cardiaque : |_|_|_| battements/min Pression artérielle: |_|_|_| / |_|_|_| mmHg

Résultat du test de grossesse (hCG) : ☐ Positif ☐ Négatif

Electrocardiogramme

Résultat : ☐ Normal ☐ Anormal *(préciser)* :

QT : |_|_|_| ms QTc : |_|_|_| ms Fréquence cardiaque : |_|_|_| battements/min

Examen physique

☐ aucune anomalie ☐ anomalie cliniquement non significative ☐ anomalie cliniquement significative

Remarques concernant l'examen physique :

VISITES FIXEES AVEC LE PARTICIPANT (5 visites minimum):

Visites n°	Visites choisies		Date	Heure
1	H0 (±0h)	■	|_|_| / |_|_| / 2016	|_|_| : |_|_|
2	H3 (±0.5h)	☐	|_|_| / |_|_| / 2016	|_|_| : |_|_|
3	H6 (±0.5h)	☐	|_|_| / |_|_| / 2016	|_|_| : |_|_|
4	H8 (±0.5h)	☐	|_|_| / |_|_| / 2016	|_|_| : |_|_|
5	H10 (±2h)	☐	|_|_| / |_|_| / 2016	|_|_| : |_|_|
6	H18 (±3h)	☐	|_|_| / |_|_| / 2016	|_|_| : |_|_|
7	H24 (±3h)	☐	|_|_| / |_|_| / 2016	|_|_| : |_|_|
8	H48 (±6h)	☐	|_|_| / |_|_| / 2016	|_|_| : |_|_|
9	H72 (±6h)	☐	|_|_| / |_|_| / 2016	|_|_| : |_|_|
10	H168 (±24h)	☐	|_|_| / |_|_| / 2016	|_|_| : |_|_|
11	H336 (±24h)	☐	|_|_| / |_|_| / 2016	|_|_| : |_|_|